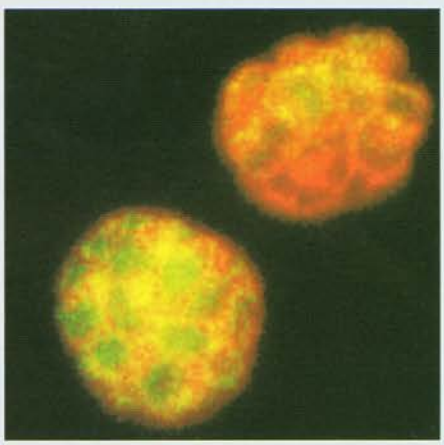
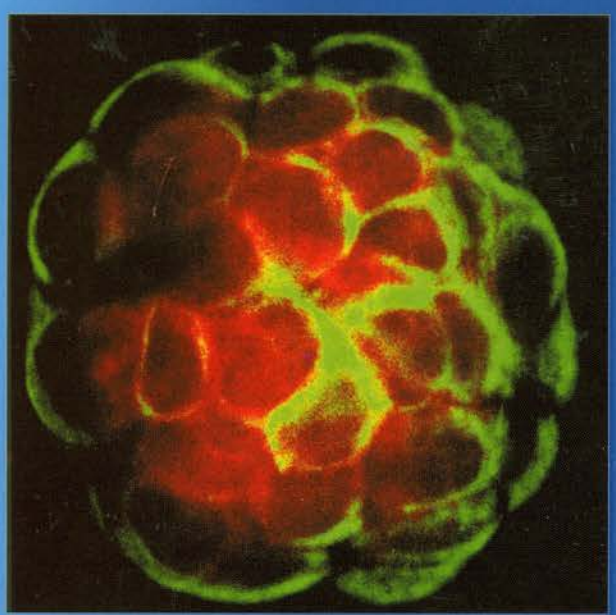
Research Reports



Cell Biology

nccs

NATIONAL CENTRE FOR CELL SCIENCE



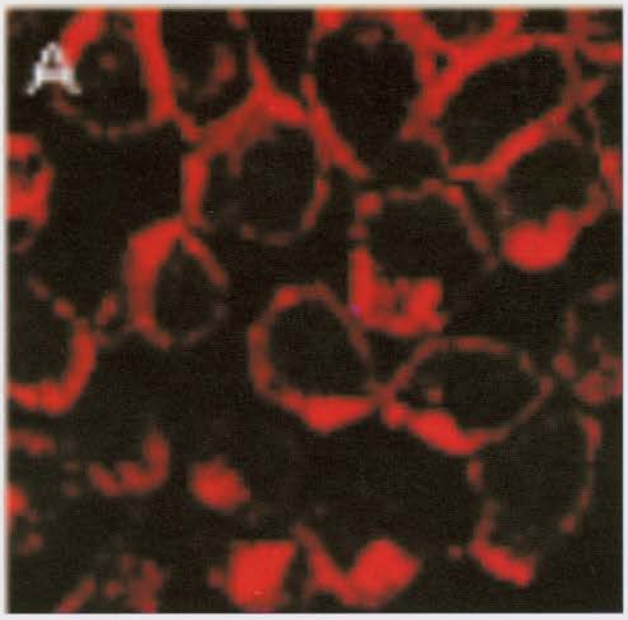
ANNUAL REPORT 2003-2004

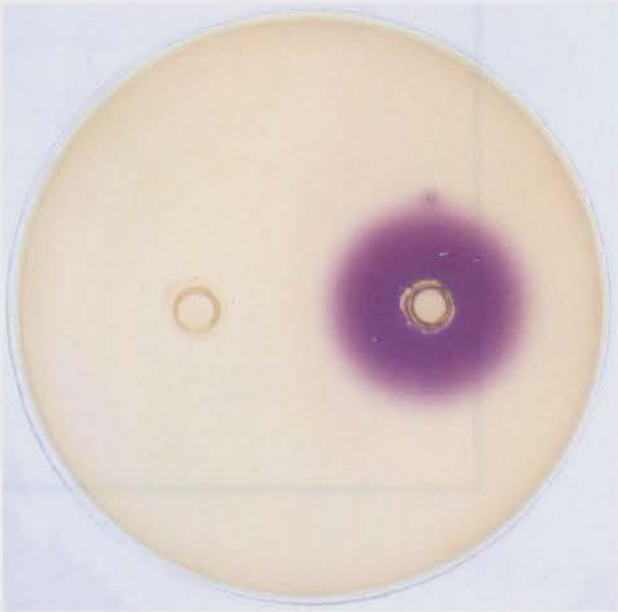
National Centre for Cell Science

Annual Report

2003 - 2004

Common thread of life...

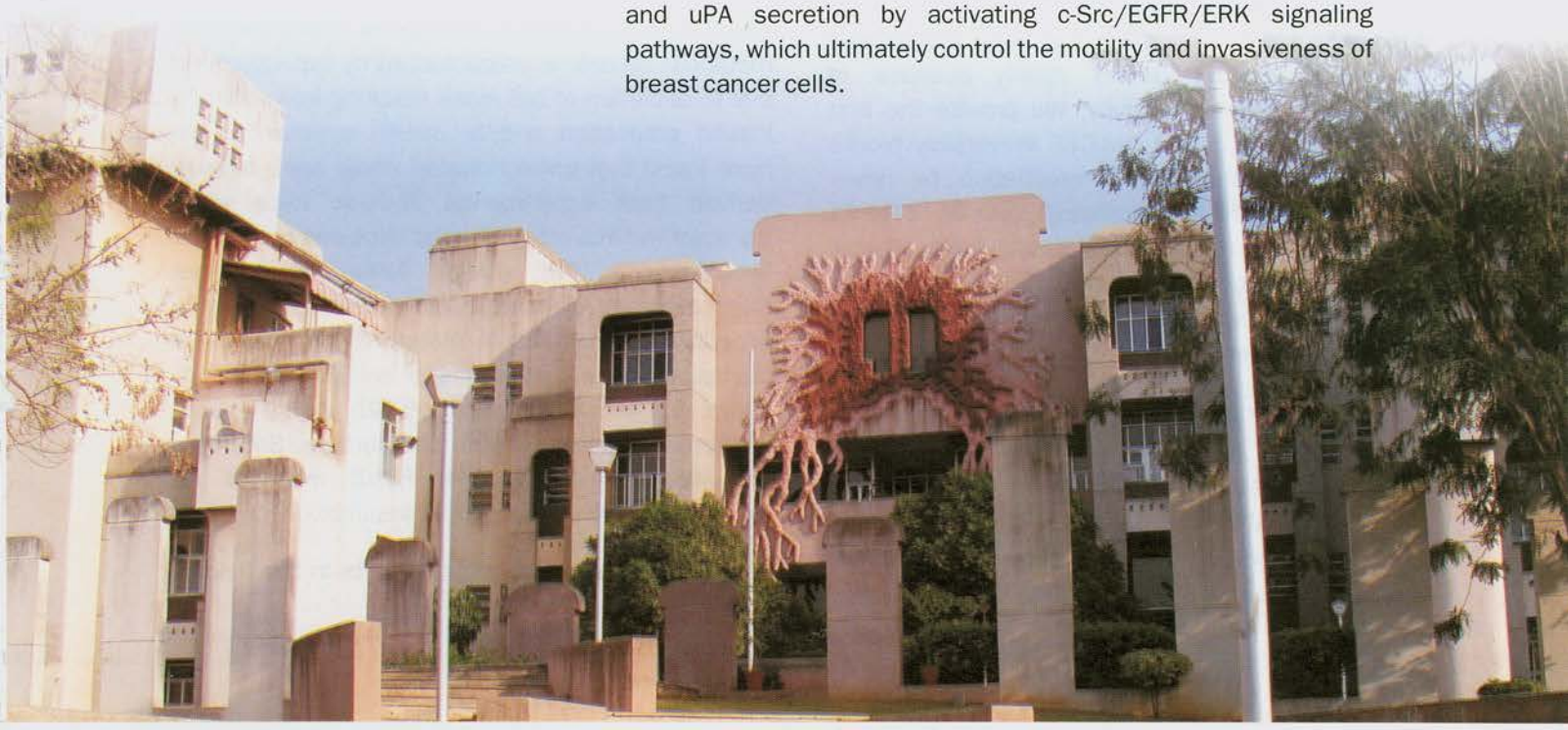




From Director's Desk

It gives me great pleasure to present the Annual report of the National Centre for Cell Science (NCCS) for the year 2003-2004. As an integral part of our mission we continue to perform research and development in various areas including cell biology, cancer biology, immunology, diabetes, signal transduction and gene regulation. We are also happy to contribute towards human resource development by virtue of teaching and training. This year we have continued to achieve significant progress in all our endeavors. NCCS also serves as a national cell repository and this year we have supplied 1017 cell lines to 163 scientific institutions within the country. In our continued efforts to establish and characterize cell lines, we have succeeded in establishment and characterization of new immortalized and primary cell lines with unique features. In our continued effort to serve the nation by imparting training in tissue culture, we have trained 10 visiting researchers at NCCS and also conducted on site training for 70 researchers in institutions located at Allahabad, Guwahati, Baramati, and Kolhapur.

Cell signaling and cell migration play a critical role in angiogenesis and metastasis. We found that Wortmannin, an inhibitor of the PI3K, blocked morphological transition and migration of SiHa cells in wound closure assay. Wortmannin also blocked EGF induced invasion by SiHa cells in ECM gel assay. Transforming Growth Factor beta (TGFβ) controls a wide range of cellular responses including cell proliferation, lineage determination, differentiation, and apoptosis. Our studies clearly show that differential activation of signal transduction pathways by TGFβ1 as a function of its concentration underlies its bi-directional effect on hematopoietic cells. We demonstrate that mammalian cells have functionally intact eNOS that is associated with sub-nuclear structures like spliceosomes, suggesting that it may perform regulatory role in splicing mechanism. We also report for the first time that osteopontin (OPN) induces $a_v b_3$ integrin-mediated AP-1 activation and uPA secretion by activating c-Src/EGFR/ERK signaling pathways, which ultimately control the motility and invasiveness of breast cancer cells.



How a-hemolysin establishes synergistic function with a protein tyrosine phosphatase is unclear. Our results suggest that the α-HL induced change in Caveolin-1 organization is distinct and clear, probably through a direct interaction with Caveolin-1 beneath the cell surface, present in the lipid rafts. During this oligomerization/assembly of α-HL, Caveolin-1 network is reorganized beneath the cell surface and this is perhaps responsible for an attenuation of receptor mediated signaling events.

In last few years, NCCS has attracted a special attention towards stem cell research in India. In this connection, continued efforts on bone marrow cryopreservation and revival of stem cells has developed better concepts. We demonstrated that addition of catalase and trehalose to freezing medium help to preserve the migration capacity of frozen $CD34^+$ cells, suggesting that additives improve the in vivo homing of frozen mouse bone marrow. Interestingly, addition of lectins to serum free medium also preserved hematopoietic progenitor cells. In the neurodegenerative disorders such as Parkinson's and schizophrenia the dopaminergic neurons are degenerated in the substantia niagra and the ventral tegmentum respectively. We have successfully generated a number of stable embryonic stem cell clones expressing the live reporter EGFP under the regulatory control of dopaminergic neuron specific promoter tyrosine hydroxylase (TH). These clones upon differentiation into neural lineage did exhibit EGFP expression.

The bone loss in many important skeletal disorders such as osteoporosis, rheumatoid arthritis, hypercalcemia of malignancy and bone metastases of various tumors occurs mainly because of increased osteoclast activity. We provide the first evidence that IL-3 and GM-CSF irreversibly blocks TNF–induced osteoclast differentiation by down-regulation of mRNA and surface expression of TNFR1 and TNFR2. Our results reveal that IL-4 acts directly on mature osteoclasts and abolish bone resorption through inhibition of NF-B activation by IL-4R mediated mechanism.

Nuclear factor kappa B (NFκB) is known to regulate a wide range of genes. Varieties of drugs that are known to affect the NFκB pathway are used in various therapies. In this regard, Carboplatin treatment is known to block the translocation of NF-κB via stabilization of the antagonistic IκBα protein. Moreover, carboplatin mediated inhibition of constitutively active NF-κB leads to Bcl-2 downregulation by direct inhibition of NFκB binding to the response element present in Bcl-2 promoter, providing a molecular mechanism of action for this antineoplastic agent.

Mitochondria are the key players in the regulation of cell death. One of the crucial steps in apoptosis induced by anticancer agents/drugs for elimination of tumors is the disruption of the mitochondrial membrane potential. Study of the downstream apoptotic pathways revealed an essential role of VDAC and Bax in altering the membrane potential and release of cytochromec during apoptosis induced by staurosporine in SK-N-MC human neuroblastoma cell line. Earlier it was shown that high glucose increases mitochondrial superoxide generation from more than one source which initiates cell death. Furthermore, mitochondrial anion channels and cytosolic dismutation to H_2O_2 may be important steps for oxidant induction of high glucose-induced cardiac cell death.

We have identified the novel 600 bp transforming gene that we cloned from mouse melanoma cells and is now identified as a member of non-coding RNAs (NCRs). The NCR found to be localized to the cytoplasm as a polyadenylated RNA. Transcript analyses by Northern hybridization with labeled riboprobe demonstrated the anti-sense expression of the gene with transcript size of 1.8 kb.

NCCS has many ongoing projects that are aimed at understanding the molecular basis of both diabetes type I and II. Diabetes mellitus is a multifactorial metabolic syndrome characterized by hyperglycemia due to depletion of cell mass resulting into deficient insulin production and/or insulin resistance. We have found that unfractionated whole bone marrow derived from experimental diabetic mice when transplanted into other diabetic mice can correct the hyperglycemia. Our studies further reveal that transplantation of bone marrow stem cells rescue experimental diabetes in mouse model. Chemical manipulation such as tagging of cell surface with 1-fluoro 2, 4-dinitrobenzene (FDNB) brings about conformational changes on cell surface. Strikingly, immunization of rats with FDNB modified rat insulinoma (RIN) cells produced insulitis.

We have number of ongoing projects in the field of

The terms of reference

- To receive, identify, maintain, store, grow and supply: Animal and human cell/cell cultures, cell lines of both existing (typed) and newly developed , hybrid cells including hybridomas; Tissues, organs, eggs (including fertilized) and embryos. Unicellular, obligate pathogens, parasites and vectors, plasmids, genes and genomic libraries.

- Develop, prepare, quality control and supply culture media, other reagents and material and cell products independently and in collaboration with industry and other organizations.

- Research and development in the above and cell cultures related materials and products.

- To establish and conduct postgraduate courses, workshops, seminars, symposia and training programs in the related fields.

- To serve as National Reference Centre for tissue culture, tissue banking and cell products and data bank etc. and to provide consultancy services to medical, veterinary and pharmaceutical institutions, public health services and industries etc. in the country.

- To provide and promote effective linkages on a contitious basis between various scientific and research agencies / laboratories and other organizations including industries working in the country.

- To participate in such a program as required in the country for the betterment of society and advancement of science and technology.

- To collaborate with foreign research institutions and laboratories and other international organizations in the areas relevant to the objectives of the facility.

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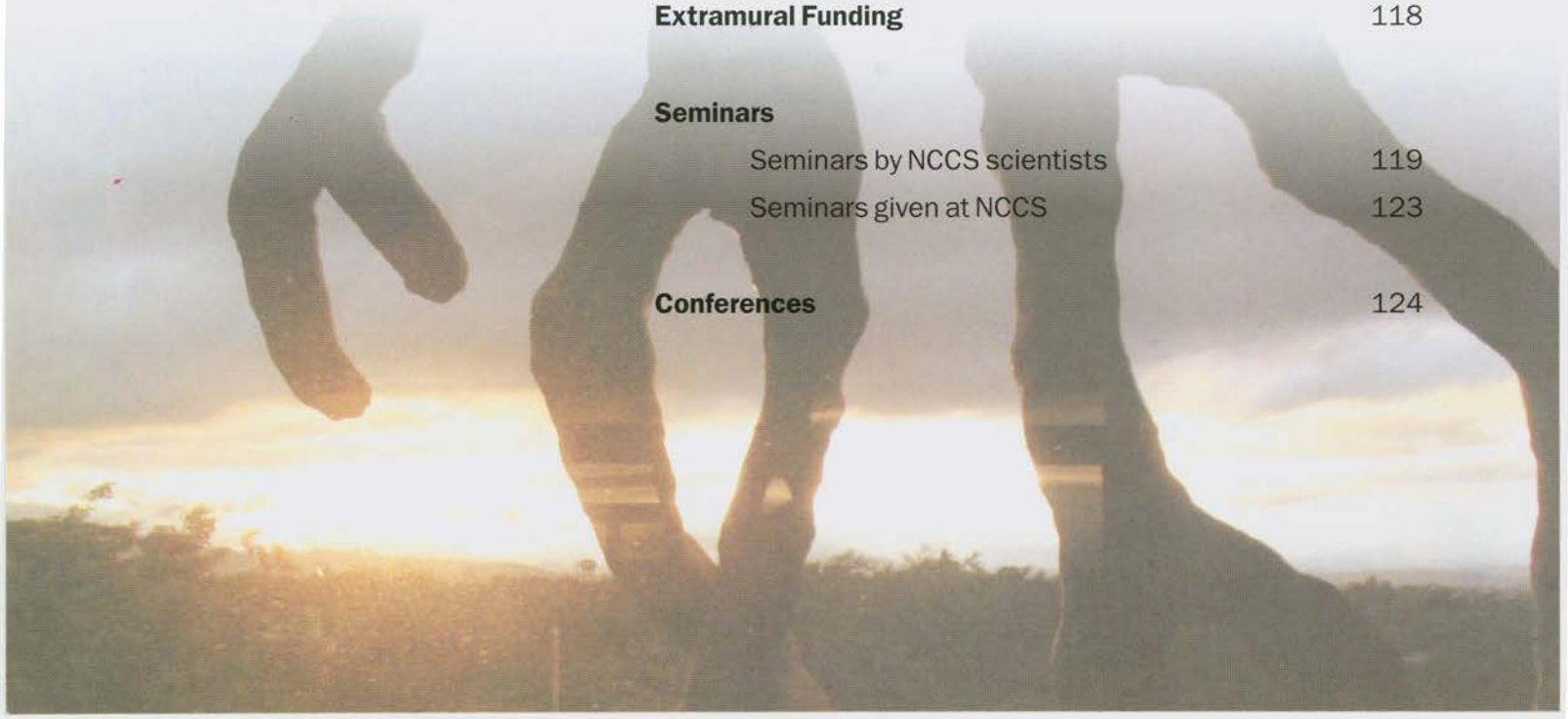

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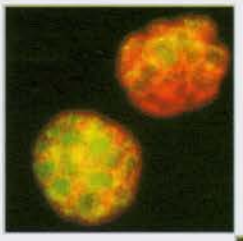

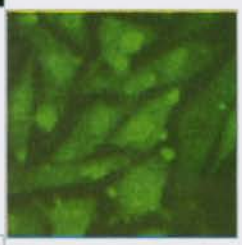

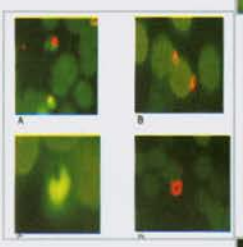

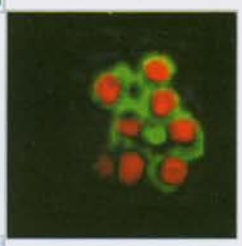

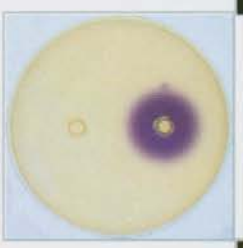

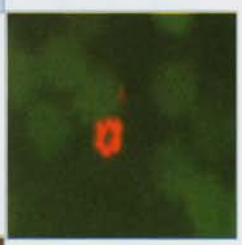

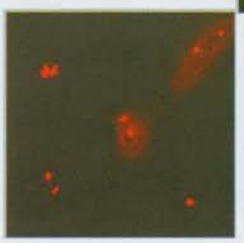

molecular parasitology. In an attempt to understand how the parasites obtain their energy, we cloned two complete coding sequences for Hexokinase isoenzymes from Drosophila. The differential expression of the two cloned enzymes indicated that the nature of 5' upstream non-coding regions may have different cis-acting elements necessary for their expression. Complete coding sequence for hexokinase gene is also cloned from the parasite Leishmania major. We isolated a novel bacterial strain, *Aeromonas culicicola,* from the midgut of the mosquito *Culex quinquefasciatus*. We are now studying the virulence factors of this strain and its pathogenic nature. We are continuing research on malarial parasites. Remarkably, we reported for the first time spontaneous development of parasites outside the human host erythrocytes. In fact we have now isolated these parasites and cultured in presence of fresh RBC and characterized by molecular tools. Macrophages play as host to Leishmania, a parasite that causes leishmaniasis in 0.5 million people annually. We demonstrate that weak CD40 signals induce extracellular stress-related kinase (ERK)-1/2-dependent IL-10 expression whereas stronger signals induce p38-mitogen activate protein kinase (p38MAPK)-dependent IL-12 production. These data unfold a novel immune evasion strategy, where a parasite differentially modulates the CD40 engaged reciprocally functioning signaling modules, and a novel strategy for defining drug target(s). These novel findings were published in Nature Medicine, 2004.

NCCS has number of ongoing projects on HIV biology. We have shown a direct interaction of the HIV-1 transactivator Tat with NFκB enhancer, a global regulatory sequence for many cellular genes both in vitro and in vivo. This interaction not only provides a novel molecular basis to explain TAR independent transactivation in HIV-1, but also point towards the potential mechanism of Tat mediated modulation of cellular genes. Our DNA immunization studies towards generation of potent HIV vaccine came out to be exciting. Using gp120 from subtype C isolate prevalent in India has revealed some startling facts about the elicitation and maturation of cytotoxic T lymphocytes. Our studies have revealed the presence of anti-HIV activity in few marine bivalves. Studies towards identification and characterization of the active components from lysates of these bivalves are underway.

We are also interested in understanding the molecular interaction between host's complement proteins with various viral proteins. Our data indicated that like host complement control proteins also possess factor I cofactor activities for complement proteins C3b and C4b and also possess decay accelerating activities for the classical as well as alternative pathway C3 convertases. We are also making efforts to determine the functional determinants of viral complement control proteins.

The role of chromatin architecture in fundamental processes involving DNA is emerging. The higher order organization of chromatin in form of loop domains is governed by MARs and their binding proteins. We have delineated the minimal domain of SMAR1, a candidate tumor suppressor, which is responsible for p53 interaction, activation and stabilization within the nucleus. Studies using SMAR1 transgenic mice suggested that apart from its role in the control of cell cycle, V(D)J recombination and T cell development, SMAR1 is also involved in the development of lymphoid organs. The T cell restricted regulator SATB1 is known to organize T cell chromatin in a unique three-dimensional architecture that circumscribes heterochromatin. We have unraveled, for the first time, primary sequence signature(s) that are embedded into cell type specific chromatin architecture. We propose that the primary sequence features such as the consensus motifs and repeating hexameric patterns project a unique chromatin context in vivo.

This year NCCS has published 35 papers in reputed peer reviewed journals. We have successfully obtained 2 US patents and also filed 6 patents. I am very happy to state that this year we have seen a substantial increase in the peer reviewed funding obtained by NCCS scientists. Our achievements are significant and we have succeeded in setting higher standards for ourselves and achieved them.

Our achievements are significant and I am confident that the progress will continue to set higher standards in coming years.

G C Mishra

Director

Repository

The repository of National Centre for Cell Science is the only repository that houses human and animal cells in India. The NCCS repository serves to receive, identify, maintain, store, cultivate and supply animal and human cell lines and hybridomas. The work done at repository mainly involves cell line procurement, expansion, cryopreservation and distribution. The repository has procured cultures from various sources within the country and abroad from 35 animal species. A major bulk of the cell lines stocked in the repository has been procured from the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Cultures (ECACC). The list of cell lines with details such as media requirements, growth conditions and its is available on demand. During the year 2003-2004, we have supplied 1017 cell lines comprising of 181 different cell types to 163 research institutions in the country. The repository has initiated programmes to develop. Immortalize and characterize cell lines from different tissue/tumour types.

Human Resource Development

One of the main objectives of the center is to enhance human resources by way of conducting symposia, workshops and tailor made programmes for individuals. To achieve this goal, the center has conducted a workshop on Production of Transgenic Mice from 13th - 17th October 2003. A total of 18 personnel were trained in the basic techniques of transgenic mice generation.

In addition, the center has also attracted about 17 summer trainees and M.Sc project students from various universities all over India. The projects were appreciated by scientists of other institutes and many of the trainees showed interest to pursue future Ph.D. programme at NCCS.

Number of NCCS scientists actively participated in various teaching activites and co-ordinating workshops at various universities, colleges and departments. These include Department of Zoology, Microbiology, Biochemistry and Biotechnology of University of Pune and also local colleges.

During this year, 21 new Junior Research Fellows joined at NCCS for Ph.D. programme. The total strength of research fellows reached to 83. In addition, currently one Research Associate has joined and continuing research at NCCS.

Additionally, we held on site workshops for training in Tissue Culture techniques as follows.

S.No.	Institute/University	Duration	No. of participants
1	North-East Hill University, Dept. of Zoology, Guwahati	15th - 20th Sept.2003	15
2	University of Allahabad, Dept. of Biotechnology, Allahabad	13th 20th Oct. 2003	15
3	Vidya Pratisthan, School of Biotechnology, Baramati	26th 30th July 2004	20
4	Shivaji University, Dept. of Zoology, Kolhapur	16th 18th Aug. 2004	20

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Establishment and characterization of cell lines from human ovarian tissues

Nawneet Kurrey, Avinash Mali, technician

Collaborators:
C. B. Koppikar, Oncosurgeon, Jehangir Hospital and Medical Centre, Pune.

Abstract and background :

Ovarian carcinoma cells form multicellular aggregates, or spheroids, in the peritoneal cavity of patients with advanced disease. The current paradigm that ascites spheroids are non-adhesive leaves their contribution to ovarian carcinoma dissemination undefined. The prognosis for patients at the stage of the disease when spheroids develop is extremely low (mean survival time < 5months). Hence, there is a need to characterize these structures and their role in metastases and drug resistance.

Aims:

Enrichment of the NCCS Cell Repository with ovarian cancer cell lines.

Work achieved:

An ascites sample derived from a patient with an advanced stage of ovarian cancer was obtained. We report here, the establishment and spontaneous immortalization of several clones from this sample. Spheroids were isolated and efforts were taken to establish these in culture as monolayers. Phenotypic heterogeneity was evident in the cultures, hence we attempted to clone out morphologically distinct sublines of cells (Fig.1). This cloning resulted in the isolation of 65 distinct clones of these, 19 clones spontaneously immortalised clones have been successfully developed, cryopreserved and stored.

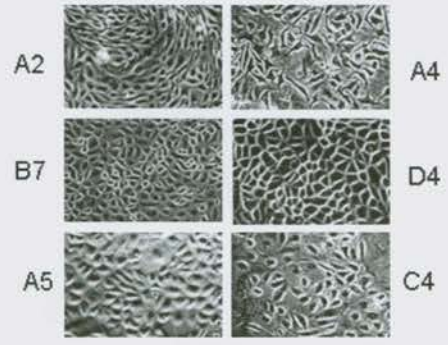

Fig. 1 Phenotypic heterogeneity in 29Asc Clones. The phenotypic heterogeneity was further investigated with respect to their growth characteristics (morphology and doubling time), tumorigenecity (in vitro soft agar and in vivo injection in nude mice), expression of surface markers (cytoskeletal components, EGFR, EMT markers, etc.).

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Role of the transcription factors Snail and Slug in ovarian cancer metastases

Amit Kumar

Collaborators:
C.B.Koppikar, Oncosurgeon, Jehangir Hospital and Medical Centre, Pune.

Abstract and background :

Epithelial mesenchymal transition (EMT) is an integral phenomenon in wound healing of normal ovarian epithelia as well as in the progression of ovarian carcinogenesis. Current understanding of ovarian metastasis regarding the molecular aspects leading to peritoneal dissemination from the primary tumor is limited. One of the major events in this process is the loosening of cell-cell contacts of the transformed cells that facilitates their invasion to the surrounding tissues and vascular channels. Recent work in other cancers, has suggested that the transcriptional regulators SNAIL and SLUG are important effectors of the process of EMT and metastasis.

Aim:

Investigation of Snail and Slug in ovarian cancer metastases

Work achieved:

Mouse snail and slug cDNA were transfected into the ovarian cancer cell line SKOV3 and clones were selected. As seen in Fig. 4A, RT-PCR analysis revealed a decrease in expression of E-cadherin, cytokeratin18 and increase in expression of Vimentin mRNA in the SKOV3 clones expressing mSnail exogenously (A2 and A12). However, the expression of endogenous Slug was unaffected in these clones. In case of SKOV3 cells expressing mSlug (Fig. 4B), RT-PCR analysis revealed a similar decrease in expression of E-cadherin and Cytokeratin18 mRNA in both stable and transient transfectants, while vimentin was upregulated only in the stable clone. Surprisingly, endogenous Snail mRNA was up-regulated in the stable mSlug clone B9. These results indicate a definite role of both Snail and Slug in mediating EMT in ovarian carcinoma.

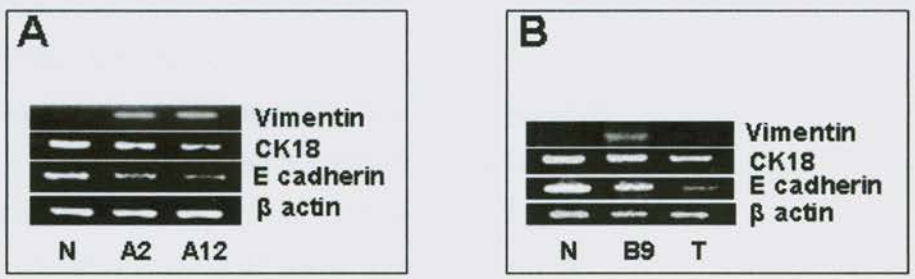

Fig. 3. RT-PCR analysis of BMT markers in mSnail and mSlugc cDNA expressing clones of SKOV3 A) N-Normal SKOV3;A2 & A12 Stable transfectants of SKOV3 expressing mSnail. B) N-Normal SKOV3;B9-Stable transfectant of SKOV3 expressing mSlung; T-Transient transfectant of SKOV3 expressing mSlug. β -actin mRNA is the internal control.

Future work:

The isolated clones are further being assessed for enhanced also tumorigenecity and metastases (if any). Further elucidation of the various pathways involving Snail and/or Slug will also be undertaken.

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Establishment and characterization of cell lines from human genetic mutants

Neeti Sharma, Avinash Mali, technician

Collaborators:
U.V.Wagh, IRSHA, Bharati Vidyapeeth, Pune, Jyoti Sharma, Bharati Hospital, Pune.
K. Prabhakara, CDFD, Hyderabad

Aims:

Enrichment of the NCCS Cell Repository with immortalised cell lines from human genetic mutants

Work achieved:

Periferal blood samples were obtained with IEC clearance, from human volunteers and clinically identified patients of specific syndromes / cytogenetic abnormalities and primary cultures were established and immortalised. The following cell lines have been established at NCCS. Approximately 10 vials of each have been cryopreserved and are being stored in LN2 containers.

Cell Line	Syndrome / Genetic Variation
GM7LBL	Balanced translocation 46XX t(2:8:10)
GM9aLBL	Balanced translocation 46XX t(11:21)
GM9bLBL	Balanced translocation 46XY t(11:21)
GM10LBL	Pericentric inversion in chromosome 4
GM11LBL	Balanced translocation 46XX t(10:12)
GM12LBL	Wilson's disease
GM15aLBL	Sickle cell disorder
GM15bLBL	Mother of child with sickle cell disorder
GM16LBL	Child with severe liver dysfunction at birth; suspected chromosomal rearrangements
GM17LBL	Cleft plate and bilateral cleft palate
GM18aLBL	Factor XIII Deficiency
GM18bLBL	Mother of child with Factor XIII Deficiency
GM21aLBL	Duchenne Muscular Dystrophy
GM21bLBL	Mother of child with Duchenne Muscular Dystrophy

Future work:

Studies on the Rubinstein-Taybi syndrome have been initiated and will be continued with the cell lines GM1LBL and GM3SF (established in the previous year).

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Structure-function analysis of eukaryotic cells: analysis of epithelial-mesenchymal transition using in vitro models.

Madhura R. Vipra, technician

Abstract and background:

Epithelial-mesenchymal transition (EMT) plays a central role in many physiological processes such as embyogenesis, wound healing and pathological processes such as tumor invasion and metastasis. Epithelial cells lose their cohesive nature and are converted into individual motile fibroblastic cells. EMT is associated with loss of epithelial features and gain of mesenchymal characteristics.

Role of EMT has been increasingly accepted in the tumor metastasis as more and more studies have reported EMT in variety of tumors such as bladder carcinoma, breast carcinoma, pancreatic cancer, uterine carcinoma and nasopharyngeal carcinoma. EMT represents the first stage in metastatic progression of tumor cells.

Many growth factors are thought to be involved in the acquisition of cell motility. Growth factors that are essential for tissue repair and morphogenesis, also play a role in invasion and metastasis of the tumor cells. Growth factors that have been shown to induce EMT both in vivo and in vitro include scatter factor (HGF), epidermal growth factor (EGF), transforming growth factor TGFβ) etc. Each factor induces a particular transduction system and affects motility of cells but the net effect seen is a consequence of interaction of various transduction systems.

Aims:

To look for epithelial-mesenchymal transition like changes in cervical carcinoma cells.

To analyze migration and invasion capacity of cells in response to growth factors.

To look for the markers of invasive capacity of the cells.

To study the cell cycle pattern of the cancer cells when induced to migrate.

Work achieved:

We have reported EMT like event in cervical carcinoma cells, SiHa, in response to EGF. EMT in SiHa was characterized by enhanced migration and change in cell shape. Cells typically underwent EMT, associated with acquisition of invasive capacities. A synergistic effect with serum was noted. EMT is associated with metastasis and characterised by acquisition of invasive potential by cancer cells. Invasive potential of SiHa cells was studied using Boyden chamber assay. We modified this assay to analyse live cells during invasion through Boyden chamber. This was the first report of its kind. Filter was coated with ECM gel and cells were seeded on the upper chamber in presence of EGF and the lower chamber was filled with FCS and EGF. Cells were stained live with vital stain Hoechst 33342 and observed during incubation period to assess the cell invasion. Cells were then observed at different levels in the gel as well as the filter using invert fluorescence microscope. Under phase contrast, cells were not very clearly visible. However, Hoechst 33342 fluorescence images from the nuclei were sharper and clearly defined, locating the cells at various focus levels. This modification also revealed cells that have traversed through the gel but not yet

reached the lower side of the filter, a significant fraction not included in the existing methods of visual counting. We further tested this method by image analysis and confocal laser scanning microscope.

SiHa cells exhibited cell shape change both in 2-D and 3-D culture condition in presence of EGF. In ECM gel assay, SiHa invaded the surrounding gel and the colonies spread through the length of the gel. Cell shape of the invading cells was clearly fibroblastoid. This assay further confirmed the invasive behaviour especially so in presence of EGF and the phenotype change in SiHa cells.

Analysis of conditioned medium form SiHa cells induced to migrate and invade in the presence of EGF showed induction of matrix metalloproteinases both MMP-2 and MMP-9. Western blotting further confirmed the induction MMPs by SiHa cells.

We further carried out tests to confirm the role of EGF in induction of migration and invasion. To determine the signaling pathways that contribute to EGF induced EMT, we examined the ability of pharmacological agents to block the changes in cell morphology and migration. We found that Wortmannin, a synthetic inhibitor of the PI3K, blocked morphological transition and migration of SiHa cells in wound closure assay. Wortmannin also blocked EGF induced invasion by SiHa cells in ECM gel assay (Figure 1). PI3K is known to be involved in the regulation of EGF mediated transcription and seems to be involved in induction of migration and invasion in SiHa.

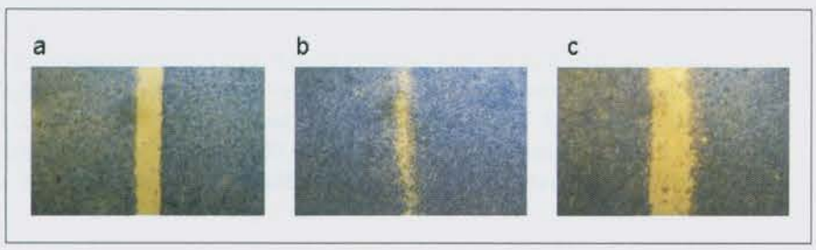

Fig. 1. Inhibition of EGF-induced migration by Wortmannin. (a) 0 hours post wound; (b) 24 hours post wound in prsence of EGF (20 ng/ml); (c) 24 hours post wound in presence of EGF (20 ng/ml) and wortmannin (10 nM).

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Molecular mechanisms behind the dose dependent differential activation of MAPK pathways induced by TGFβ1 in hematopoietic cells

Anuradha Vaidya
George Fernandes, technician

Abstract:

Transforming Growth Factor beta (TGFβ) controls a wide range of cellular responses including cell proliferation, lineage determination, differentiation, apoptosis etc and figures prominently in animal development. It is considered as a pleiotropic factor because it can exert a positive or negative effect on various cellular processes depending on developmental stage of the target cell, its microenvironment and also its biochemical make up. It has been shown to have a strong inhibitory effect on hematopoietic stem cell proliferation and differentiation. We have earlier shown that TGFβ1 exerts a bidirectional effect on hematopoietic cell proliferation as a function of its concentration (Kale 2004). Though it acted as an inhibitor at high concentrations, at low concentrations it stimulated the stem/progenitor cells. We also provided evidence that the differential activation of Mitogen Activated Protein Kinase pathways was responsible for the observed bi-directional effect. In the present study we examined the molecular mechanism behind this phenomenon. We observed that the high inhibitory concentrations of TGF β 1 induced a strong phosphorylation of SMAD 3 and also activated stress kinase related transcription factors namely c-Jun and ATF-2. On the other hand low stimulatory concentrations acted in SMAD 3 independent pathway and activated STAT proteins.
Our results clearly show that differential activation of signal transduction pathways by TGFβ1 as a function of its concentration underlies its bi-directional effect on hematopoietic cells.

Aims:

To understand the molecular mechanism behind the dose dependent differential activation of MAPK by TGFβ1 in hematopoietc cells.

Work achieved:

Bidirectional effect of TGFβ1 on the growth of KG1 a cells: In our earlier experiments we had reported that low concentrations of TGFβ1 induced p44/42 MAPK activation while high inhibitory concentrations induced p-38 activation in KG1a cells and also its bi-directional or biphasic effect on colony formation (CFU) of hematopoietic cells as a function of its concentration. We had observed that low TGF induced stimulation of CFU while high TGF exhibited the classical inhibitory effects. In this set of experiments we examined whether TGFß1 has a similar bidirectional effect on growth of KG1a cells.

We incubated serum starved KG1a cells in complete medium (IMDM + 20% FBS) supplemented or not with various concentrations of TGFβ1 as indicated. Viable cell count was taken after every 24 hours by Trypan Blue dye exclusion method. It was observed that the cells in all sets were >97% viable. The low concentration of 10 pg/ml induced an increased proliferation of KG1a cells while high concentrations of 10 ng/ml induced inhibition (Fig 1). The concentrations of 20 pg/ml and 5ng/ml exhibited intermediate curves between 10 pg/ml-control and 10ng/ml-control respectively (data not shown).The results indicated that the bidirectional or biphasic effect of TGFβ1on CFU formation of HSPC was also reflected in cell line model making KG1 a suitable model system to elucidate down stream effects of TGFβ1 in hematopoietic cells as a function of its concentration.

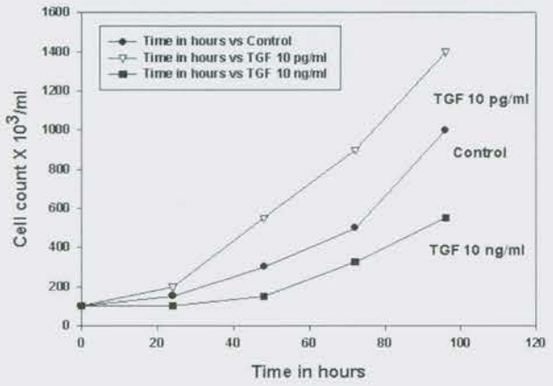

Fig. 1. Dose dependent bi-directional effect of TGF β1on the growth of KG1α cells: Serum starved KG1α cells were seeded in complete medium at a density of 1 X 10^5 cells/ml in 35 mm dishes and TGFβ1 was added to the dishes at indicated concentrations. Viable cell count was taken after every 24 hours. Data for only control, 10 pg/ml TGF β1and 10 ng/ml TGF β1 have been shown here for simplification. Similar results were obtained in three independent experiments.

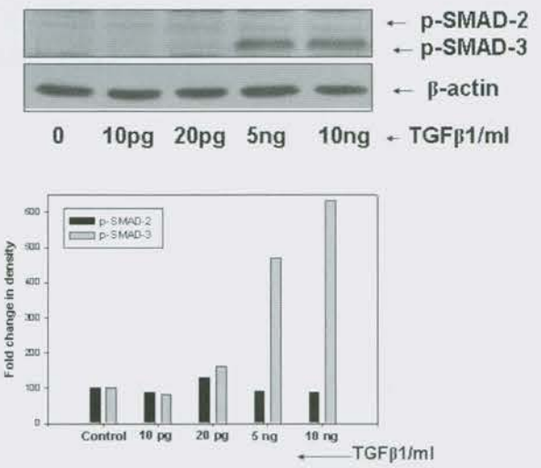

Fig. 2. SMAD3 is specifically activated by high concentrations of TGFβ1: Serum starved KG1 cells were treated with various doses of TGFβ1 for 2 hours. Western blot experiment was carried out with the lysate prepared from these cells as described. The blot was sequentially probed with antibodies to p-SMAD 2/3 and β-actin. The densitometric values of p-SMAD 2/3 were normalized with those of β-actin. Percent change was calculated by taking value of control lane as 100%.

Signaling mechanisms induced by high concentrations of TGFβ1 are mediated through SMAD 3: SMAD proteins are important mediators of signaling mediated by TGF β family members. It is also known that SMAD 2 and 3 are specifically involved in TGFβ1 pathway. We, therefore, looked for the phosphorylation status of these proteins in KG1a cells in response to various concentrations of TGFâ1. The serum starved KG1a cells were treated with TGFβ1 for 2 hours and the cells were lysed in RIPA buffer. Equal amount of proteins were separated by electrophoresis and were transferred to PVDF membrane. The membranes were then probed with anti p- SMAD 2/3. The actin levels were used as loading controls.

It was observed that high concentrations of TGFβ1 namely 5ng and 10 ng per ml specifically induced a strong phosphorylation of SMAD 3 (Fig 2). The increase was also dose dependent as at 10 ng/ml the level of phosphorylation was even higher (632 % of control) than that seen at 5ng/ml (471 % of control). At 20 pg/ml concentration a marginal increase in phosphorylation of SMAD 3 was seen (161 % of control) while at 10 pg/ml concentration there was a slight decrease (81% of control). SMAD 2 was not phosphorylated by any of the concentration used at least at 2 hours after stimulation with TGF β1.

The results clearly indicate that high concentrations of TGFβ1 induce a strong and sustained phosphorylation of SMAD3.

Dose dependent enhancement of TAB1-TAK1 binding: TAB 1 and TAK 1 play an important role in TGFβ1 mediated signaling. Upon TGFβ1 stimulation the TAB1 protein is thought to be activated, an event that results in its binding to the serine/threonine kinase domain of TAK 1. We, therefore, assessed the levels of TAB1 bound TAK1 in KG1a cells treated with different doses of TGFβ1. Equal protein lysates were immuno-precipitated with anti-TAB-1 antibody. The precipitate was run on 12.5% SDS-poly-acrylamide gel and separated proteins were electrically transferred on PVDF membrane. The membrane was probed with anti TAK 1 antibody. The levels of immuno-precipitated TAK1 with anti TAK1 antibody from identical protein samples were considered for normalization of values.

As seen in Fig 3 (panel a and d) increasing levels of TAK1 were co-immuno precipitated with anti TAB1 antibody with increasing concentration of TGFβ1 (280 and 281% of control for 5 and 10ng/ml respectively vs. 195 and 191% of control by 10 and 20 pg/ml respectively). The TAB 1 bound TAK was found to be phosphorylated on serine residues when the blot was reprobed with anti-p-Serine antibody (Fig 3 panel b). The total amount of TAK 1 protein was similar in all sets as seen in the blot of TAK 1 IP probed with anti TAK 1 antibody from equivalent

samples (Fig 3 panel c). The results indicate that the extent of association of TAB1 and TAK 1 may be dependent on the levels of TGFβ1 present in the system. Since TAK 1 acts upstream of stress kinases the result indicates that perhaps increased activity of TAK1 may be responsible for activation of stress kinases seen in response to high TGFβ1 concentrations.

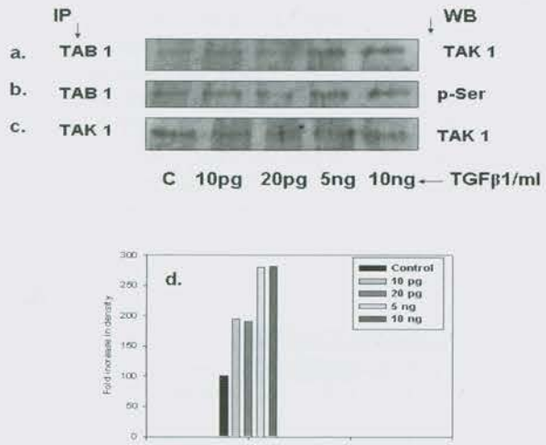

Fig. 3. Dose dependent increased association of TAK 1 and TAB 1 by TGFβ1: Cell lysate of KG1 cells treated or not with TGFβ1 was subjected to immuno-precipitation with either anti TAK 1 or anti TAB 1 antibodies using equivalent protein aliquots. Immune complexes were captured on Protein A/G sepharose and the beads were boiled in 2X sample buffer. The proteins were resolved on 12.5% acrylamide gel and blotted on PVDF membrane. The blots were sequentially probed with anti -TAK1, anti TAB 1 and anti p-ser antibodies. Densitometric calculations were done as described by taking TAK 1 IP-TAK-1 probing as normalizing values.

Activation of stress kinase related transcription factors by high doses of TGFβ1: ATF2 and c-Jun are two important transcription factors activated down stream of stress kinases. We looked for the phosphorylation of these two factors in response to various concentrations of TGFβ1. As seen in Fig 3 (a, b) there was a TGFβ1 dose dependent increase in the levels of these factors supporting the earlier data. A strong phosphorylation of ATF 2 was induced by 10 ng/ml of TGFβ1 (553% of control).

Since c-Jun is activated by both stress kinases namely p38 and SAPK/ JNK we examined the levels of p-SAPK/JNK in KG1 cells as a function of TGFβ1 concentration. As seen in Fig 4c the levels of p-SAPK/JNK did not increase to a very great extent by high TGF (nearly same as control by 5ng/ml and (141% of control by 10 ng/ml TGFβ1). In our earlier experiments the levels of phospho-p-38 in high TGF treated cells was much higher (165 % and 255 % of control by 5 and 10 ng/ml TGFβ1 respectively). The data indicate a predominant role of p38 MAPK in the TGFβ1 mediated activation of ATF-2 and c-Jun with a minor contribution from SAPK/JNK.

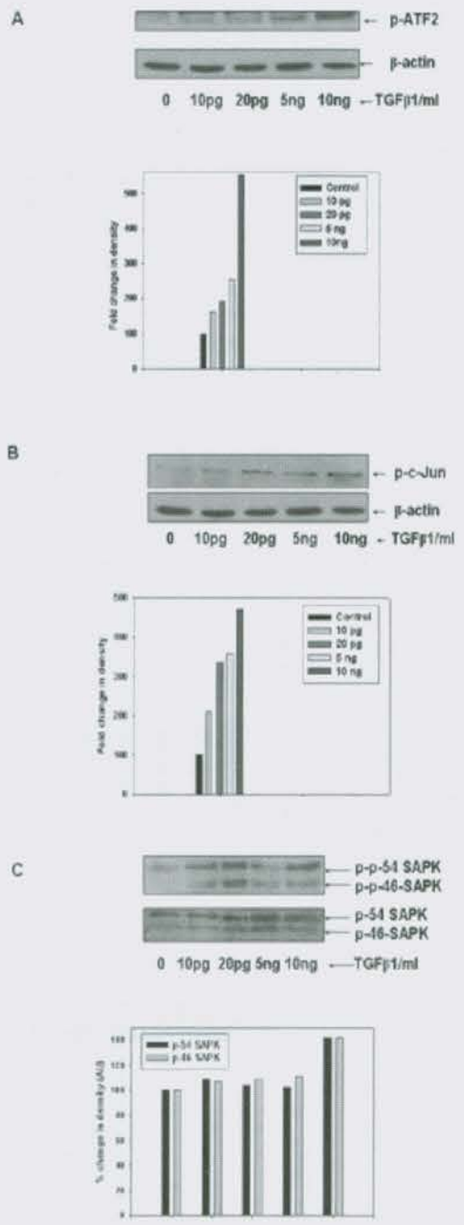

Fig. 4. TGFβ1 at high concentrations activates stress responses in hematopoietic cells: Blots of protein lysates prepared from KG1-a cells exposed to various concentrations of TGFβ1 were probed with antibodies to p-ATF2 (A), p-c-Jun (B) and p-SAPK/JNK (C). The densitometric values for p-ATF2 and p-c-Jun were normalized against those of β-actin. Values for native SAPK/JNK were used for normalization of p-SAPK/JNK activation. Percent change was calculated by taking control values as 100%.

Enhanced phosphorylation of STAT proteins by low concentrations of TGFβ1: STAT proteins play an important role in the proliferation and differentiation of hematopoietic stem cells in response to growth factor and cytokine signaling. These transcription factors also form down stream targets of various signaling pathways. Our results show that TGFβ1 has a dose dependent effect on proliferation of hematopoietic cells and perhaps this was a result of different activation of MAPK pathways in these cells. In this set of experiments we examined whether phosphorylation of STAT proteins namely STAT 1,2,5 and 6 were differentially modulated by TGFß1.Western blot analysis was carried out as described before and the blots were sequentially probed with antibodies to various phosphorylated forms of STAT proteins. Levels of β-actin in respective lanes were used to normalize the values.

It was observed that STAT 1/β was constitutively phosphorylated in KG1a cells and the levels were not affected by various doses of TGFβ1 (Fig 5a). The levels of STAT 3,5 and 6 were, however, affected by TGFβ1 in a dose dependent fashion. Levels of phospho-STAT 3 were enhanced (132% of control) by 10 pg/ml concentration and showed a decrease with increasing concentrations of TGFβ1. (Fig 5b) Levels of phospho-STAT 5 were increased by low TGF (Fig 4c; 236% and 196% with 10 and 20 pg/ml TGFβ1 respectively) while the levels were similar to controls at higher concentrations. Lower concentrations also induced enhanced phosphorylation of STAT 6 in KG1-a cells (Fig 4d; 154% and 124% with 10 and 20 pg/ml TGFβ1 respectively) while higher concentrations resulted in decease in the phospho-STAT-6 (67% and 54% of control for 5 and 10 ng/ml TGFβ1 respectively).

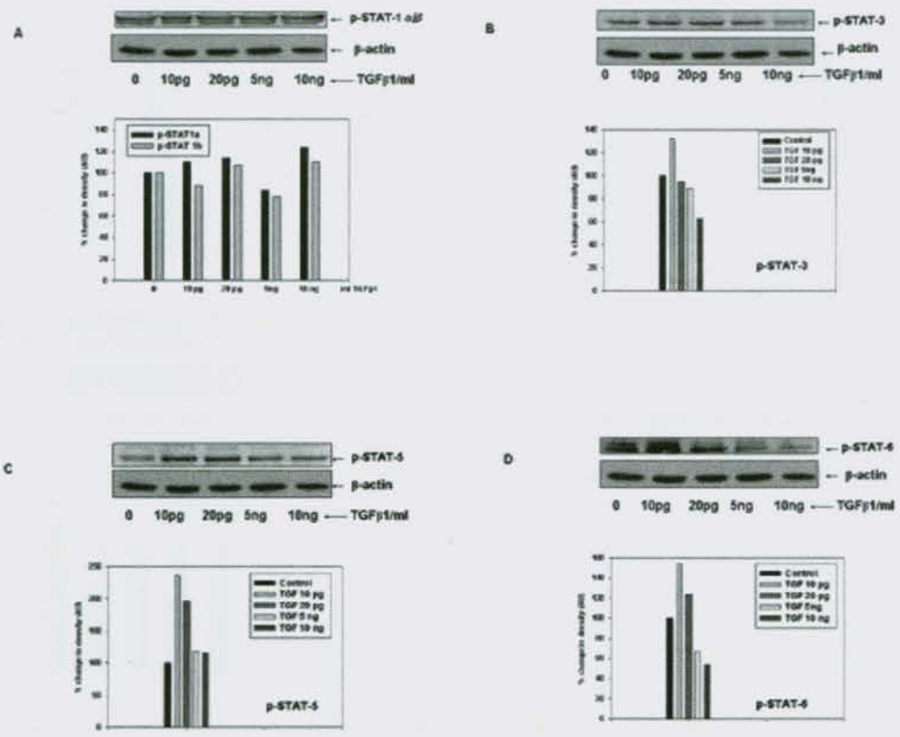

Fig. 5. Differential modulation of STAT proteins by TGFβ1 as a function of its concentration the blots of KG1a cell lysates exposed to various concentrations of TGFβ1were sequentially probed with antibodies to various phosphorylated STAT proteins as indicated Signals obtained with b-actin were taken for normalization and % change was calculated by taking control value 100% (A) p-STAT 1 a/β (B) p-STAT 3 (C) P-STAT 5 and (D) p-STAT 6.

The data show that activation of STAT proteins is also modulated by TGFβ1 in a concentration dependent manner and low concentrations of TGFβ1 may act in concert with cytokine signaling to give better proliferation of hematopoietic cells.

Future work:

We propose to carry out analysis of signaling pathways involved in various physiological processes of hematopoietic cells like proliferation, differentiation, apoptosis, migration etc.

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Studies on nuclear localization of eNOS in mammalian cells.

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Collaborators:
L. C. Padhy, TIFR, Mumbai

Abstract and background:

Nitric oxide (NO) is one of the gaseous signaling molecules and has been shown to play important regulatory role in the physiological processes like blood pressure, regulation of vascular smooth muscle tone, proliferation, cell-mediated immunity, and inflammation. We have demonstrated that NO plays a deterministic role in the regulation of hematopoietic stem cells. We made an interesting observation that eNOS was localized to the nuclei of stromal cells in addition to its usual known localization in the plasma membrane caveolae and ER-Golgi. We then examined the issue as to whether the presence of nuclear eNOS is a specific property of marrow derived stromal cells or is it a general phenomenon using a panel of different cell lines such as HEK293, CHO, MCF-7, K562 and KG1a. We observed that the nuclear eNOS was present in all the cell lines we examined. These results indicated that nuclear eNOS may be a general property of mammalian cells and may have a role in cellular physiology in a much wider context.

Aims:

1. Studies on nuclear eNOS (NOS III), especially the mechanisms responsible for its targeting to nucleus and to specific subnuclear regions.
2. Identification of the role of the nuclear NOS in cellular physiology.
3. Molecular mechanisms of nitric oxide mediated signaling in mammalian cells.

Work achieved:

Subnuclear localization of eNOS:

Using immuno-fluorescence studies for eNOS protein in various cell lines, we show that it is present in the nucleus and is seen specifically in the nuclear speckles. To see its interaction with other proteins we carried out double staining experiments. The pair of secondary antibodies was carefully chosen to avoid interference. Scanning was done in sequential mode to avoid bleed through and this was also confirmed by single stained slides and other controls where one of the primary antibodies was eliminated. In these studies carried out so far indicated that eNOS found to be associated with spliceosomes and colocalized with splicing factor (Fig1). We are carrying out co-immuno precipitation experiments to support these observations.

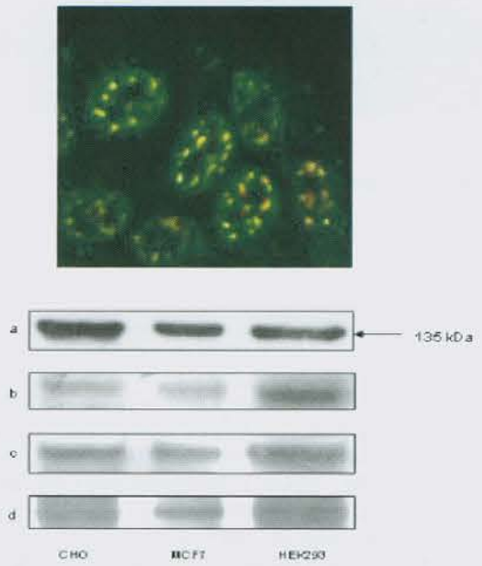


Fig. 1. & 2. Double immunofluorescence of nuclei isolated from MCF-7 cells showing colocalization of eNOS (green) and splicing factor (red). Western blot analysis of nuclear lysates of CHO, MCF 7, HEK 293 separated on 6% SDS-PAGE and probed with the following. A) anti N-terminal eNOS,b)anti c-terminal eNOS,c)anti middle antigen eNOS and d)phosphorylated (Thr 495) form of anti eNOS. The band indicates 135 kDA eNOS.

We then wanted to examine the issue whether the nuclear eNOS is an intact protein or not by carrying out western blot analysis of the protein isolated from the nuclei of various cells. The lysates prepared from nuclei of MCF-7, CHO and HEK cell lines were loaded on 6% poly acrylamide gel and the separated proteins were transferred electrically to PVDF membrane. The blots were probed with antibodies to various regions of eNOS to detect the protein using appropriate secondary antibodies tagged to HRP. The signal was detected by chemiluminescence kit (Cell Signaling) and captured on Konica X-ray films. It was observed that a 135 kD band of eNOS protein was detected by all three antibodies used indicating that the eNOS present in the nucleus is an intact protein (Fig 2).

Nuclear eNOS is enzymatically active:

In order to show that the nuclear eNOS detected by immunological methods is indeed an active enzyme, we studied eNOS activity using a fluorescent dye called DAF-FM. The isolated nuclei from MCF-7 cells were incubated with buffer containing various cofactors and substrate L-arginine in a black micro-titer 96 well plate. Then the fluorescent dye was added. In case of wells where nuclei have been added there is increase in fluorescence (excitation wavelength 485 and emission at 510 nm) as compared to the blank (DAF + Buffer). This clearly shows that enzymatically active molecule is present in the nucleus. Similar experiment was carried out with different protein concentrations of MCF7 nuclear lysate. It was observed that as the protein concentration increased there was proportional increase in fluorescence (data not shown). In the wells where a known concentration of nuclear protein was added along with an NO scavenger (CPTIO), there was considerable decrease in fluorescence indicating the specificity of the activity (Fig. 3 and 4).

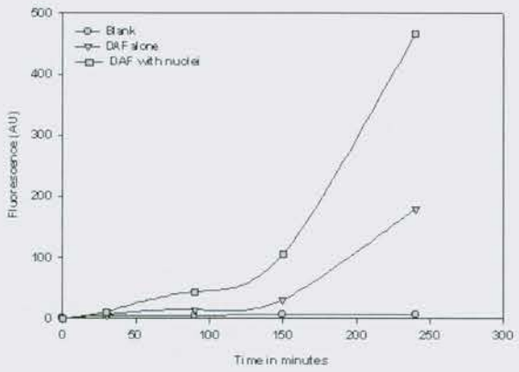


Fig. 3. MCF 7 nuclei incubated with DAF-FM show increased fluorescence indicating NOS activity.

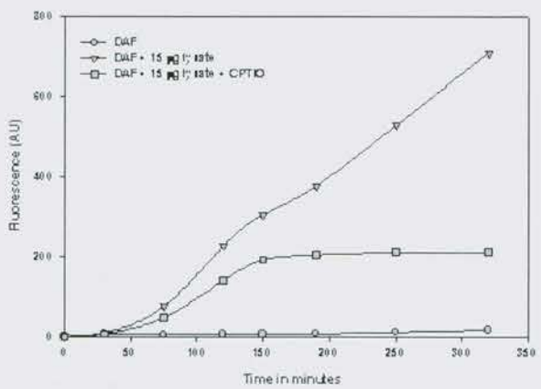


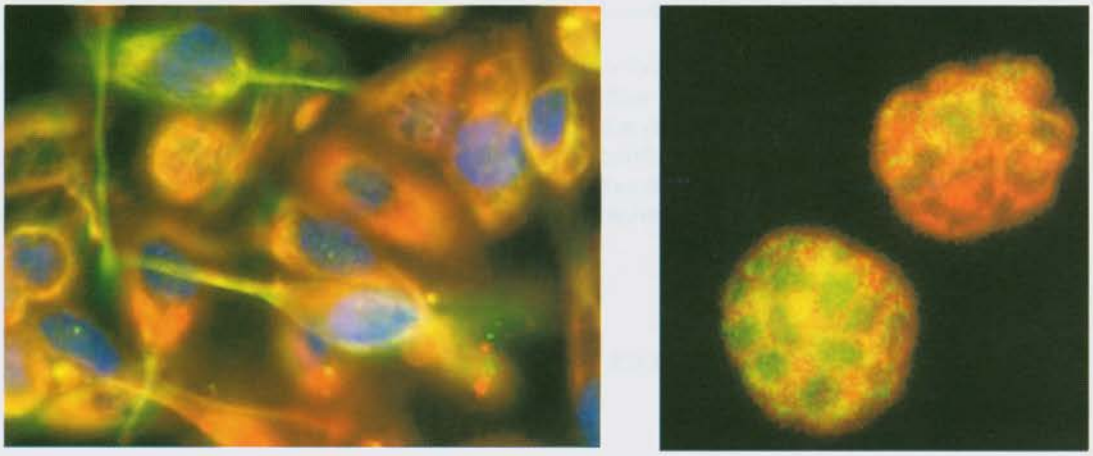
Fig. 4. Fluorescence of DAF-FM in presence of nuclear lysate of MCF-7 cells.

The results indicate that the nuclei of mammalian cells have functionally intact nuclear eNOS in steady state condition and it is associated with sub-nuclear structures like spliceosomes. Since it was found to co-localize with splicing factor we speculate that it may have some regulatory role in splicing mechanism. We are investigating this issue further.

Future work:

We propose to investigate the mechanism of nuclear localization of eNOS, its exact role in cellular physiology and its regulation by cellular signaling machinery.

One of the 19 clones viz. 29-A2 was found to be tumorigenic in nude mice, even at early passages. However, another clone viz. 29-A4, that earlier was untransformed, progressed to a transformed phenotype at later passages, that was more aggressive than the A2 clone. These two clones also have retained the capacity to form spheroids in culture and express both Cytokeratin 18 as well as Vimentin in monolayers and embryoids (Figs. 2 and 3) that reflects on their mesothelial origin.



Figures 2 and 3 : Cytokeratin (red) and Vimentin (green) Immunofluorescence in A4 monolayers and embryoids respectively. Nuclei stained with DAPI (blue).

Future work:

The in vitro experimental system thus developed is one of its kind developed so far for ovarian cancer and hence will be used in studies relating to the origin, development and progression of ovarian carcinoma. The heterogeneity seen in the various clones will also being analysed at the molecular level.

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Regulation of Caveolin-1 expression in breast cancer-cells by Estrogen receptor mediated signaling

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Collaborators:
L. C. Padhy, TIFR, Mumbai

Abstract and background:

In recent years greater attention has been focused on the function of caveolae in the signal transduction. Caveolin-1, the scaffolding protein of caveolae was found to interact with a whole series of lipid-modified proteins that are involved in signal transduction processes. It is via this interaction that Caveolin-1 suppresses the functions of hetero-trimeric G-proteins, protein kinase C isoforms and eNOS etc. Proteins participating in cell proliferation (growth factor receptor [e.g.-EGFR], Src, tyrosinkinases, H-Ras) also are shown to accumulate in the caveolae.

There are reports that suggest that Caveolin 1 is involved in the negative regulation of the cell cycle. Since the continuous activation of signal molecules leads to cell transformation, their binding to Caveolin-1 might have a suppressive effect. The abundance of caveolae is an important characteristic of non-transformed terminally differentiated cell populations. In contrast there is a dramatic drop in Caveolin-1 synthesis and consequently, in the number of caveolae present in oncogenically transformed cells. In support of this observation it has been shown that the recombinant expression of Caveolin in various transformed cell-lines inhibits their growth, while the down regulation of caveolin-1 by the introduction of a caveolin-1 anti-sense vector induces tumor.

Earlier we observed that MCF-7 cell line expresses mRNA for all three isoforms of Caveolin, namely Caveolin 1, 2 and 3. However, the protein levels of Caveolin were quite low in comparison with the normal breast epithelial cells. The presence of estrogen receptor (ER) in these cells and also that of Caveolin 1, although at low levels, made this system ideal to examine the possible correlation of expression of these two molecules.

Aims:

Our aim of the project is to examine the regulation of Caveolin expression by estrogen receptor signaling. Our working hypothesis is that there is a reciprocal relationship between ER activity and Caveolin expression.

Work achieved:

Increased expression of Caveolin 1 after transfection of antisense ER constructs in MCF7 cells:

MCF7 (T1) & MCF7 (T2) stable cell line have been generated after transfection of ER antisense constructs in MCF7 cells (Courtesy Dr Indraneel Mittra, TMH, Mumbai). Increased Caveolin-1 expression was seen in transfected cells as compared to control (Fig 1). The results supported our working hypothesis. The transfected cells also showed increased phosphorylation of p44/42 MAPK. We are investigating the role of various signaling pathways involved in the regulation of Caveolin-1.

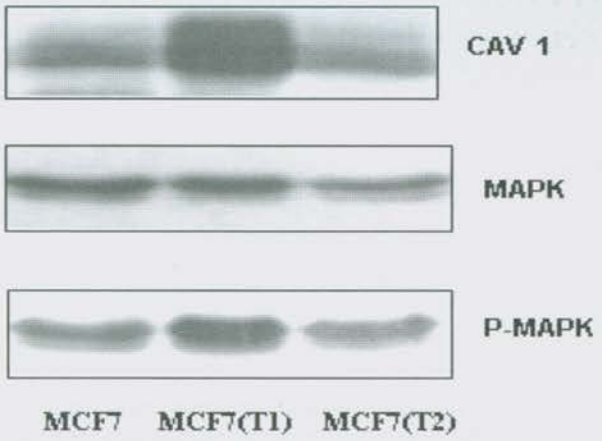

Fig.1. Higher expression of Caveolin-1 in MCF 7 cells transfected with antisense estrogen receptor constructs (T1 and T2).

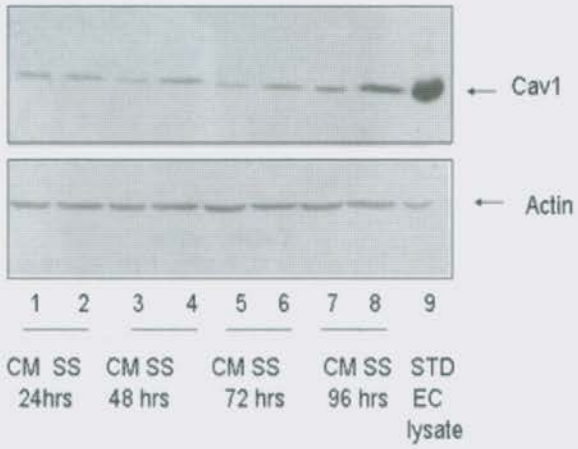

Fig. 2. Effect of serum starvation on Caveolin 1 expression in MCF 7 cells.
CM= complete medium wth serum
SS = Serum staved.

Effect of serum starvation on caveolin-1 protein expression: The MCF 7 cells were serum starved for different time period by culturing the cells in medium devoid of serum. After 24,48, 72 and 96 hrs cells were lysed and equal protein samples were resolved on 12.5% polyacrylamide gel. They were transferred on PVDF membrane and the blots were probed with antibody to Caveolin1. Levels of actin were used to normalize the values. As seen in the figure 2 levels of Caveolin-1 increase with time under serum starved conditions.

Future work:

Regulation of Caveolin-1 expression by modulators of estrogen signaling, cell cycle and cell signaling pathways will be examined.

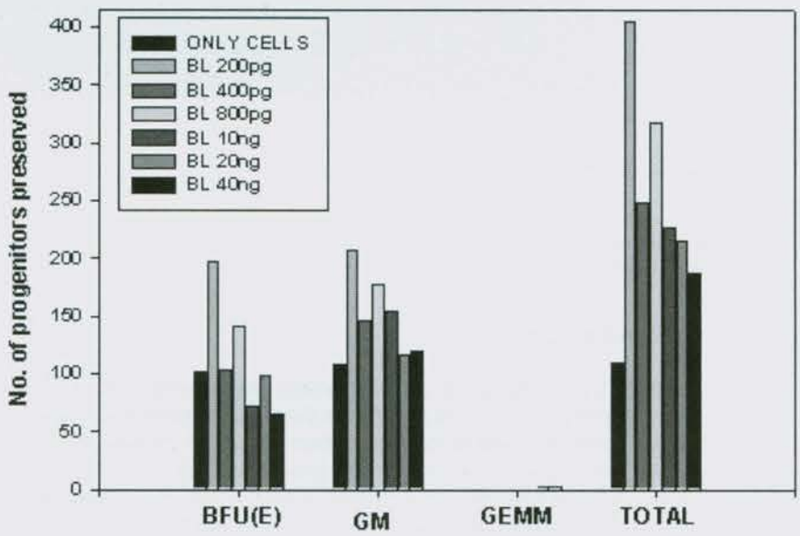

Fig 1: Effect of Banna lectin (BL) on the preservation of various progenitors.

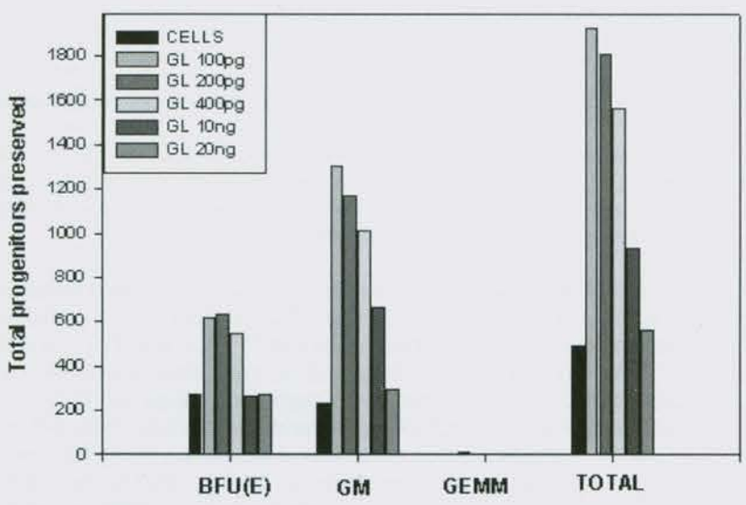

Fig. 2. Effect of Garlic lectin (GL) on the preservation of various progenitors.

Future work:

1. Examination of the activity of AL and DL using colony formation assays.
2. Use of LTC-IC assays to detect early stem/progenitors after incubation with lectins.
3. Identification of signaling pathways induced by these lectins in hematopoietic cells. Since the primary cells isolated from cord blood are not sufficient for biochemical analysis we are exploring the possibility of using TF1 cell line as a model system.

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Studies on cryopreservation of haematopoietic cells.

Lalita M.Sasnoor, SRF, Nikhat Firdaus Siddiqui, technician

Collaborator : Dr. V. P, Kale, NCCS

Abstract and background:

This is an ongoing project of NCCS. Here our aim has been to optimize the freezing protocols to get improved recovery of hematopoietic cells in terms of functionality and viability after freezing.

We have used antioxidant and membrane stabilizers as additives in the conventional freezing medium and tested its effect on in vitro and in vivo engraftment.

Aims:

1. To optimize freezing protocols so as to get improved recovery of haematopoietic cells in terms of viability and functionality.
2. To preserve quality of graft as close to the fresh cells as possible.
3. To study the quality of frozen cells by various in vitro and in vivo assays.

Work achieved:

Hematopoietic cells were frozen with or without a combination of catalase and trehalose as additives in the freezing medium. The revived cells were subjected to various assays to test homing properties of frozen graft.

The experiments on in vitro and in vivo homing of frozen hematopoietic cells were carried out .

FLT3 responsiveness:

FLT3 is an early acting cytokine and has been recently reported to play an important role in homing of hematopoietic cells to the bone marrow. We therefore studied the resonsiveness of frozen hematopoietic cells to this cytokine by in vitro CFU assays using methyl cellulose cultures. Figure one summarizes the results of one representative experiment. Here we report increased responsiveness by cells (MNCs/CD34+ cells) that were frozen with a combination of catalase and trehalose in the conventional freezing medium . Cells that were frozen without additives in the conventional freezing medium served as control.

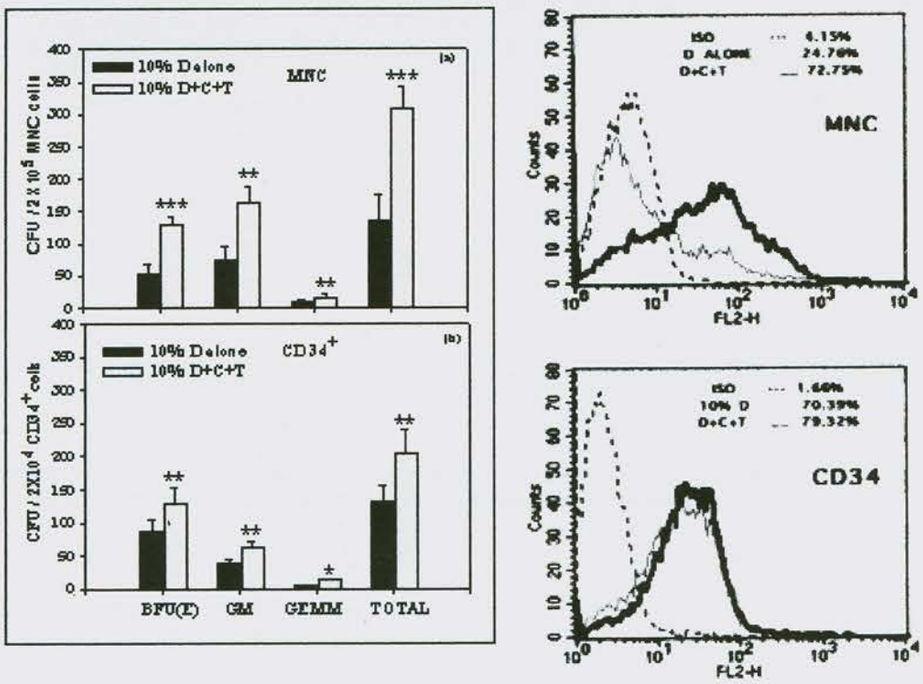

Fig.1. Responsiveness of frozen MNC/ CD34$^+$ cells to FLT-3 increases in the test cells: MNCs/CD34$^+$ cells were plated in methyl cellulose cultures for CFU assay. Colonies formed were scored on 14th day. The culture plates contained optimum concentrations of GMCSF, IL-3, EPO and SCF and sub-optimal concentration of FLT-3. In both MNC(a) and CD34$^+$ (b) cells colonies formed by test groups were higher in number than the control groups **p=0.01, ***p=0.001. FACS analysis showed higher expression of FLT3R on MNCs (c) and CD34$^+$ in the test group (d)

Adhesion of frozen cells to ECM proteins:

KG1a cells were used as model of hematopoietic cells .These were frozen with or without additives to conventional freezing medium containing 10% DMSO. The revived cells were labeled with radioactive chromium Cr^{51} and allowed to adhere to ECM coated plates for 2hrs.The radioactivity in the adherent and non adherent fraction was measured separately and the percent adherence was calculated by standard formula. It was found that when cells were frozen with additives they showed significantly increased adhesiveness to all ECMs tested i.e. fibronectin, vitronectin, collagen I and collagen IV (Fig. 2).

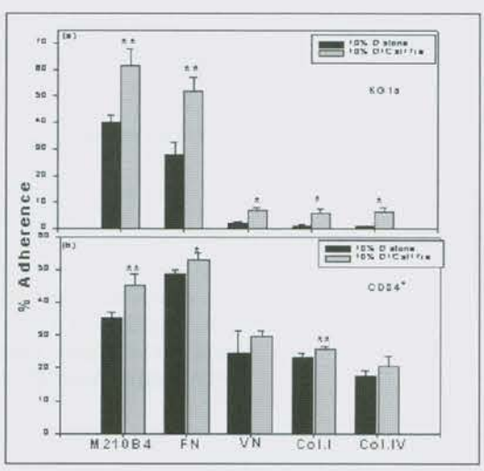

Fig. 2. Increased adhesion of KG1a (a) cells and CD34+ cells(b) frozen with additives than those without additives to M210B4 and ECM: KG1a cells/CD34$^+$ were frozen with or without catalase and trehalose in the conventional freezing medium. Revived cells were labeled with Cr^{51} and allowed to adhere for 2hrs at 37^0 C on M210B4 layer or plates coated with ECM proteins- FN, VN, Col I, and Col IV. Radioactivity in the adherent and non-adherent fraction was measured as described in methods. % adhesion of test KG1a cells was more significant than % adhesion of control cells on both M210B4 and ECM *p=0.05, **p=0.01. (a)The increase in adhesion of test CD34+cells was statistically significant on M210B4, FN and Col I. (b).

Chemotaxis assay:

Migration of frozen CD34$^+$ cells in a transwell chamber assay in response to SDF-1a:

A direct link between in vitro motility of CD34$^+$ cells towards SDF-1α and in vivo stem cell function has been demonstrated. Thus the fraction of HSC which migrates in response to chemotactic cues of bone marrow microenvironment forms an important component of the graft material for successful transplantation procedure.

We therefore studied in vitro migration of frozen CD34$^+$ cells in response to SDF-1α by transwell migration assay to examine the issue whether the migration ability of early HSC population is better preserved in the medium supplemented with catalase and trehalose as additives. It was found that significantly higher number of cells ($p<0.01$) could be recovered from migrated compartment when the cells were frozen with additives than those frozen without additives, thus indicating that catalase and trehalose in freezing medium help to preserve the migration capacity of frozen CD34$^+$ cells towards SDF-1α.

In vivo experiments to study engraftment of frozen mouse bone marrow:

Mouse bone marrow cells were frozen with or without additives to conventional freezing medium. The revived cells were transplanted in irradiated syngeneic hosts. The engraftment potential of frozen cells was quantitated by various parameters like % survival, MNC recovery, neutrophil and platelet counts at different time intervals for 60 days. One representative data is depicted in Fig.3. The data is suggestive that additives improve the in vivo homing of frozen mouse bone marrow.

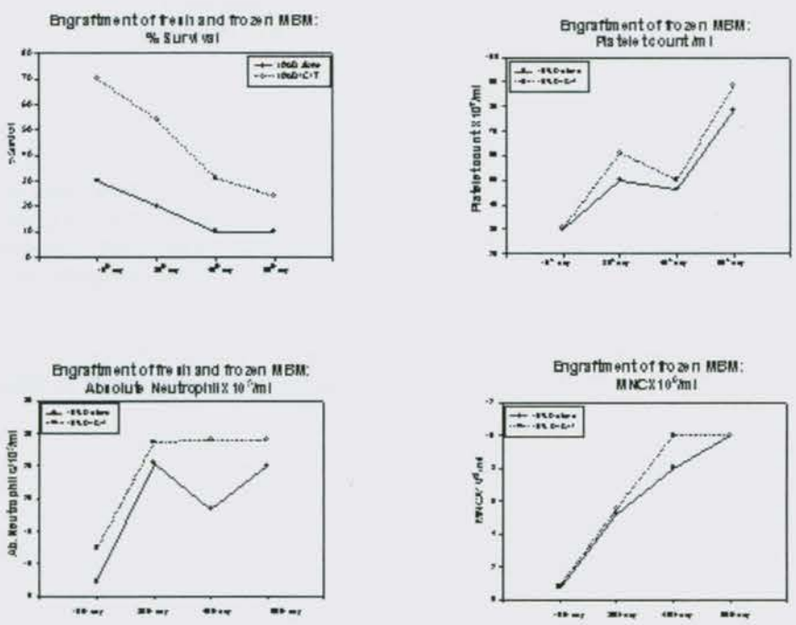

Fig. 3. Long term Engraftment of frozen mouse bone marrow cells: MBM was frozen with or without additives(catalase and trehalose) in freezing medium.The revived marrow was infused in irradiated syngenic host and engraftment was quantitated by parameters like % survival, neutrophil,platelet and MNC recovery at different time points. Results show better engraftment by cells frozen with additives as compared to control.

Future work:

1. To study by multiple parameters the role of apoptosis in damaging the cells during freezing and in vitro expansion of hematopoietic cells.
2. Attempt to prevent apoptosis during the above mentioned processes by addition of antiapoptotic agents and other stress relieving agents.
3. Standerdization of pre CFU-S assay using Swiiss albino mice. Repeating experiments on C57BL6 Ly5.1 and Ly 5.2 mice to detect engraftment chimera.

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Identification and characterization of lectins having stem cell preservation activity.

Ashwini Hinge

Collaborators:
A. Surolia, IISc, Bangalore., L. S. Limaye, NCCS

Research report:

Abstract and background: Hematopoietic Stem Cells (HSCs) are primitive type of cells that can either self renew or differentiate into mature functional blood cells. HSC are known to undergo rapid differentiation events in culture in the presence of various growth factors and thus ultimately get exhausted. In the absence of growth factors these cells wither off. In a recent report a lectin, named as FRIL, isolated from Dolichos lablab was found to have stem cell preservation ability. It was shown that this lectin preparation could preserve the stem cell activity in culture in the absence of growth factors. We, therefore, initiated the present project to screen lectin preparations having such activity. Such preparations would be useful not only in the clinical practices but also for laboratory investigations.

Aims:

To identify and to characterize lectins having stem-cell preservation activity.

Work achieved: We have selected following lectins viz. Dolichos lablab (DL), Banana lectin(BL), Artocarpus integrifolia lectin(AL) and Garlic lectin (GL) for the present study as they have mannose binding properties similar to FRIL. The lectins were isolated, purified and characterized in Prof. Surolia's lab.

The $CD34^+$ cells isolated from cord blood were incubated in serum free medium with or without the addition of lectins. The concentrations of lectin to be used were determined after testing their toxicity on TF1 cell line. The cultures were incubated at 37°C for 10, 20 and 30 days and at each time point cells were harvested and subjected to colony formation in methyl cellulose using a combination of growth factors (SCF,GM-CSF,IL3 20 ng/ml and Epo 2U/ml). Cells without any addition were used as negative control and cells incubated with FLT3L were used as positive control. The plates were incubated at 37 ^{0}C / 5% CO_2 for 14 days in a humidified atmosphere and then they were scored in terms of different colonies formed as BFU-E, CFU-GM and CFU-GEMM using standard morphological criteria. The number of progenitors preserved at the end of the incubation period was calculated by comparing to the values obtained with fresh cells.

Preliminary experiments carried out with BL and GL (used at 100-200 pg/ml) show that these lectins preserve the progenitor cells in culture for at least days. (Fig 1 and 2). The effect was more appreciable on GM and BFU-E type of progenitors. The formation of GEMM type of colonies was very poor. Further experiments to examine the effect on these early stem/progenitor cells by using long term culture system (LTC-IC) are in progress.

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Standardization of technology of bone marrow cryopreservation

Nikhat Firdaus Sidiqqui, technician

Collaborators:
R.L.Marathe, Haematology Head, Jehangir Hospital, Pune, S. G. A. Rao, CRI, Mumbai.

Abstract and background:

This technology was standardized at NCCS and transferred to AFMC. A programmable freezer was purchased and shifted to AFMC under this scheme. They are setting up the laboratory at AFMC and have carried out transplantations using frozen samples. However these samples were frozen in -80°C as they do not have liquid nitrogen facility. They are submitting proposal for installation of liquid nitrogen plant.

Aims:

1. Standerdization of technology of bone marrow cryopreservation
2. Transfer of technology to hospitals that need it.
3. Help the hospital staff in case of any trouble shooting.

Work achieved:

The technology of cryopreservation of bone marrow and Cord blood was standardized by NCCS and CRI and NIV and transferred to AFMC. From Oct 2002 till May2004 using this technology AFMC has cryopreserved 3UCB, 1BM, 6 Autologous MPBL 2Allogenic MPB samples.The samples were frozen at -80°C.The samples were used for transplantation of patients suffering from ALL, AML, CML, juvenile CML,Hodgkin's disease, non Hodgkin's lymphoma multiple myeloma.and germ cell tumor.

Future work:

Help and guide the AFMC, Transfusion department staff in case of any trouble shooting.

stable ES cell clones. The rational being during the differentiation of ES cells into cardiomyocytes the respective promoters should be active only in the specific cell types and in turn would activate the EYFP expression in cell type (cardiomyocytes) specific manner that should correlate with the endogenous cardiac gene expression. The stable clones were generated by G418 selection and induced to differentiate into cardiac lineage and the fluorescence was monitored under the microscope. These clones were characterized by rhythmic beating upon differentiation and the fluorescence was restricted to beating areas only and thus proving the authenticity of these clones (Fig. 2A). The cardiomyocytes specific expression of EYFP was further ascertained by immunocytochemical approach using a-actinin antibody. As could be seen in Fig. 2B the EYFP expressing cells were also a-actinin positive. We are currently engaged in manipulation of the culture conditions to enrich these myocyte populations and purify these by both genetic selection and fluorescence activated cell sorting (FACS). This would facilitate us to analyze the signaling cascade and differentially expressed gene expression profile during cardiomyocyte development.

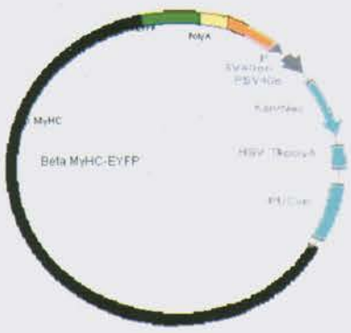

Fig. 1. The beta MHC-EYFP vector construct used to generate stable ES cell clones.

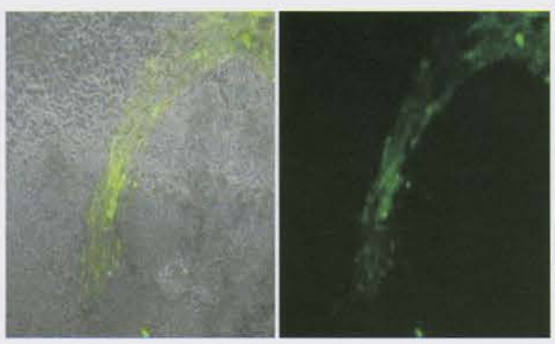

Fig. 2A. Expression of EYFP under the regulatory control of beta myosin heavy chain promoter in differentiating cardiomyocytes.

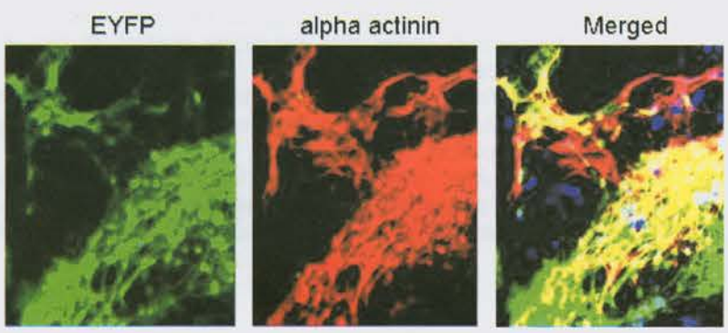

Fig. 2B. Immunostaining of cardiac specific proteins to characterize the stable clones.

Neurogenesis: In the neurodegenerative disorders like Parkinson's and schizophrenia the dopaminergic neurons are degenerated in the substantia nigra and the ventral tegmentum respectively. The conventional therapy using L-DOPA, however, has not been proved substantial in curing this disorder. Hence, the cell replacement therapy could be an ideal alternative to the conventional one placing these ES cell derived dopaminergic neurons into limelight for their use in therapeutic transplantation; as there exists a dearth in obtaining these neurons. In this regard, the present study focuses on (i) establishing the culture conditions for efficient generation of functionally active dopaminergic neurons from transgenic ES cell derived clones (ii) their purification and functional characterizations and (iii) exploring the therapeutic potential by transplanting those into the Parkinsonian model for treating Parkinsonism in future.

We have successfully generated a number of stable ES cell clones expressing the live reporter EGFP under the regulatory control of dopaminergic neuron

specific promoter tyrosine hydroxylase (TH). These clones upon differentiation into neural lineage did exhibit EGFP expression and further characterization of these cells was attempted by immunostaining with markers like dopamine transporter, Pitx3, Nurr1, D2R, MAP2 and TH etc. Monitoring the EGFP expression profile during differentiation revealed that the dopaminergic neurons started appearing after 3-4 days of plating and the optimum generation of these neurons was observed by 2 weeks of plating (Fig. 3) that remained constant there after without further increase in the number. The EGFP expressing cells were MAP2 positive indicating their neuronal identity and some of these cells were expressing Nurr1 (Fig 4A), the marker for dopaminergic neuron specified population and D2R (Fig 4B), the receptor present in the dopaminergic neurons. Preliminary attempt to analyze these neurons by reverse phase HPLC also showed dopamine release indicating their functional behaviour. We are currently trying to purify these neurons and these will be subjected to electrophysiological characterizations and transplantation studies.

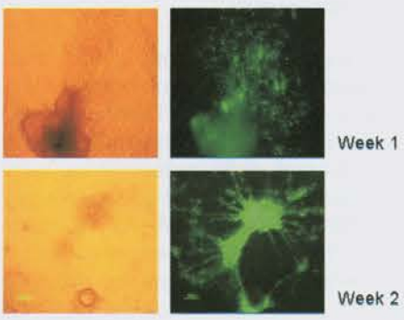

Fig. 3. EGFP expression profile differentiation of TH-EGFP ES cell clones showing optimum generation of dopaminergic neurons by 2nd week postplating.

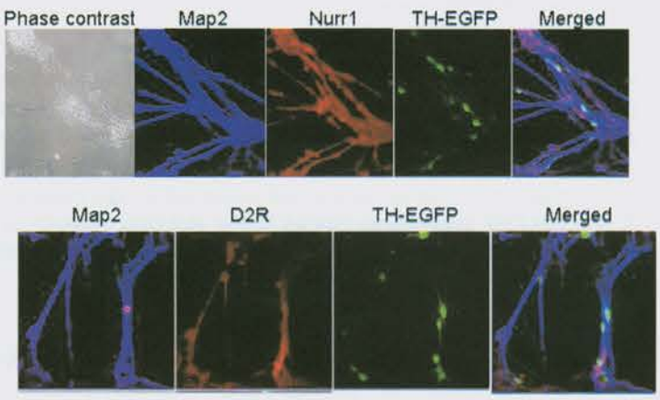

Fig. 4. The EGFP expressing dopaminergic neurons express (A) Nurr1 and (B) D2receptor (D2R).

Future Work:

1. Optimization of cardiomyogenesis from ES cells in vitro.
2. Purification of cardiomyocytes and investigation of their cardiac chamber specific expression pattern.
3. Investigation of differentially regulated gene expression profile during cardiomyogenesis.
4. Investigation of signaling cascade involved during cardiomyogenesis and neurogenesis.
5. Electrophysiological characterization and transplantation of the neural progenitors and dopaminergic neurons into Parkinsonian animal model and exploring their potential in functional recovery by the behavioral studies.

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Embryonic stem cells as a developmental paradigm to explore early neuro- and cardiogenic proceedings

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Collaborators:
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Abstract and Background:

The embryonic stem (ES) cells derived from the inner cell mass of the blastula stage embryo bear the complete repertoire of the complex organizational blueprint of an organism. These fascinating cells are bestowed with pluripotent characteristics and are capable of differentiating in vitro into any cell type of the body recapitulating the in vivo events in a relatively precise manner. Hence, the ES cell system could serve as an ideal in vitro model in gaining insight into the early developmental events and additionally could also be explored for its potential application in cell replacement therapy. Using the ES cell model we have attempted to investigate the early neurogenic and cardiogenic proceedings in vitro. We have generated a number of stable ES cell clones and taking advantage of the live reporter based cell trapping approach we could successfully monitor "live" the differentiation events. This strategy has helped us in both qualitative and quantitative detection and characterization of tissue specific progenitor population and their differentiated derivatives.

Aims:

To unravel the complex temporo-spatial cell fate decision machinery from uncommitted ES cells, the major focus of our group has been,

(i) To establish stable transgenic ES cell clones using live reporter gene expression under the regulatory control of tissue-specific promoters/ enhancers.
(ii) To differentiate the ES cells into cardiac and neural lineages and understand the underlying molecular basis of lineage commitment and specification.
(iii) Manipulate extrinsic factors for the efficient generation of proliferative neural progenitors, differentiated neurons with special reference to the dopaminergic neuronal subtypes and cardiomyocytes from ES cells in vitro.

Work achieved:

Cardiomyogenesis: Cardiovascular diseases are one of the major causes of death in infants and adults alike and the improper functioning of the cardiomyocytes results in the diseased state. Efforts are being made to explore the transplantation therapy approach as a suitable alternative for treating these disorders. In many cardiac diseases in fact the embryonic genetic program is reactivated. Hence, understanding the early embryonic cardiac developmental pattern would help gaining insight into the molecular basis of these diseases. Hence, we have chosen the ES cell model to generate the cardiomyocytes in vitro and to investigate the molecular basis of cardiac lineage specification and cardiomyocytes development.

Embryonic stem cells differentiate into a heterogeneous population of cells. Hence, to demarcate the cardiac population in these heterogeneous mass we have introduced the live reporter (EYFP) under the regulatory control of supposedly atrial chamber specific alpha myosin heavy chain (a-MyHC) and ventricular specific beta myosin heavy chain (b-MyHC; Fig. 1) promoters and successfully generated a number of

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Cellular and molecular mechanisms involved in the regulation of osteoclast differentiation and activation by cytokines

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Satish Potey, technician

1. Role of IL-3 and GM-CSF on TNF-α-induced osteoclast differentiation

Abstract and background:

Osteoclasts, the multinucleated cells that resorb bone, differentiate from hemopoietic precursors of monocyte/macrophage lineage, which also give rise to macrophages or dendritic cells. Differentiation of osteoclasts depends primarily on two critical cytokines, M-CSF and receptor activator of NF-κB (RANK) ligand (RANKL) produced by stromal cells/osteoblasts. This is evident by the osteopetrotic phenotype of the M-CSF-deficient op/op mouse, and the RANKL-deficient mouse that lacks osteoclasts. Although RANKL is essential and necessary for osteoclast differentiation, TNF-α also induces osteoclast differentiation from M-CSF-dependent bone marrow-derived macrophages in a RANKL-independent manner. TNF-α has been implicated in the bone loss that accompanies many inflammatory diseases such as rheumatoid arthritis, peridontitis and orthopedic implant loosening. TNF-α also plays important role in estrogen deficiency-induced bone loss in postmenopausal osteoporosis. However, little is known about the factors that regulate TNF-α induced osteoclast differentiation.

IL-3 and GM-CSF regulate osteoclast differentiation in mouse hemopoietic tissue. Recently, we have shown that IL-3 acts directly on osteoclast precursors and irreversibly inhibits RANKL-induced osteoclast differentiation by inhibiting NF-κB activation (Khapli et al. 2003, J. Immunol. 171:142-151). In this study we have clarified the mechanism by which IL-3 and GM-CSF inhibits TNF-α induced osteoclast differentiation in mouse osteoclast precursors. We provide the first evidence that IL-3 and GM-CSF irreversibly blocks TNF-α induced osteoclast differentiation by down-regulation of mRNA and surface expression of TNFR1 and TNFR2.

Aims:

1. To identify the role of IL-3 and GM-CSF on TNF-α induced osteoclast differentiation in stromal and lymphocytes-free cultures of osteoclast precursors.
2. To investigate the mechanism(s) by which IL-3 and GM-CSF acts on TNF-α induced osteoclast differentiation.

Work achieved:

IL-3 and GM-CSF are potent inhibitors of TNF-α induced osteoclast differentiation

We show here that both IL-3 and GM-CSF potently inhibits TNF-α induced osteoclast differentiation. Similar to the action on RANKL-induced osteoclast differentiation, IL-3 and GM-CSF inhibited TNF-α induced osteoclast differentiation by directly acting on osteoclast precursors. As shown in Fig. 1 IL-3 and GM-CSF significantly inhibits expression of osteoclast specific genes such as cathepsin K, c-Src and tartrate-resistant acid phosphatase (TRAP). TNF-α and RANKL are abundant in sites of inflammatory bone erosions and strong synergism has been shown between RANKL and TNF-α for increased osteoclastogenesis. This synergy involves enhanced

activation of intracellular signaling pathways necessary for osteoclastogenesis. In this study IL-3 and GM-CSF significantly inhibited the synergistic effect of RANKL and TNF-α suggesting the potent inhibitory action of IL-3 and GM-CSF on in vitro osteoclastogenesis. IL-3 and GM-CSF showed inhibitory action only when added during initial stages of osteoclast differentiation and the inhibitory effect is decreased when addition of IL-3 and GM-CSF is delayed. Antibodies against IL-3 and GM-CSF completely neutralized the inhibitory effect of IL-3 and GM-CSF conditioned medium respectively suggesting that IL-3 and GM-CSF are the only factors responsible for inhibition of osteoclast formation. We also found that IL-3 and GM-CSF does not act on mature osteoclasts. The inhibitory effect of IL-3 and GM-CSF on TNF-α induced osteoclast differentiation was irreversible.

IL-3 and GM-CSF down-regulates expression of TNFR1 and TNFR2

Next, we investigated the mechanism involved in action of IL-3 and GM-CSF on TNF-α induced osteoclast differentiation. TNF-α induces a number of biological responses by recognizing two cell surface receptors termed as TNFR1 and TNFR2 (also called type 1 or p55 and type 2 or p75 receptors). It has been reported that TNFR1 and TNFR2 differentially impact osteoclastogenesis and TNF-α stimulates osteoclast formation in $TNFR2^{-/-}$ but fails to stimulate osteoclastogenesis in $TNFR1^{-/-}$ mice. Therefore, we first evaluated the mRNA expression levels of TNFR1 and TNFR2 genes in IL-3 or GM-CSF treated cultures. Expression of TNFR1 and TNFR2 was significantly down regulated by IL-3 and GM-CSF. Furthermore, by FACS analysis we confirmed that IL-3 and GM-CSF also inhibits surface expression both TNFR1 and TNFR2 at 48h and inhibitory effects were maintained for 96h (Fig. 2).

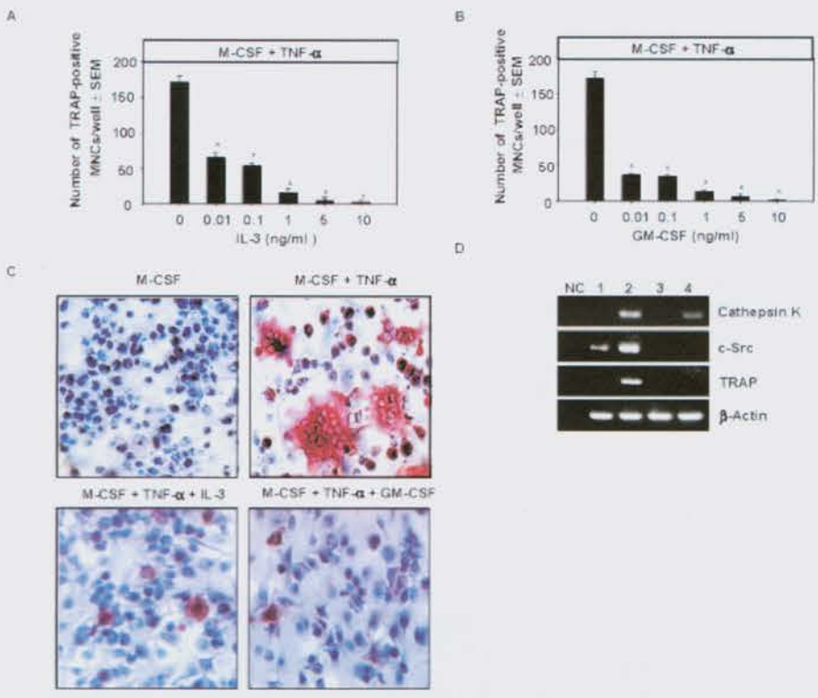


Fig. 1. Effect of IL-3 and GM-CSF on TNF-α induced osteoclast differentiation in osteoclast precursors. Osteoclast precursors were incubated in 96-well plates in the presence of M-CSF (30 ng/ml) and TNF-α (40 ng/ml) in the absence or the presence of increasing concentrations of IL-3 (A) or increasing concentrations of GM-CSF (B). After 5 days the number of TRAP-positive multinuclear cells (MNCs) was counted. In both (A) and (B) results are expressed as the mean ± SEM of six cultures per variable. *, p 0.01 vs. cultures with M-CSF and TNF-α . Similar results were obtained in three independent experiments. C, TRAP staining of osteoclast precursors incubated in the presence of M-CSF with or without TNF-α , and M-CSF, TNF-α and IL-3 (1 ng/ml) or GM-CSF (1 ng/ml). Magnification, X 20. D, RNA was extracted from osteoclast precursors treated with M-CSF with or without TNF-α , and M-CSF, TNF-α and IL-3 (5 ng/ml) or GM-CSF (5 ng/ml) for 5 days and subjected to RT-PCR analysis for Cathepsin K, TRAP, c-Src, and -actin genes. NC-nonloading control, Lane 1, M-CSF; lane 2, M-CSF and TNF-α ; lane 3, M-CSF, TNF-α and IL-3; lane 4, M-CSF, TNF-α and GM-CSF. Similar results were obtained in two independent experiments.

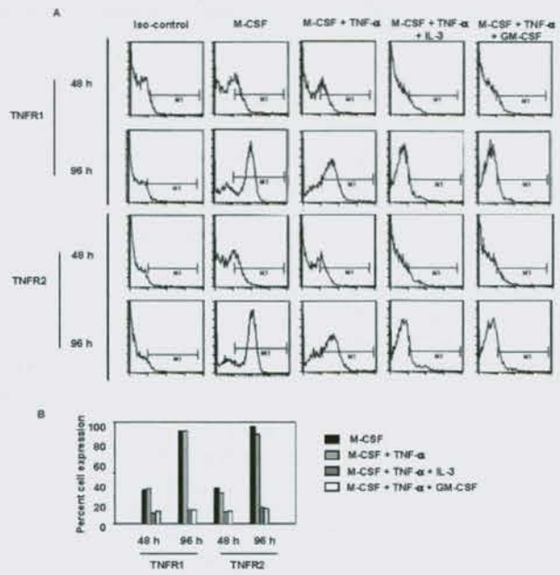


Fig. 2. Effect of IL-3 and GM-CSF on surface expression of TNFR1 and TNFR2. A, Osteoclast precursors were incubated for 48 and 96 h in the presence of M-CSF (30 ng/ml); M-CSF and TNF-α (40 ng/ml) with or without IL-3 (5 ng/ml) or GM-CSF (5 ng/ml). Cells were washed, fixed and blocked with mouse Fc block. Cells were treated with primary antibodies TNFR1 and TNFR2 and then secondary FITC or PE labeled antibodies, and acquired and analyzed by FACS Vantage. B, Represents the percent cell expression from cultures in (A). Results are representative of two independent experiments.

Characteristics of IL-3 and GM-CSF treated osteoclast precursors

The effect of IL-3 and GM-CSF on osteoclast differentiation was irreversible. Therefore, we characterized the nature of cells treated with IL-3 or GM-CSF. As shown in Fig. 3 cells incubated with IL-3 showed formation of few clusters of mononuclear cells, whereas many such clusters were seen in the presence of GM-CSF. These clusters were loosely adherent and morphologically looked like dendritic cells clusters. Further characterization of IL-3 and GM-CSF treated cells for macrophage and dendritic cells specific antigens is in progress.

Future work:

We will further investigate the in vivo role of IL-3 on bone resorption and bone formation in mice.

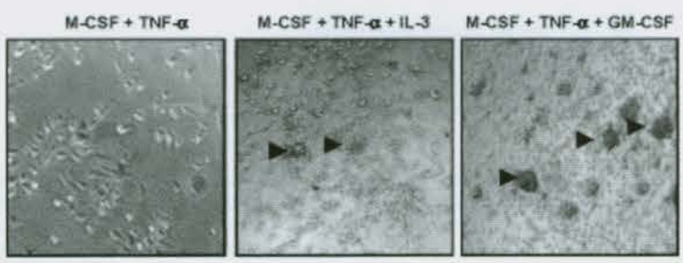


Fig. 3. Characterization of cells induced by IL-3 and GM-CSF in the presence of TNF-α. Osteoclast precursors were incubated with M-CSF (30 ng/ml) and TNF-α (40 ng/ml) without or with IL-3 (5 ng/ml) and GM-CSF (5 ng/ml) for 5 days. Magnification, X 4.

2. Role of IL-4 on bone resorption by mature osteoclasts

Abstract and background:

The bone loss in many important skeletal disorders such as osteoporosis, rheumatoid arthritis, hypercalcemia of malignancy and bone metastases of various tumors occurs mainly because of increased osteoclast activity. The study of molecular mechanisms by which cytokines regulate bone resorption by mature osteoclasts has been limited because of difficulties in obtaining sufficient number of mouse mature osteoclasts.

IL-4 is an important immune cytokine that regulates function of lymphocytes and macrophages, and also regulates osteoclastogenesis and bone resorption. Much recent work has clarified the role and molecular mechanisms by which IL-4 inhibits osteoclast differentiation. However, the role and mechanism of IL-4 action on bone resorption by mature osteoclasts is not known. In this study using enriched population of mature osteoclasts we investigated the molecular mechanisms involved in action of IL-4 on bone resorption by mature osteoclasts. We show that IL-4 acts directly on mature osteoclasts and inhibits bone resorption through inhibition of RANKL activated NF-κB.

Aims:

1. To study the effect of IL-4 on mature osteoclasts and its function.
2. To investigate the mechanism(s) involved in action of IL-4 on bone resorption by mature osteoclasts.

Work achieved:

IL-4 inhibits bone resorption by mature osteoclasts

We prepared large number of mature osteoclasts induced by RANKL and show that IL-4 with increasing concentration inhibited the RANKL-induced bone resorption by mature osteoclasts (Fig 1). IL-4 also decreased the individual pit size suggesting that each osteoclast is defective in terms of resorptive activity. The direct effect of IL-4 on mature osteoclasts was confirmed by using anti-mouse IL-4 antibody, which neutralized the inhibitory effect of IL-4 on bone resorption. We provided definitive evidence that IL-4R is expressed on mature osteoclasts. IL-4 inhibited TRAP expression in mature osteoclasts without affecting the multinuclearity of cells. Majority of multinuclear cells (MNCs) in the presence of IL-4 were TRAP-negative. The number of TRAP-negative MNCs in the culture was also increased with increasing concentrations of IL-4.

IL-4 inhibits actin ring formation induced by RANKL

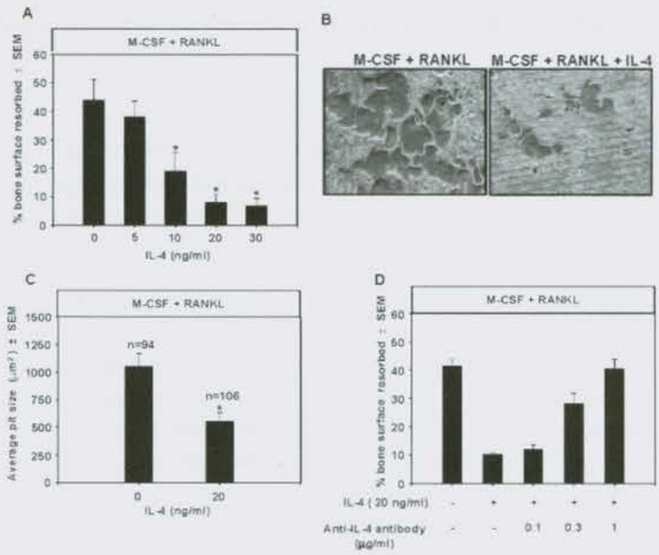


Fig. 1. Effect of IL-4 on bone resorption by mature osteoclasts. Mature osteoclasts prepared on bone slices were incubated with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the absence or presence of increasing concentrations of IL-4. After 48 h bone slices were examined by reflected light microscopy (A) and scanning electron microscopy (B, Magnification, X 250). Results in (A) are expressed as the mean ± SEM of 6 cultures per variable. *, p 0.01 vs. control. Similar results were obtained in three independent experiments. C: Pit size (μm^2) was measured by software in scanning electron microscope. *, p 0.01 vs. control. D) Effect of anti-IL-4 neutralizing antibody on IL-4 inhibited bone resorption. Mature osteoclasts were incubated for 48 h with M-CSF and RANKL in the presence or absence of IL-4 (20 ng/ml), or M-CSF, RANKL, IL-4 and increasing concentrations of neutralizing antibody. Similar results were obtained in two independent experiments.

There is an excellent correlation between actin ring formation and bone resorption. IL-4 treated mature osteoclasts showed a reduced intensity and the disruption of actin ring structures induced by RANKL (Fig 2). The disrupted actin rings and diffuse cytoplasmic staining observed in the presence of IL-4 reflects the improper assembly of actin rings. Thus, in our study decreased TRAP expression and structural disturbances in actin rings by IL-4 contributed largely to the reduced bone resorption by mature osteoclasts.

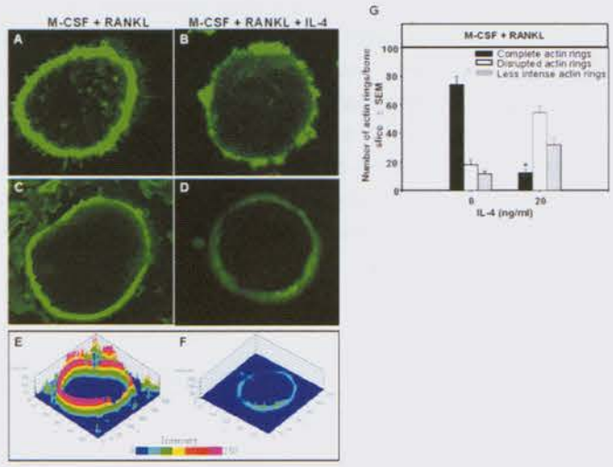


Fig. 2. Effect of IL-4 on actin ring formation by mature osteoclasts. Mature osteoclasts prepared on bone slices were preincubated for 1 h at 37°C in MEM + 10% FBS and then incubated further for 6 h with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the absence or presence of IL-4 (20 ng/ml). Bone slices were fixed, incubated in 1 g/ml FITC-conjugated phalloidin and actin rings were visualized using a confocal microscope. A and B shows the structure of actin rings. C and D shows the intensity of actin rings (Magnification, X 360). E and F are the graphical presentation of C and D respectively, analyzed by Zeiss LSM 510 software. G- Number of complete, disrupted and less intense actin rings in mature osteoclasts incubated with RANKL in the presence or absence of IL-4. Results are from 6 cultures per variable in 3 experiments. *, p 0.01 vs. control.

IL-4 prevents RANKL-induced nuclear translocation of NF-κB subunit

Expression of RANK on mature osteoclasts not only supports its activation but also provide the evidence that RANK is a receptor for signal transduction for activated osteoclasts. Also RANK-dependent signaling is essential for osteoclast cytoskeleton organization and resorption function. In this study we show that IL-4 does not inhibit RANK expression on mature osteoclasts. Therefore, to investigate further the molecular mechanisms of IL-4 action we examined the effect of IL-4 on NF-κB activation in mature osteoclasts. NF-κB activation is essential for the osteoclast differentiation and its role has been implicated in bone resorption. In our study IL-4 prevented the nuclear translocation of p65 NF-κB subunit induced by RANKL. In conclusion, our results reveal that IL-4 acts directly on mature osteoclasts and inhibits bone resorption through inhibition of NF-κB activation by IL-4R mediated mechanism.

Future work:

We will further study the in vivo role of IL-4 on RANKL stimulated hypercalcemia in mice.

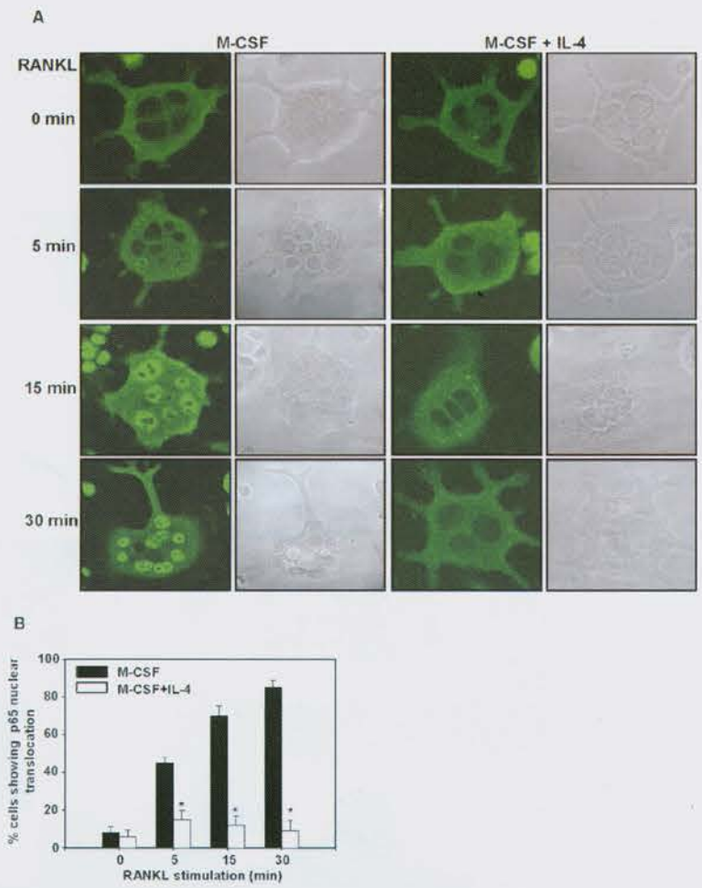
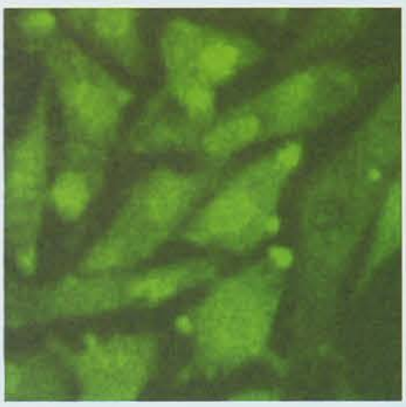


Fig 3. Effect of IL-4 on RANKL-induced nuclear translocation of p65 NF-B subunit in mature osteoclasts. A) Mature osteoclasts prepared on glass Lab-Tek chamber slide were preincubated for 2 h in the presence of M-CSF (30 ng/ml) with or without IL-4 (20 ng/ml) and stimulated with RANKL (30 ng/ml) for 5, 15 and 30 min. MNCs were analyzed for nuclear translocation of p65 by immunofluorescence using confocal microscope. B) Number of MNCs showing p65 nuclear translocation was scored. Results are representative of three independent experiments. *, p 0.01 vs. cultures with M-CSF.

Research Reports



Cancer Biology

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Chemo sensitivity of cancer cells to drugs: mechanism of cell death and role of tumor suppressor p53

Rishi Raj Chhipa, Sandeep Singh, Ankur Kumar Upadhyay ,
Vijay Kumar M.V., technician

Background

The success of chemotherapy in the treatment of certain leukemias and testicular cancer has been dramatic. For rest of the other cancers the success for the treatment has been less than expected. Ideally, chemotherapeutic drugs should specifically target only cancerous cells and should decrease tumor growth by inducing cytotoxic or cytostatic effects, without affecting the normal cells. In reality, the effectiveness of chemotherapy has suffered due to lack of specificity, rapid drug metabolism, and both intrinsic and acquired drug resistance. More over, the mechanisms of chemotherapeutic drugs mediated cells killings have not been totally investigated. Therefore understanding the molecular events that contribute or enhance drugs induced cell death will not only help in explaining the relationship between cancer genetics and chemotherapy drugs but also improve sensitivity as well as specificity of the treatment. Also majority of human cancers are difficult to treat, especially in their advanced metastatic forms. One of the major hurdles in treating cancers cells is development of resistance to chemotherapeutic drugs. Over the past few years the concept of combining chemotherapeutic agents to increase cytotoxic efficacy has greatly evolved. The rationale for combination therapy is centered on attacking different biochemical targets simultaneously. Moreover, malfunction of specific genomic "care taker" systems leading to increased mutability of human genome is a major factor in cancer development. One of the most prominent members of these systems is the tumor suppressor p53 gene. Studies with mouse cells have provided evidence of drug resistance after p53 inactivation, but the extrapolation of these results to humans is far from straight forward. The requirement of wild-type p53 for apoptosis after genotoxic damage caused by anticancer agents, including irradiation has been demonstrated in tissues of lymphoid origin. However, the influence of p53 on apoptosis in malignant tissues of non-hematological origin is by no means clear. Therefore p53 is one of the ideal targets for studying its roles in cancer therapeutics employed for solid tumors.

Aims

1. To better understand oral cancers susceptibility to chemotherapeutic drugs - Investigations on HPV E6 positive cells as a model.
2. To investigate potential usefulness of combination therapy in breast cancers - Combitorial effect of doxycycline and cyclophosphamide, on human breast cancer cells both in vitro and in vivo.
3. p53: It's role in cancer cells.

Work achieved:

Oral Cancers Susceptibility to Chemotherapeutic Dugs -Investigations on HPV E6 Positive Cells as a Model

NF-κB family of proteins plays important role in inflammatory responses, cell growth, differentiation, regulation of apoptosis and neoplastic transformation in mammals.

These transcription factors include RelA (p65), RelB, c-Rel, p50 (NF-kB1), and p52 (NF-κB2) proteins, that form homo- or hetro-dimer among each other. The most common form of NF-κB consists of hetro-dimer of p50 and p65 proteins. In normal cells, NF-κß is mostly retained in cytoplasm as an inactive complex through direct binding of inhibitory molecule, IκB (inhibitor of NF-κB). Exposure of cells to variety of stimuli including, inflammatory cytokines, mitogens, growth factors, infectious agents, irradiation and stress related agents, leads to activation of NF-κB through generation of signals that are transmitted via cascade of interacting kinases. That results in phosphorylation and degradation of IκBa leading to translocation of active NF-κB to the nucleus.

Constitutively high level of NF-κB activity is detected in many hematopoetic and solid tumors because of chromosomal aberration in NF-κB family proteins, hyper-activation of NF-κB signaling pathway and/or mutation in various regulatory molecules. Additionally, constitutive NF-κB activity is connected with multiple aspects of oncogenesis and also for resistance to chemotherapy or radiation. Therefore, inhibitors of active NF-κB may function as better cancer preventive compound either alone or in combination, resulting in enhanced apoptotic potential of chemotherapeutic drugs. More studies are needed to understand the role of NF-κB on the efficacy of various chemotherapeutic drugs for different tumors. Carboplatin is widely used drug for the treatment of various cancers, either alone or in combination with other drugs or radiation. It is a second-generation platinum compound that may be classified as a nonclassical alkylating agent. It forms interstrand cross-links and intrastrand adducts with DNA by reacting with N7 guanine residues. Alkylation at N7 position of guanine causes depurination and leads to strand breakage. Based on our observations that NF-κB is constitutively active in HEp-2 and the fact that carboplatin treatment induces apoptosis in these cells we investigated if NF-κB is involved. The objective of the study was to elucidate the role of NF-κB in carboplatin mediated cell death of HPV-18 E6 positive cancer cells. Our data demonstrate for the first time that HPV-18 E6 positive HEp-2 cells express constitutively high level of active NF-κB, which is essential for the survival of these cells. Carboplatin treatment blocks the translocation of NF-κB via stabilization of IκBα protein. Moreover, carboplatin mediated inhibition of constitutively active NF-κB leads to Bcl-2 downregulation by direct inhibition of NF-κB binding to the response element present in Bcl-2 promoter.

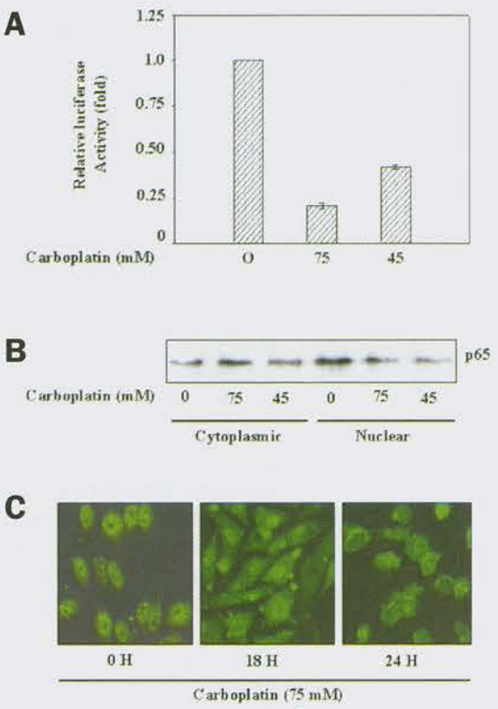

FIG. 1. Effect of carboplatin on constitutive NF-κB activity and p65 in HEp-2 cells.
(A) HEp-2 cells were transfected with pNF-κB-Luc construct and treated with indicated concentration of carboplatin for 24 h. Equal amount of protein was analyzed for NF-κB luciferase reporter activity. Luciferase activity (normalized to β-galactosidase activity) is reported as fold of arbitrary relative light units (mean ± SDs). (B) HEp-2 cells were treated with indicated concentration of carboplatin for 24 h. Cytoplasmic and nuclear extracts were prepared. Equal amount of protein were resolved on 10% SDS-PAGE gel, electro transferred on nitrocellulose membrane and probed for p65. (C) HEp-2 cells were incubated alone or with carboplatin (75 mM) for indicated time and then analyzed for the distribution of p65 by confocal analysis. Green stain indicates the localization of p65.

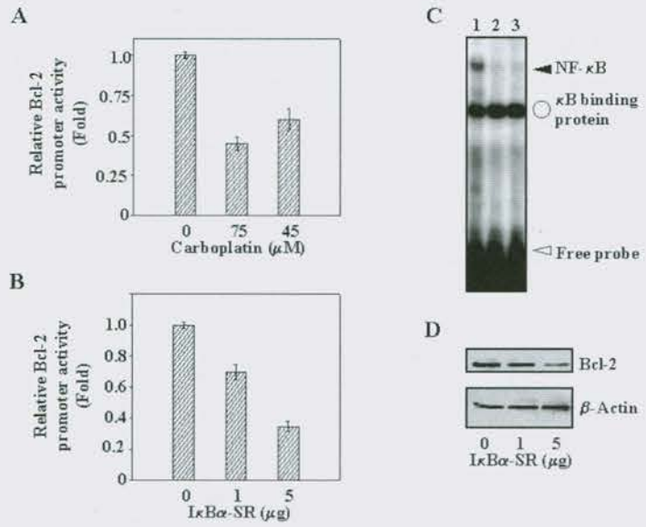

Fig. 2. Effect of carboplatin mediated inhibition of NF-kB on Bcl-2 in HEp-2 cells.
(A) HEp-2 cells were transfected with full-length Bcl-2 promoter reporter construct Bcl-2-FL and treated with indicated concentration of carboplatin for 24 h. Luciferase activity (normalized to b-galactosidase activity) is reported as fold of arbitrary relative light units (mean ± SDs). (B) HEp-2 cells were co-transfected with full-length Bcl-2 promoter reporter construct Bcl-2-FL and IκBα-SR. Equal amount of protein was tested for Bcl-2 promoter luciferase activity. Luciferase activity (normalized to b-galactosidase activity) is reported as fold of arbitrary relative light units (mean ± SDs). (C) HEp-2 cells were treated with 75 (lane 3) and 150 μM (lane 2) of carboplatin for 24 h and nuclear extracts were prepared. Equal amount 5 μg nuclear protein was tested for NF-κB DNA binding activity by EMSA using g 32P-labeled DNA probe having the κB motif of the Bcl-2 promoter. The circle indicates the position of a constitutively κB region binding protein, and the triangle indicates the position of unbound oligonucleotides. NS; non-specific binding (D) HEp-2 cells were transfected with indicated concentration of Iκβα-SR. Cell lysates were prepared and analyzed for Bcl-2 (upper panel) by western blotting. Blot was stripped and reprobed for β-actin.

Doxycycline Potentiates Antitumor Effect of Cyclophosphamide in Mice

The concept of combining chemotherapeutic agents to increase cytotoxic efficacy has evolved greatly over past several years. The underlying rationale is the realization that except for Burkitt's lymphoma and Choriocarcinoma, individual chemotherapeutic agents for the majority of tumors have not increased cure rates in the treatment of cancer. However with the advancements in our understanding the effects of chemotherapeutic drugs on cancer cells, it is becoming apparent that combination therapy of two or more drugs may have greater beneficial effects on cancer treatment and management. Cyclophosphamide (CPA) is a cell cycle-dependent DNA and protein-alkylating agent that has a broad spectrum of activity against variety of neoplasms, and is widely used in the clinical management of human malignancies including breast cancer. In conventional chemotherapy, CPA is one of the most commonly employed drugs and is used in high dose regimen to treat metastatic breast cancer. In this setting the therapeutic efficacy of this drug is limited by host toxicity as a result of the systemic distribution of activated drug metabolites that have significant cytotoxic effects including cardiac and renal toxicity. Therefore drugs that could potentiate CPA antineoplastic effect would be of significant importance and will lessen the toxic effects associated with high dose CPA treatment. Doxycycline (DOX), a commonly used antibiotic has antitumor activity against several malignancies. Recently it has been reported that DOX has potential treatment value in bone metastasis of breast cancer cells. DOX inhibits these effects by inhibiting MMPs, not only in breast cancer cells but also in human endothelial, prostate cancer, osteocarcinoma cells of patients. Moreover, it also inhibits cell proliferation and induces apoptosis in various cancer cells. All these studies demonstrate that this well tolerated antibiotic may be effective in treatment of various human cancers, either alone or in combination therapy. DOX is potentially beneficial in bone metastasis of breast cancer cells and CPA is an important component of chemotherapeutic regimen for treatment of breast cancers. Therefore we postulated that this combination treatment might enhance antitumor effect of CPA on breast cancer cells in vivo. We found that DOX significantly enhances the tumor regression activity of CPA on xenograft mice model of MCF-7 cells. In addition, we explored the molecular basis of syngerstic effect of CPA and DOX, and investigated mechanism of action both in vitro and in vivo. Our results raise the possibility that this combination chemotherapeutic regimen may lead to additional improvements in treatment of breast cancer.

Characterization of a Unique Isogenic MCF-7T Cell Line: Role of p53

p53 is the most commonly mutated gene in human cancer and the p53 pathway is involved in vast majority of tumors without mutations in p53, this p53 protein becomes an ideal target for studying its role in cancer therapeutics. Major mechanisms through which p53 function is controlled are regulation at protein levels, control of the localization of the p53 protein and modulation of the activity of p53, particularly its ability to function as a sequence specific transcription factor. p53 is a multifaceted transcription factor regulating multiple cellular processes including cell cycle progression, apoptosis, DNA repair and differentiation. After DNA damage, p53 levels increase and mediate multiple cellular responses i.e., (a) G1 arrest via transcriptional induction of p21, a CDK inhibitor; (b) DNA damage repair via transcriptional induction of GADD45; and (c) induction of apoptosis in some cell types, if the damage is excessive. In addition, p53 status in a cell has been implicated in the outcome of cancer cells treated with various cancer therapeutic namely irradiation and chemotherapy. So far results have been controversial. The requirement of wild-type p53 for apoptosis after genotoxic damage caused by anticancer agents, including irradiation has been demonstrated in tissues of lymphoid origin. However, the influence of p53 on apoptosis in malignant tissues of non-hematological origin is by no means clear. p53 is an ideal target for studying its roles in cancer therapeutics employed for solid tumors. To undertake this work, an appropriate isogenic cell lines based system exhibiting low p53 transactivation activity was developed. MCF-7T clone was developed from MCF-7 by process of selection for a stable clone for Tetracycline based gene regulators system. This clonal cell lines has been partially characterized and compared with parental MCF-7 cells. MCF-7T cell line has consistent basal epithelial phenotype, morphology, and cell proliferation rate and estrogen receptor expression similar to MCF-7 cells. Interestingly, MCF-7T was more sensitive to same DNA damanging chemotherapentic drugs.

Future Work:

- Studies on HPV E6 positive cancer cells will be expanded to include other cancer types.
- Loss of tumor formation ability of MCF-7T will be investigated and p53 involvement will be studied.

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Understanding mechanisms of transformation elicited by a novel 600bp. non-coding RNA gene - M3TR

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Abstract and background:

Noncoding RNAs are known to perform multiple tasks in living cells that range from the storage and transfer of genetic information to the catalysis of biochemical reactions. The transcripts produced by the NCR's may function directly as structural, catalytic or regulatory RNA's rather than being expressed as mRNA's that encode proteins. The important NCR's known to play important roles in cell and developmental biology in various species include Pgc required for germ cell formation in Drosophila, BCl, Bsr and Ntab involved in neuronal development in rats and human Bic which is strongly up-regulated in B-cell Lymphomas and roX1/2 and Xist/Tsix involved in dosage compensation in insects and mammals respectively. Many of the NCR's are also expressed as antisense transcripts. The recent studies have revealed that the introns and NCR's carry out the majority of transcription of the genomes of humans and other species and form a second tier of gene regulation which are necessary for differentiation and development. Our group has identified and cloned a novel 600bp gene from mouse melanoma cells that is a member of a family of expanding family of untranslated RNA's or non-coding RNA's (NCR) and possesses transforming function.

Aims:

1. Expanding the repertoire of gene expression studies for the 600bp NCR using nested primers in various tissues and cell types.

2. Cloning the human 600bp homologue of M3TR from SK-N-MC Neuroblastoma cells and transcript analyses.

3. Understanding the mechanism of transformation elicited by the 600bp NCR and define the Signal transduction mechanisms elaborated by this category of molecules.

Work achieved:

Our group identified and cloned a novel 600bp gene from mouse melanoma cells that is a member of a family of expanding family of untranslated RNA's or non-coding RNA's (NCR) . The NCR gene interestingly possesses transforming function as assayed by tumorigenicity assays on ectopic expression in primary, diploid and established cell-lines. The gene sequence of this molecule was found to be highly conserved across mouse, rat and human species. Gene expression studies demonstrated its expression predominantly to the transformed neuro-epithelial cell-lines like SK-N-MC, LN18, SK-N-SH and haematopoietic cell-lines like Jurkat, HUT 78 and Raji. We cloned the human 600bp homologue from SK-N-MC cells and found it to have similar transforming function like the mouse gene. The NCR was found to be localized to the cytoplasm as a polyadenylated RNA. Transcript analyses by Northern hybridization with labeled riboprobe demonstrated the anti-sense expression of the gene with transcript size of 1.8 kb. Our studies with Inter-simple Sequence Repeat PCR using CG repeat primers demonstrated that transfection of 600bp construct either transiently or stably induced genomic instability in the chromosome and consequently depicted accumulation of wild-type p53 to the nucleus. Experiments are underway to understand if this molecule can function as a SiRNA and decipher its role in furthering our knowledge about the mechanisms employed by the NCR's in gene regulation.

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Studies on a bi-potent cell-line with stem cell features derived from a human neuro-epithelial tumor

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Abstract and background :

Gliomas are the most common of primary brain tumors and account for more than 40 % of all CNS neoplasms. An understanding of the mechanism(s) of tumor progression from grade 1 anaplastic astrocytoma (AA) to grade 4 GBM using a series of experimental approaches would be of importance in limiting advance of the disease and prolonging survival. One of the major issues addressed regarding the origin of gliomas and other brain tumors is whether these tumors arise from stem cells or derived from adult differentiated cells which due to some reason undergo dedifferentiation and begin to recapitulate development . we have developed a a novel model system generated from human malignant glioma comprising two cell lines - Human Neural Glial Cell-line HNGC-1 and HNGC-2 which express neural stem cell-like characters. This model system, comprising of 2 cell-lines with features representing two sequential stages -Grade 1 astrocytoma and Grade 4 Glioblastoma in gliomagenesis form an excellent system for studying the mechanisms related to transformation and in furthering the knowledge in unraveling the signal transduction pathways in gliomagenesis.

Aims :

1. Study of the molecular mechanisms involved in the pathways leading to cell transformation of HNGC-1 to HNGC-2.

2. Cytogenetic studies of the chromosomal re-arrangements in the HNGC-1 and HNGC-2 cell-lines using methodologies spectral karyotyping (SKY)

3. Identification and analyses of genes important in cell survival, differentiation and maturation into specific cell types of the glial or neuronal lineage.

Work achieved:

We developed two cell-lines HNGC-1 and HNGC-2 from a glioma patient. The HNGC-1 cell line is slow growing, contact inhibited, non-tumorigenic and is non-invasive, whereas HNGC-2 is a rapidly proliferating, anchorage independent, highly tumorigenic and invasive cell-line. The proliferation of cell lines was found to be independent of the addition of exogenous growth factors. Interestingly, the HNGC-2 cell-line displayed a near-haploid karyotype except for a disomy of chromosome 2. The two cell-lines expressed the neuronal precursor and progenitor markers vimentin, nestin, MAP-2, NFP160 as well as glial differentiation protein S100â. The HNGC-1 cell-line also expressed markers of mature neurons like Tuj1 and GFAP, an astrocytic differentiation marker, hence contributing towards a more morphologically differentiated . phenotype with a propensity for neural differentiation in vitro. Additionally, over-expression of EGFR, c-erbB2 and loss of fibronectin was observed only in the HNGC-2 cell-line implicating the significance of these pathways in tumor progression. This in vitro model system assumes importance in unraveling the cellular and molecular mechanisms in differentiation, transformation and in gliomagenesis.

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Molecular mechanism of osteopontin (OPN) regulated cell motility, c-Src dependent epidermal growth factor receptor transactivation and urokinase type plasminogen activator (uPA) secretion through activation of transcription factor, AP-1

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Pallavi Bapat, Shalini Jain, Sailaja Badi, Sutirtha Datta, Anuradha Bulbule, technician

Collaborators:
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Project: Identification of Bioactive Compound from Marine Sources.

Background and Abstract:

We have recently reported that osteopontin (OPN) stimulates cell motility and nuclear factor κB-mediated secretion of urokinase type plasminogen activator (uPA) through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells. However, the molecular mechanism by which OPN activates AP-1 and regulates uPA secretion through activation of c-Src and EGF receptor (EGFR) in breast cancer cells is not well defined. Here we report that OPN induces $\alpha_v\beta_3$ integrin-mediated c-Src kinase activity and c-Src dependent EGFR transactivation. Furthermore, OPN induces $\alpha_v\beta_3$ integrin/EGFR regulated MEK dependent ERK1/2 phosphorylation, AP-1-mediated uPA secretion and cell motility. These data demonstrated that OPN regulates $\alpha_v\beta_3$ integrin-mediated c-Src dependent EGFR transactivation and AP-1-mediated uPA secretion through activation of MAP kinase in breast cancer cells.

Aims:

(a) To examine whether OPN induces $\alpha_v\beta_3$-mediated c-Src kinase activity in low invasive MCF-7 and highly invasive MDA-MB-231 cells.

(b) To study whether OPN regulates EGFR transactivation through c-Src kinase mediated pathway.

(c) To investigate whether OPN stimulates EGF receptor-mediated ERK1/2 and AP-1 activation in these cells.

(d) To analyze whether OPN enhances c-Src/AP-1 activation, uPA secretion and cell motility.

(e) To establish a functional molecular link between OPN-induced c-Src dependent EGFR transactivation and ERK/AP-1-mediated uPA secretion and whether all of these ultimately control the motility of breast cancer cells.

Work achieved:

We have shown that OPN induces $\alpha_v\beta_3$ integrin-mediated c-Src kinase activity in both highly invasive (MDA-MB-231) and low invasive (MCF-7 breast cancer cells. Ligation of OPN with $\alpha_v\beta_3$ integrin induces kinase activity and tyrosine phosphorylation of EGFR in MDA-MB-231 cells and wild type EGFR transfected MCF-7 cells and this was inhibited by dominant negative form of c-Src (dn c-Src) indicating that c-Src kinase plays crucial

role in OPN-induced EGFR phosphorylation. OPN induces association between $\alpha_v\beta_3$ integrin and EGFR on the cell membrane in a macromolecular form with adaptor protein c-Src in these cells. Furthermore, OPN induces $\alpha_v\beta_3$ integrin/EGFR-mediated ERK1/2 phosphorylation and AP-1 activation. Moreover, OPN also enhances uPA secretion and cell motility in these cells. OPN-induced ERK phosphorylation, AP-1-activation, uPA secretion, cell motility and invasion were suppressed when cells were transfected with dn c-Src or pretreated with $\alpha_v\beta_3$ integrin antibody, c-Src kinase domain inhibitor (pp2), EGFR tyrosine kinase inhibitor (PD153035), MEK-1 inhibitor (PD98059) and enhanced when cells were transfected with wild type EGFR. To our knowledge, this is the first report that OPN induces $\alpha_v\beta_3$ integrin-mediated AP-1 activation and uPA secretion by activating c-Src/EGFR/ERK signaling pathways and further demonstrates a functional molecular link between OPN-induced integrin/c-Src-dependent EGFR tyrosine phosphorylation and ERK/AP-1-mediated uPA secretion, and all of these ultimately control the motility and invasiveness of breast cancer cells.

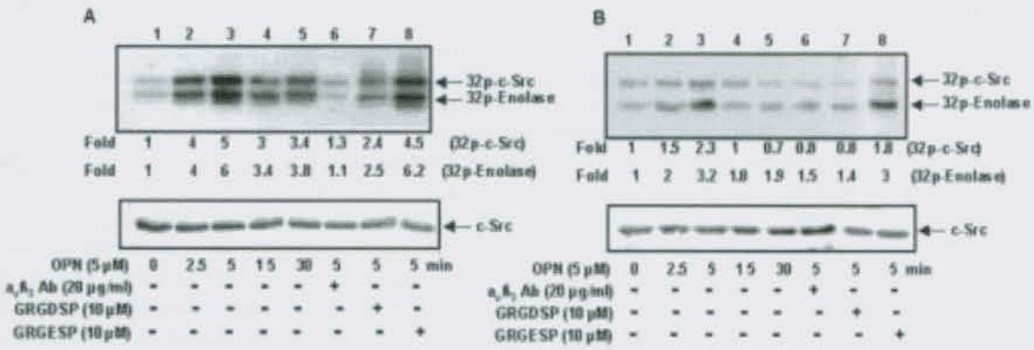

Fig. 1. OPN stimulates the autophosphorylation and kinase activity of c-Src. Both MDA-MB-231 (A) and MCF-7 (B) cells were either treated with 5 μM OPN for 0-30 min or pretreated with anti- $\alpha_v\beta_3$ integrin blocking antibody or RGD peptide and then treated with OPN. Cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-c-Src antibody and half of the immunoprecipitated samples were used for kinase assay using enolase as substrate (upper panels of A and B, lanes 1-8). The remaining half of the immunoprecipitated samples was analyzed by Western blot using anti-c-Src antibody (lower panels of A and B, lanes 1-8). The arrows indicate the specific bands of 32p-c-Src, 32p-enolase and c-Src. All these bands were quantified by densitometric analysis and the -fold changes are calculated.

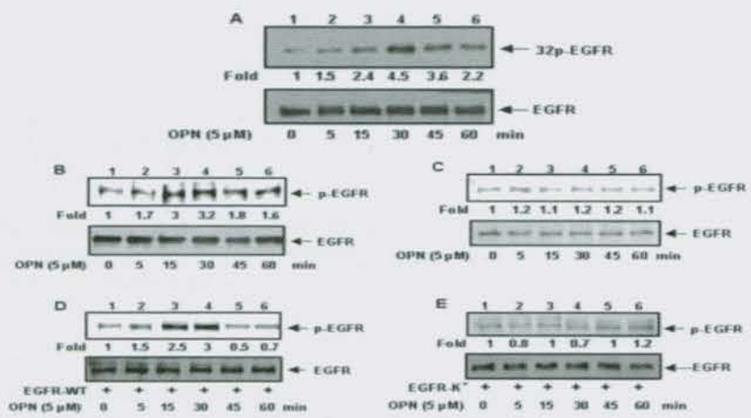

Fig. 2. OPN induces kinase activity and tyrosine phosphorylation of EGFR. A, EGFR kinase activity. MDA-MB-231 cells were stimulated with 5 μM OPN for 0-60 min, immunoprecipitated with anti-EGFR antibody. The half of the immunoprecipitated samples were used for kinase assay (upper panel, lanes 1-6) and remaining half was used for Western blot using anti-EGFR antibody (lower panel, lanes 1-6). **B and C,** EGFR tyrosine phosphorylation. Both MDA-MB-231 (B) and MCF-7 (C) cells were treated with 5 μM OPN for 0-60 min. Cell lysates were immunoprecipitated with anti-EGFR antibody, and the immunocomplexes were analyzed by Western blot with anti-phosphotyrosine antibody (lanes 1-6). **D and E**, MCF-7 cells were transfected with wild type EGFR (EGFR-WT) (D) or with kinase inactive mutant of EGFR (EGFR-K) (E) in presence of LipofectAMINE Plus and then treated with 5 μM OPN for 0-60 min. Cell lysates were immunoprecipitated with anti-EGFR antibody and immunoblotted with anti-phosphotyrosine antibody (lanes 1-6). These blots were reprobed with anti-EGFR antibody (B-E, lower panels). Note that maximum EGFR phosphorylation was observed at 30 min in MDA-MB-231 and EGFR-WT transfected MCF-7 cells. All these bands were quantified by densitometric analysis and the -fold changes are calculated.

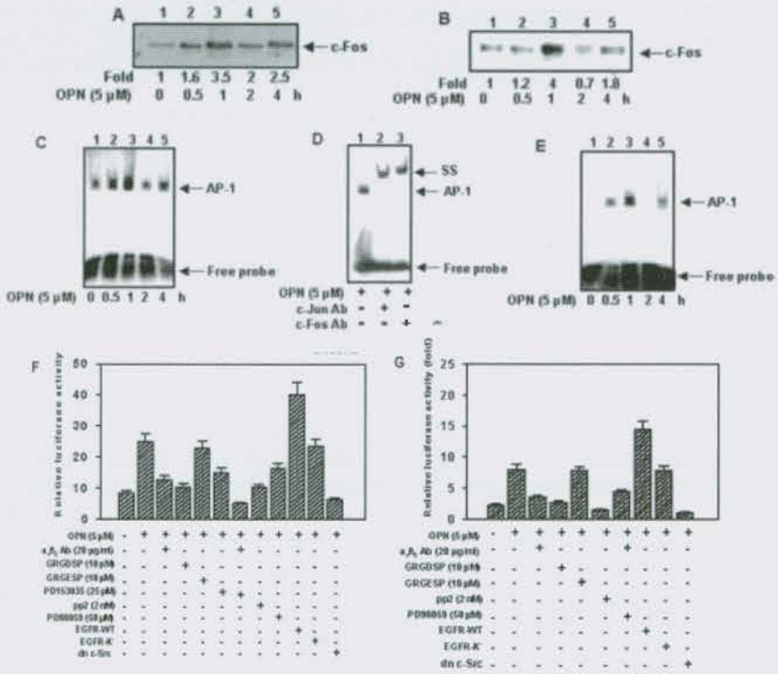

Fig. 3. A and B, OPN enhances c-Fos expression in breast cancer cells. MDA-MB-231 (A) and MCF-7 (B) cells were treated with OPN (5 μM) for 0-4 h. The nuclear extracts were prepared as described under "Experimental Procedures". The level of c-Fos in the nuclear extracts was analyzed by Western blot using anti c-Fos antibody (panels A and B, lanes 1-5). Note that maximum level of c-Fos was observed at 1h in both these cells. **C and E**, OPN induces AP-1-DNA binding. Both MDA-MB-231 (C) and MCF-7 (E) cells were treated with OPN (5 μM) for 0-4 h. The nuclear extracts were prepared and analyzed by EMSA. The arrow indicates the AP-1 specific bands. Note that maximum AP-1-DNA binding was also observed at 1 h in both these cells (lanes 1-5). D, supershift assay. The nuclear extracts from OPN-treated MDA-MB-231 cells were incubated with anti c-Jun or anti c-Fos antibody and then analyzed by EMSA (lanes 1-3). The results shown in A-E represent three experiments exhibiting similar effects. **F and G,** luciferase reporter gene assay. MDA-MB-231 (F) and MCF-7 (G) cells were transiently transfected with luciferase reporter construct (pAP-1-Luc) with LipofectAMINE Plus. Transfected cells were either stimulated with OPN (5 μM) for 6 h or pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP (10 μM), GRGESP (10 μM), PD153035 (25 pM) (MDA-MB-231 alone), pp2 (2 μM) or PD98059 (50 μM) and then treated with 5 μM OPN. In other experiments, cells were individually cotransfected with EGFR-WT, EGFR-K or dn c-Src in presence of pAP-1-Luc and then treated with 5 μM OPN. The cell lysates were used to measure the luciferase activity. The values were normalized to Renilla luciferase activity. The -fold changes were calculated, and mean ± S.E. of triplicate determinations is plotted.

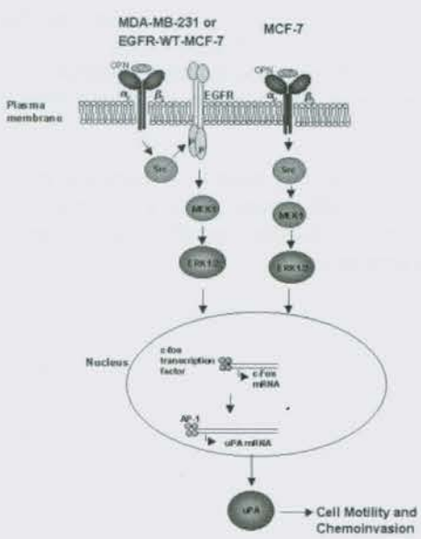

Fig. 4. Molecular mechanism of OPN-induced c-Src-mediated EGFR dependent/independent and ERK-regulated AP-1 activation, uPA secretion, and cell motility in breast cancer cells. OPN induces αvβ3 integrin-mediated c-Src kinase activity. In MDA-MB-231 cells, this leads to EGFR transactivation, ERK ½ phosphorylation and AP-1 activation which further stimulates uPA secretion, cell migration and invasion. However, in MCF-7 cells, OPN can directly induced c-Src-mediated ERK1/2 phosphorylation, AP-1 activation, uPA secretion, cell migration and invasion in absence of EGFR activation. PD153035, pp2, PD98059 and dn c-Src specifically disrupt these OPN-regulated c-Src/EGFR/ERK-dependent AP-1-mediated signaling pathways.

Future work

The signaling pathways by which upstream kinases regulate OPN-induced transcription factor activation and transcription factor-mediated gene expression will be studied.

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Role of mitochondrial proteins and molecular mechanisms in staurosporin induced apoptosis in neuroblastoma cells.

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Mitochondria are the key players in the re ulation of cell death. One of the crucial steps in apoptosis induced by anticancer a ents/dru s for elimination of tumors is the disruption of the mitochondrial membrane potential due to the formation of the permeability transition pores (PTP). The mitochondria respond to apoptotic stimuli by releasin a number of soluble apoptosis inducin proteins such as cytochrome c, apoptosis inducin factor Smac/Diablo and certain endonucleases from the intermembrane space into the cytosol. The PTP is proposed to be composed of the volta e dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and the mitochondrial matrix protein cyclophillin D. VDAC, localized in the outer mitochondrial membrane, forms the main interface between the mitochondrial and the cellular metabolisms. The activity of VDAC is believed to be directly modulated by the proteins of the Bcl-2 family.

In our earlier studies, we demonstrated that STS induced apoptosis in SK-N-MC cells is mediated primarily throu h the mitochondrial pathway. The objective of the study was to underline the role of the PTP proteins in staurosporine induced apoptosis in context with proapoptotic protein Bax, mitochondria and release of cytochrome c.

The experimental desi n involved induction of apoptosis in a human neuroblastoma cell line SK-N-MC cells in the presence or absence of inhibitors bon krekic acid, a li and for ANT, cyclosporine A a cyclophillin D inhibitor and DIDS, an anion channel blocker that inhibits VDAC. The effect was analyzed by sequential parameters such as breakdown in membrane potential, release of cytochrome c, activation of caspase cascade, and estimation of apoptosis by TUNEL assay. We observed that the addition of DIDS prevented the breakdown of membrane potential with STS treatment. In contrast, BA and CsA demonstrated no such protective effect (Fi .1A B). Studies elucidatin the down stream pathways revealed that DIDS completely blocked the cytochrome c release from mitochondria and activation of caspases (Fi .2A B). Investi ation of Bax expression showed translocation of Bax to the mitochondria that was hindered on treatment with DIDS su estin an association between the VDAC and Bax. Our data provides evidence for the essential role of VDAC and Bax in alterin the membrane potential and release of cytochrome c durin apoptosis induced by STS in SK-N-MC cells.

The study assumes importance in view with several lines of evidence implicatin the essential role of VDAC in apoptosis. Thus VDAC could be considered as a potential tar et for chemotherapeutic a ents, thou h further investi ation is necessary to provide evidence for the precise role in apoptosis.

Future studies are aimed at investi atin the involvement of bak, a propapototic molecule either directly or indirectly in Bax-VDAC interaction in apoptosis in our experimental system.

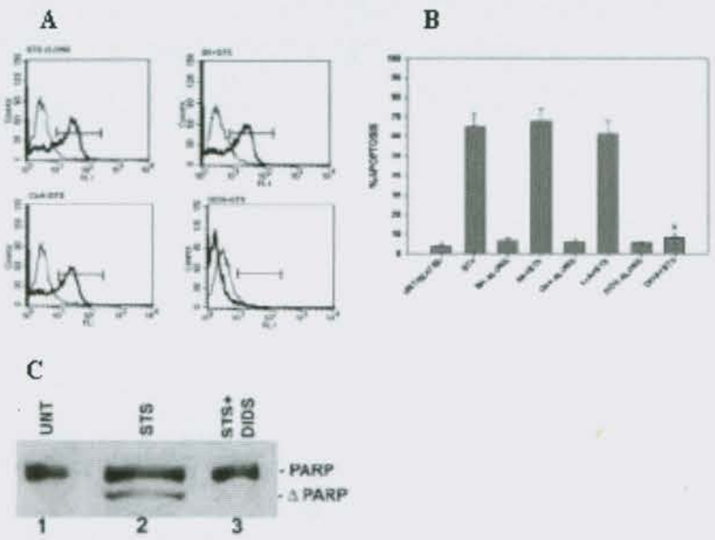

Fig. 1. Effect of inhibitions. BA,CsA and DIDS on STS induced by TUNEL assay A) Representative panel depicted with controls (thin lines) and treated (bold lines). B) Data represented as mean ± SD from three independent experiments. C) Expression of cleaved PARP in control and STS treated cells in the presence or absence of DIDS.

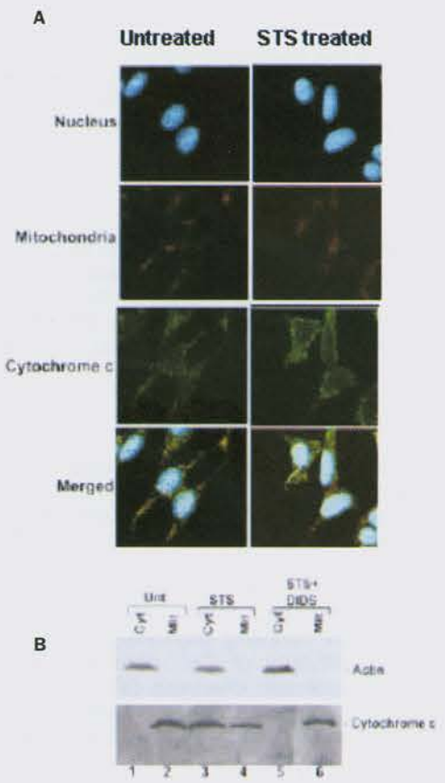

Fig. 2A. A representative panel depicting the cytochrome relese from mitochondria in STS treated cells visualized by confocal laser scanning microscopy. B. Expression of cytochrome C in mitochondrial and cytosolic fractions of untreated and cells treated with STS in the presence or absence of DIDS. Actin was used as loading controls in cytosolic fractions.

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Investigation of mitochondrial dysfunstion, oxidative damage and apoptotic cell death stress mechanism as a critical pathway in cardiomyocyte death induced by hyperinsulinemia/hyperglycemia.

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Abstract and background

Cell death, as a comprehensive consequence of myocardial abnormalities, is an important cause of various cardiomyopathies. Diabetes-induced cell death has been observed in multiple organs in vivo and in endothelial cells in vitro). Recent studies showed that the incidence of apoptosis increases in the heart of patients with diabetes and STZ-induced diabetic animals. The increased myocardial apoptotic cell death is related directly to hyperglycemia.
Mitochondria play an important role in apoptosis under a variety of proapoptotic conditions, such as oxidative stress. Among apoptotic stimuli, reactive oxygen species (ROS) have been shown to cause mitochondrial cytochrome c release and activation of caspase-3. A correlation between ROS generation and the pathogenesis of various diabetic complications has been observed. However, the source and mechanism of ROS induction are unclear. There are no studies on the direct measurement of ROS generation during high glucose treatment. Such direct measures are needed to clarify important questions that remain regarding the role of ROS as inducing agents, including their source, where they are metabolized, and the relative contributions of different oxidant species to the high glucose-induced cardiac cell death.
The purpose of our study is therefore to investigate the role of mitochondrial ROS in the induction of high glucose-induced cardiac cell death, and to clarify which ROS are required for the cell death response. For this study, we are using the embryonic, rat, cardiac muscle cell line, H9C2/in H9c2 cardiomyocytes and chick cardiomycytes, to study contractile activity.

Aims:

To investigate the role of mitochondrial ROS in the induction of oxidative myocardial injury, and to clarify which ROS are required for the cell death response. This study is designed to elucidate the impact of high glucose concentration mediated radical production in H9c2 (a clonal myogenic cell line derived from embryonic rat ventricle that can serve as a surrogate for cardiac or skeletal muscle in vitro) and STZ diabetic mice.

Objectives

- To investigate the role of mitochondrial ROS in the induction of hyperglycemia-induced myocardial apoptosis .
- To clarify which ROS are required for the cell death response (signaling pathways) including their source using STZ diabetic mice and H9c2 rat cardiac myoblast cells.
- To characterize the mitochondrial Ca^{2+} influx and efflux mechanisms in cardiac muscle cells and determine how these mechanisms regulate excitation-contraction coupling ultimately leading to new insights for therapy, the involvement/role of Ca^{2+} channels and the various subtypes of voltage-sensitive calcium channels (VSCCs) viz L-type (nifedipine-sensitive), N-type (omega-conotoxin GVIA-sensitive), and N- and P/Q-type (omega-conotoxin MVIIC-sensitive) will be studied using specific inhibitors.

Work achieved

We are testing whether exposure to high glucose induces cardiac cell death by way of generating ROS in rat cardiac myoblast H9c2. We exposed cardiac myoblast H9c2 cells to D-glucose in a final concentration of 22 and 33 mmol/l in cultures for in vitro treatment with high levels of glucose according to previous publications and exposed to 5.5 mmol/l D-glucose as control for 96 h. Exposure to high glucose increased apoptotic cell death from 4.5% to 39.95%. In the high glucose treated chick cardiomyocytes, increase in contractile activity (beats/min) was observed up to 60 hrs. compared with control cultures. During induction, ROS oxidation of the probe dichlorofluorescin (sensitive to H_2O_2) increased ~17-fold, DHE (sensitive to $O_2^{\cdot-}$) increased ~2-fold but not DHR-123 (sensitive to ONOO) as well as generation of NO (data shown earlier). Conversely, the DCF oxidation signal during hyperglycemia was attenuated with the antioxidant enzymes SOD, Catalse and reduced glutathione (GSH) and with the thiol reductant 2-mercaptopropionylglycine, L-NA and aspirin. The NAD(P)H oxidase inhibitor diphenyleneiodonium diphenyleneiodonium, cytosolic Cu,Zn-superoxide dismutase inhibitor diethyldithiocarbamic acid, and the anion channel inhibitor 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate, all of which also abrogated cell death. ROS generation during high glucose treatment was attenuated by a site I electron transport inhibitor rotenone, but not by a site III electron transport inhibitor myxothiazol. We conclude that high glucose increases mitochondrial superoxide generation from more than one source which initiates cell death. Furthermore, mitochondrial anion channels and cytosolic dismutation to H_2O_2 may be important steps for oxidant induction of high glucose-induced cardiac cell death.

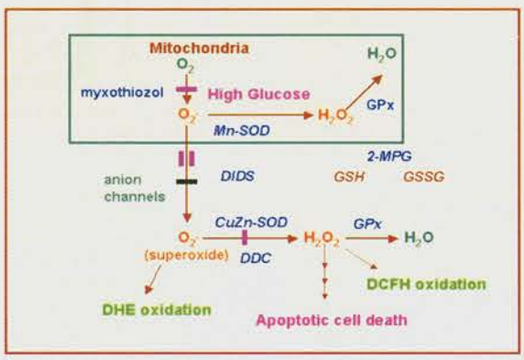


Fig. 1. Possible pathway of ROS generation and metabolisam during high Glucose treatment in H9c2 cells.

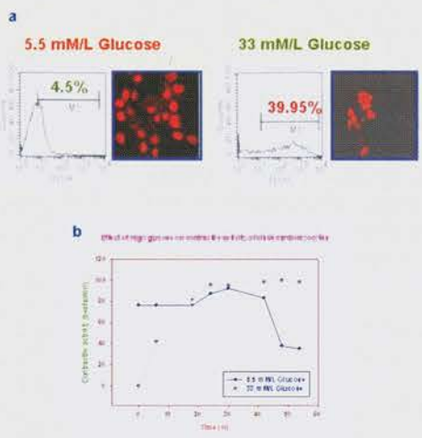


Fig. 2. Cell Death and Contractile Function in high glucose treated Versus control Cells – As reported previously, cell death in this model of high glucose treatment H9c2 cells occurred primarily after 96 h treatment. (a) After 96 h of treatment, cell death (PI uptake) in the present study was 39.95% in high glucose treated cells compared to controls (4.5%). (b) In the high glucose treated chick cardiomyocytes, increase in contractile activity (beats/min) was observed up to 60 hrs. compared with control cultures. Thus, treatment with high glucose significantly increased cell death and enhanced the contractile activity.

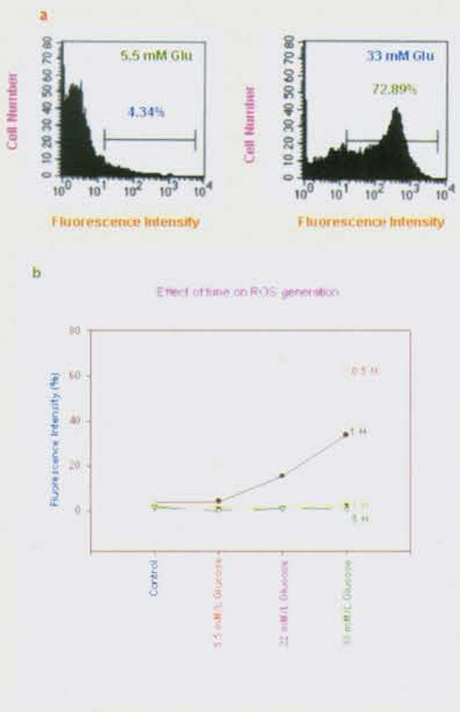


Fig. 3. Intracellular ROS Generation during the high glucose induced cell death - We next tested the role of ROS generation during high glucose treatment (a) DCF fluorescence during high glucose treatment. Brief exposure to high glucose caused a rapid and significant increase in ROS generation compared with controls (b) Of note, ROS generation increased during the initial 1 h treatment, and decreased thereafter.

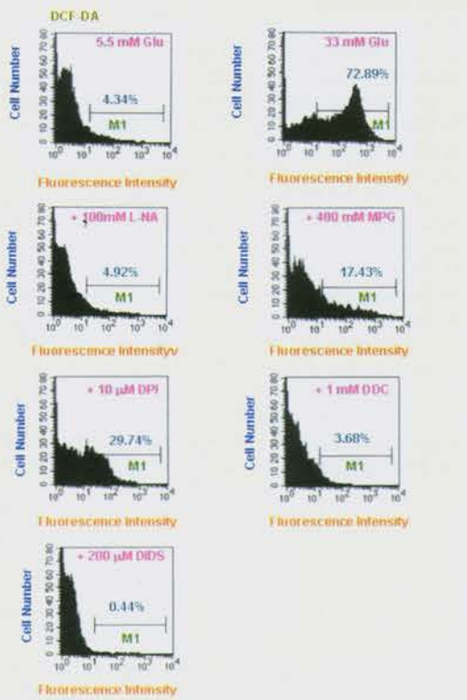
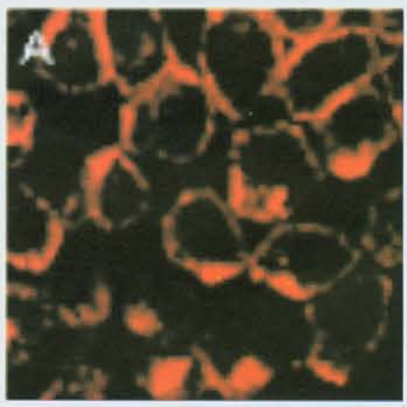


Fig. 4 Attenuation of ROS generation during high glucose treatment. The thiol reductant 2-mercaptopropionyl glycine, the inhibitor of nitric oxide and superoxide formation from NOS, L-NA and the COX-1 inhibitor aspirin as well as the NAD(P)H oxidase inhibitor diphenyleneiodonium, cytosolic Cu,Zn-superoxide dismutase inhibitor diethyldithiocarbamic acid, and the anion channel inhibitor 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate, all of which abrogated cell death. ROS generation during high glucose treatment was attenuated by a site I electron transport inhibitor rotenone, but not by a site III electron transport inhibitor myxothiazol.

Future work

1. Characterization of high glucose induced mitochondrial Ca2+ influx and efflux mechanisms in cardiac muscle cells and determine how these mechanisms regulate excitation-contraction coupling ultimately leading to new insights for therapy using various specific calcium anion channel blockers.
2. Study signaling pathways involved both in vitro and in vivo using specific inhibitors.
3. Study the effect of good glycemic control and therapeutic potential of multiple anti-oxidants on oxidative and nitrative stress in diabetic mice/rat.

Research Reports



Signal Transduction

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How α-hemolysin establishes the synergistic function with a Protein Tyrosine Phosphatase?

Ravi Vijayavargia, Anag Sahasrabuddhe, Ira Surolia
Anil Lotke, technician

Abstract and background

Information about the molecules that aid α-hemolysin (α-HL) of Staphylococcus aureus during its initial stages of binding and assembly at the surface of nucleated cells is not clear. Our studies show that the á-HL interacts with the Caveolin-1 present in A431 cells for its assembly. The information obtained by us suggests that the conformational changes required to form the heptameric pore are triggered with the help of Caveolin-1 binding motif of α-HL as the motif deficient mutants bind to the target cell surface, but do not undergo further conformational changes to form a heptameric pore. Moreover, mutants fail to behave like dominant negative mutants of α-HL indicating that the motif of each monomer is involved in initial conformational changes of α-HL. We have earlier shown that the α-HL assembly leads to signal attenuation. Detailed understanding of this phenomenon is very important as the identity of receptor-attenuating molecules for most receptor tyrosine kinases is still not known. Hence, the information on molecules that associate or work or supposed to work in synergy with EGFr are of tremendous importance for various reasons viz. (i) to know their role in signal transduction and cell proliferation (ii) to serve as starting steps for rational design of new generation molecules.

Aims:

1. After the removal of phosphorylation signal from EGFr, are the downstream signals affected? If so which ones are affected and what is the time frame at which these occur?

2. Which phosphatase is participating in the dephosphorylation of EGFr?

3. If phosphatase activation is positive, how such activation is taking place?

Work achieved

α-HL partitions into lipid rafts after binding to target cells:

In order to find out the molecule that aids α-HL during its assembly, we asked the question where does α-HL target itself on A431 cell surface?. We have chosen well established procedures to isolate lipid rafts of α-HL treated A431 cells using the detergent free, carbonate buffer approach. The lipid rafts of α-HL treated A431 cells were isolated by ultracentrifugation under sucrose density gradient. As shown in Fig. 1A the key components that authenticate the raft fractions viz. Caveolin-1 and EGFr are observed between the fractions 4 and 9, which is in accord with the studies published so far. Also α-HL is significantly concentrated between the fractions 4 and 9, very much like Caveolin-1 and EGFr. The amount of α-HL associated with the rafts is about 25-30% protein bound to cell surface. These results indicate the propensity of staphylococcal α-HL to localize in the lipid rafts at cell surface

Fig. 1A. α-HL is present in Caveolin-1 enriched membrane fractions: The Caveolin-1 containing fractions were obtained by ultracentrifugation. At the end of ultracentrifugation, the protein pellets were denatured in Laemmli sample buffer and split into three equal parts. α-HL panel was obtained by autoradiography. EGFr, Caveolin-1 panels were obtained by probing with appropriate antibodies. b-actin panel was obtained by probing with anti-b-actin after stripping the anti-Caveolin-1 antibody. The numbers on top of the lanes indicate the fraction numbers. C represents whole cell lysate. **1B:** Immuno-precipitation of [^{35}S-Met.] labeled α-HL with anti-Caveolin-1 antibody: A431 cells were treated with [^{35}S]-Methionine labeled α-HL as described in the methods section and then lysed in two different lysis buffers viz. IPA buffer and CHAPS. Lane 1 shows freshly translated [^{35}S]-Methionine labeled á-HL . Lanes 2 & 4 are controls in which only protein G agarose beads were added (anti-Caveolin-1 antibody was absent) and Lanes 3 & 5 shows the presence of [^{35}S]-Methionine labeled α-HL pulled down by anti-Caveolin-1 antibody.

Further proof of the interaction of α-HL with Caveolin-1 was obtained by pull down assay, shown in Fig. 1B. The data in Fig. 1B were obtained by treating A431 cells with [^{35}S]-methionine labeled α-HL and the cell lysate was immunoprecipitated with anti-Caveolin-1 antibody, which clearly shows the presence of α-HL. We have also proved that the α-HL does not immuno precipitate with the anti-EGFr, which is well known to interact with the Caveolin-1, under similar conditions.

We have also analyzed the binding of scaffolding domain peptide of Cav-1 to α-HL by surface plasmon resonance. In these experiments, we have immobilized the biotinylated Cav-1 peptide on a streptavidin coated sensor chip SA and various concentrations of α-HL were flown through the chip. The sensograms in Fig. 2 show a time dependent rise and diminution in RUs when α-HL and buffer, respectively, were passed over Caveolin-1 peptide coated chip highlighting again the interaction between α-HL with the scaffolding domain of Caveolin-1. In addition, this data suggests that the signals observed in ELISA experiments is not due to any non-specific interactions as the residuals errors observed for the sensograms is well within ±2 RUs. Moreover, Denatured α-HL showed no change in response units when it was passed over the immobilized Cav-1 peptide surface under the identical conditions attesting to the specificity of Cav-1 peptide-α-HL interaction. A preliminary analysis of this interaction suggests a two step process. Remarkably, α-HL dissociates very slowly (κ_{-1} ~10^{-4} sec^{-1}) from its complex with Cav-1 peptide indicating that the complex, of α-HL and Cav-1 peptide has exceptional stability under the physiological conditions.

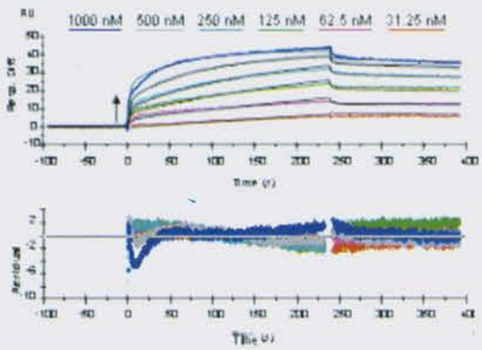

Fig. 2. Surface plasmon resonance traces of α-HL binding to Cav-1 peptide: Biotinylated Cav-1 peptide was immobilized on a streptavidin coated SA sensor chip. Various concentrations of α-HL in HBS-EP buffer were passed simultaneously through both flow cell 2 and flow cell 1, while flow cell 1 is blank. The arrow indicates the direction of increasing concentrations of α-HL and various concentrations are represented by indicated colours. The Black curves overlapping the sensograms are obtained by global fitting of the sensograms. Lower panel: The residuals of the fitted curves (± 2 Ru).

Colocalization of α-HL and Caveolin-1: We then examined whether or not α-HL co-localizes with Caveolin-1 on A431 cells upon binding. As shown in Fig. 3D we observe co-localization of α-HL with Caveolin-1 on the cell surface, which is easily noticeable. Interestingly, upon the α-HL treatment, a lot of clustering of the label associated with Caveolin-1 is seen on the cell surface. Normal cells, for example, show an even distribution of Caveolin-1 before α-HL treatment (3E). However, upon α-HL treatment, redistribution or clustering of Caveolin-1 is seen very prominently (Fig. 3D Vs. 3E). It is clear from the published work that Caveolin-1 is distributed evenly after EGF stimulation in A431 cells. All these results suggest that the α-HL induced change in Caveolin-1 organization is distinct and clear, probably through a direct interaction with Caveolin-1 beneath the cell surface.

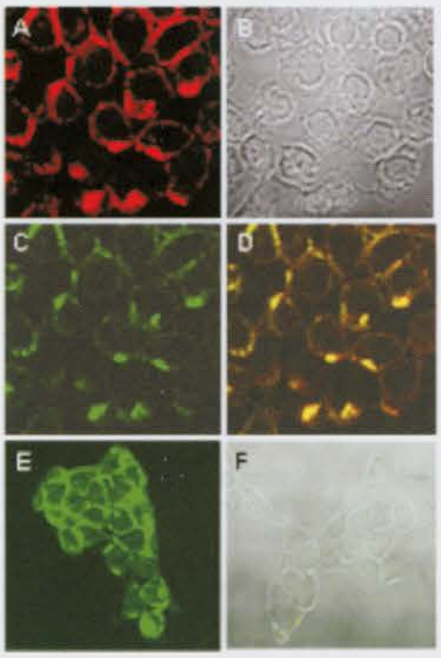

Fig. 3. Visualization of co-localization of α-HL with Caveolin1 using Confocal Microscopy: Representative panels of the colocalization of α-HL with Caveolin-1 on A431 cells in which cells were treated with Rhodamine conjugated α-HL for 15 min at room temperature and then the specimens were processed for specific staining. (A) Rhodamine conjugated α-HL alone (B) shows cells in phase contrast, (C) fluorescent staining of Caveolin-1 with anti-Caveolin-1 antibody. (D) overlay of panel A and C to show colocalisation of α-HL with Caveolin-1. (E) Distribution of Caveolin-1 on A431 cell surface before α-HL treatment. (F) Negative control for anti-Caveolin-1 antibody. All the panels were obtained with an identical settings.

In summary, our studies clearly illustrate that the assembly of α-HL on nucleated A431 cells invoke interaction with Caveolin-1 present in the lipid rafts. Moreover, our study shows that the interaction of the 9 amino acid motif of α-HL with Caveolin-1 results in a strong adherence of the heptameric oligomers of α-HL to cell surface. Thus, α-HL is distinct from the other members of pore forming family of toxins viz. Aerolysin, CsαHL etc. in a significant way essentially due to its Caveolin-1 dependent assembly. During this oligomerisation/assembly of α-HL, Caveolin-1 is reorganized beneath the cell surface and that reorganization of Caveolin-1 network is perhaps responsible for an attenuation of receptor mediated signaling events.

Future work:

Our future work involves the investigation of changes in the integrity and lipid raft network of mammalian cells after the assembly of α-HL by immuno electron microscopy.

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Studies on role of protein tyrosine kinase, p56lck in regulation of cell motility and nuclear factor κB-mediated secretion of uPA through tyrosine phosphorylation of IκBα

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Pallavi Bapat, Shalini Jain, Sailaja Badi, Sutirtha Datta, Anuradha Bulbule, technician

Collaborators:
C.G. Naik and Dr. P. S. Parameswaran NIO, Goa.
Project: Identification of Bioactive Compound from Marine Sources.

Background and Abstract:

Nuclear factor κB (NFκB) plays major role in regulating cellular responses due to environmental injuries. Lck, a member of the Src family non-receptor protein tyrosine kinase, is expressed in T cells, B cells as well as breast cancer cell lines. However, the molecular mechanism by which $p56^{lck}$ in presence of reactive oxygen species (ROS) generated by hypoxia/reoxygenation (H/R) regulates NF-κB activation and modulates the expression of downstream genes, which are involved in breast cancer cell motility is not well defined. We have demonstrated that H/R induces $p56^{lck}$ dependent NF-κB transactivation through tyrosine phosphorylation of IκBα. H/R also enhances the interaction between SH2 domain of Lck and tyrosine phosphorylated IκBα. Lck induces NF-κB dependent uPA promoter activity and cell motility through secretion of uPA in presence of H/R. Taken together, $p56^{lck}$ in presence of H/R regulates NF-κB activation, uPA secretion and cell motility through tyrosine phosphorylation of IκBa.

Aims:

(a) To investigate whether reactive oxygen species (ROS) generated by hypoxia/reoxygenation (H/R) regulates $p56^{lck}$ activation and $p56^{lck}$ dependent NF-κB activation through tyrosine phosphorylation of IκBα,

(b) To examine whether $p56^{lck}$ in presence of H/R induces NF-κB dependent uPA promoter activity,

(c) To delineate whether $p56^{lck}$ regulates H/R-induced cell motility and uPA secretion through activation of NF-κB and to demonstrate whether this pathway is independent of IKK / IκBα-mediated signaling pathway.

Work Achieved

We investigated the involvement of protein tyrosine kinase, $p56^{lck}$ in the redox regulated activation of NF-κB following H/R in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. We report that H/R induces tyrosine phosphorylation of $p56^{lck}$, nuclear translocation of NF-κB, NF-κB-DNA binding and transactivation of NF-κB through tyrosine phosphorylation of IκBα. Transfection of these cells with wild type Lck but not with mutant Lck F394 followed by H/R induces the tyrosine phosphorylation of IκBα and transcriptional activation of NFκB and these are inhibited by Lck inhibitors. In vitro kinase assay demonstrated that immunoprecipitated $p56^{lck}$ but not Lyn or Fyn directly phosphorylate IκBα in presence of H/R. Pervanadate, H_2O_2 and H/R induce the interaction between Lck and tyrosine phosphorylated IκBα and this interaction is inhibited by SH2 domain inhibitory peptide suggesting that tyrosine phosphorylated IκBα interacts with SH2 domain of

Lck. Luciferase reporter gene assay indicated that Lck induces NFκB dependent uPA promoter activity in presence of H/R. Furthermore, H/R stimulates the cell motility through secretion of uPA. This is the first report that $p56^{lck}$ in presence of H/R regulates NFκB activation, uPA secretion, and cell motility through tyrosine phosphorylation of IkBa and further demonstrates an important redox-regulated pathway for NFκB activation following H/R injury that is independent of IKK/IκBα-mediated signaling pathways.

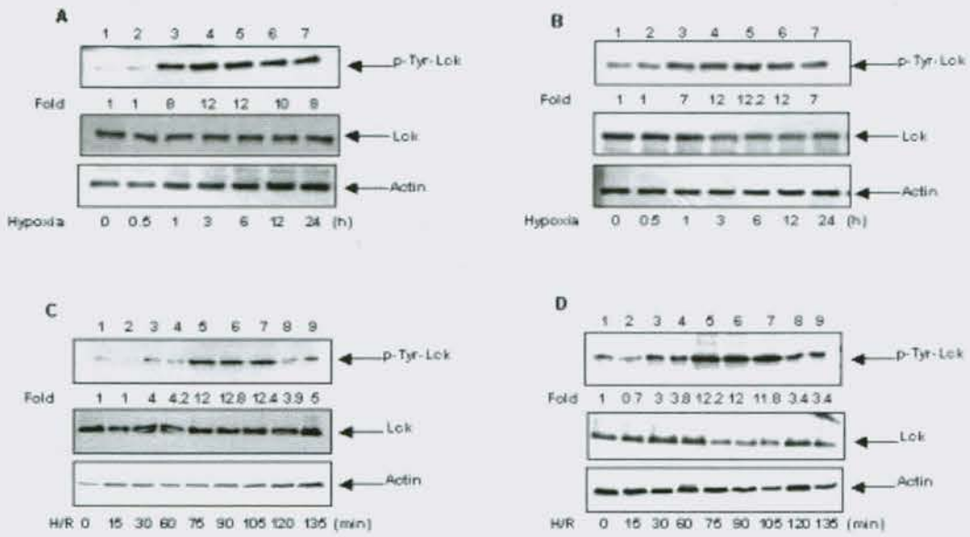

Fig. 1. H/R induces tyrosine phosphorylation of Lck. **A and B,** both MCF-7 (panel A) and MDA-MB-231 (panel B) cells were exposed with hypoxia for 0 to 24 h and reoxygenated for 90 min. Cell lysates containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-Lck antibody and half of the immunoprecipitated samples were analyzed by Western blot using mouse monoclonal anti phosphotyrosine antibody. The remaining half of the immunoprecipitated samples were immunoblotted with anti-Lck antibody. Note that maximum H/R induced Lck phosphorylation was observed between 3-6 h (upper panels of A and B, lanes 1-7) whereas the expression of non-phospho Lck was unchanged (middle panels of A and B, lanes 1-7). Actin was used as loading control (lower panels of A and B). **C and D,** both MCF-7 (panel C) and MDA-MB-231 (panel D) cells were exposed with hypoxia for 3 h and reoxygenated for 0-135 min. Equal amount of total proteins from cell lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody. Half of the immunoprecipitated samples were analyzed by Western blot using anti-phosphotyrosine antibody (upper panels of C and D, lanes 1-9), the remaining half of the samples were immunoblotted with anti-Lck antibody (middle panels of C and D, lanes 1-9). Note that maximum tyrosine phosphorylation of Lck is observed when cells are reoxygenated for 75 min and sustained upto 105 min (upper panels in C and D, lanes 5-7). The expression of non-phospho Lck under these conditions was remained same (middle panels of C and D, lane 1-9). Actin was used as loading control (lower panels of C and D). All of these bands were quantified by densitometric analysis and the values of -fold changes are calculated.

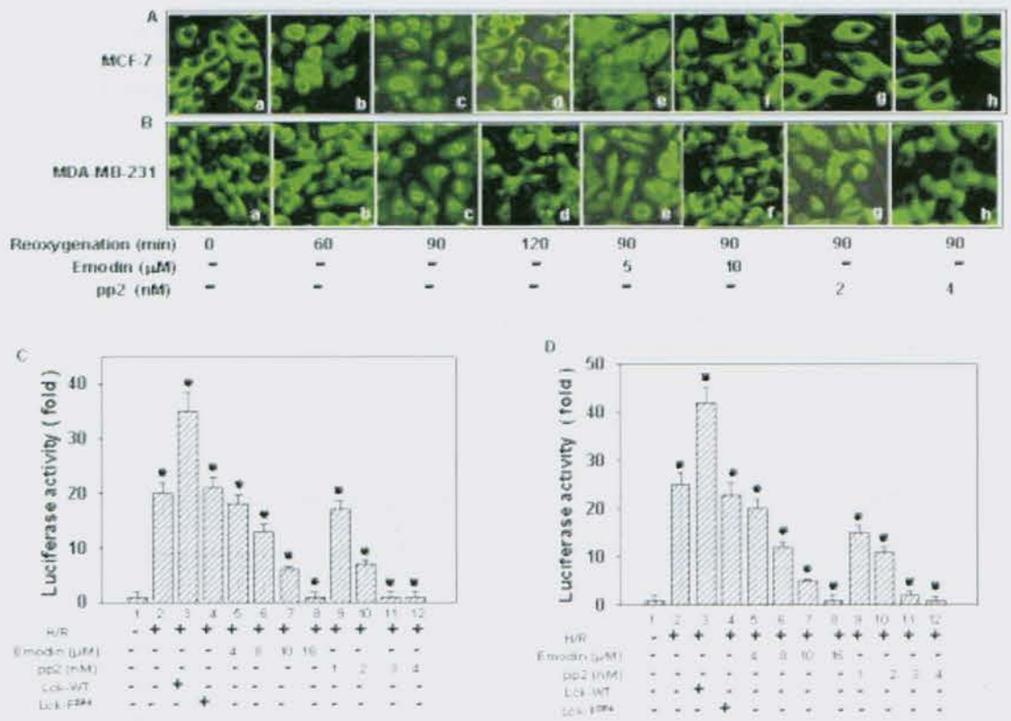

FIG. 2 A and B, H/R induces Lck-mediated nuclear translocation of p65 subunit of NFκB. MCF-7 (A) and MDA-MB-231(B) cells were induced by hypoxia for 3 h and reoxygenated for 0-120 min. In separate experiments, these cells were individually pretreated with different concentrations of emodin (0-10 μM) and pp2 (0-4 nM) for 6 h, induced by hypoxia for 3 h and reoxygenated for 90 min. These cells were fixed, incubated with rabbit polyclonal anti-NFκB, p65 antibody, incubated further with FITC-conjugated anti-rabbit IgG and analyzed under confocal microscopy. panel a, untreated cells; panel b, reoxygenation for 60 min; panel c, reoxygenation for 90 min; panel d, reoxygenation for 120 min; panel e, treated with emodin (5 μM) and then with H/R; panel f, treated with emodin (10 μM) and then with H/R; panel g, treated with pp2 (2 nM) and then with H/R and panel h, treated with pp2 (4 nM) and then with H/R. Note that there was H/R-induced translocation of p65 at 90 min in both MCF-7 and MDA-MB-231 cells but it was shuttled back at 120 min. Both emodin and pp2 inhibited the H/R-induced nuclear translocation of p65. **C and D, H/R stimulates Lck-mediated NFκB transactivation.** Both MCF-7 (C) and MDA-MB-231 (D) cells were transiently transfected with luceferase reporter construct (pNFκB-Luc) in the presence of LipofectAMINE Plus as described under "Experimental Procedures". In separate experiments, these transfected cells were treated with Lck inhibitors (emodin or pp2). In another experiments, these cells were transfected with wild type Lck or mutant Lck F394 along with pNFκB-Luc. The transfection efficiency was normalized by cotransfecting the cells with pRL vector, induced by hypoxia for 3 h and reoxygenated for 24 h and luciferase activity was measured. The cells transfected with wild type Lck followed by induction with H/R showed maximum pNFκB luciferase activity compared with mutant Lck F394 transfected cells or cells transfected with LipofectAMINE Plus alone. Both emodin and pp2 dose dependently suppressed the H/R-induced pNF B luciferase activity (panels C and D). The values were normalized to Renilla luciferase activity. The -fold change were calculated, and the results are expressed as the means ± S.E. of three determination. The values were also analyzed by Student's t test (*, $p < 0.001$).

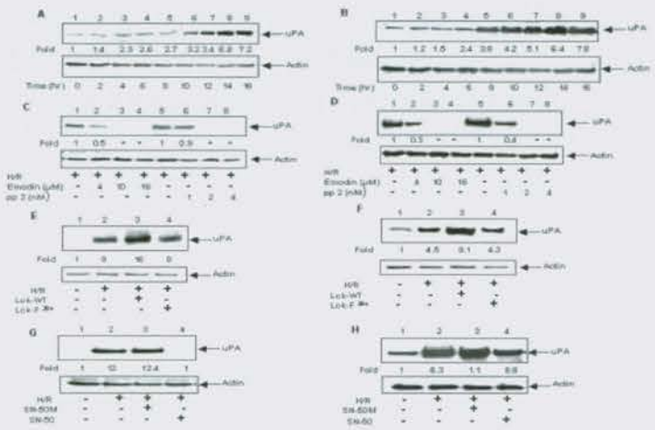

FIG. 3 A and B, H/R induces uPA secretion in breast cancer cells. Both MCF-7 (A) and MDA-MB-231 (B) cells were induced by hypoxia for 3 h and reoxygenation for 0-16 h, lysed and the level of uPA in the lysates were detected by Western blot analysis using anti-uPA antibody (upper panels of A and B, lanes 1-9). **C and D, Lck inhibitor (emodin or pp2) suppresses H/R-induced uPA secretion.** Both MCF-7 (C) and MDA-MB-231(D) cells were pretreated with different doses of Lck inhibitors (emodin and pp2) and then induced by hypoxia for 3 h and reoxygenated for 16 h. The level of uPA in the cell lysates was detected by Western blot analysis (upper panels of C and D, lanes 1-8). Both emodin and pp2 suppressed the H/R-induced uPA secretion in a dose dependent manner. **E and F, over expression of Lck enhances H/R-induced uPA secretion.** Both MCF-7 (E) and MDA-MB-231 (F) cells were transiently transfected with wild type Lck or mutant Lck F394, induced by H/R as described above and the level of uPA in the cell lysates were detected by Western blot analysis. Wild type Lck (upper panels of E and F, lane 3) but not Lck F394 (lane 4) induced the uPA secretion indicating that tyrosine 394 is involved in this process. The level of uPA in H/R induced cell lysates (lane 2) was almost identical as mutant transfected cells (lane 4) whereas almost no uPA expression was detected in non-H/R-induced cells (lane 1). **G and H, effect of NFκB modulators on H/R-induced uPA secretion.** Both MCF-7 (G) and MDA-MB-231 (H) cells were treated with NFκB inhibitory peptides (SN-50 and SN-50M). These cells were induced by H/R as described above and uPA level was detected by Western blot analysis. The H/R-induced uPA expression (upper panels of G and H, lane 2) is blocked by SN-50 peptide (lane 4) but not with control SN-50M peptide (lane 3). The level of uPA was absent in non-induced MCF-7 cells (lane 1) whereas significant level of uPA was detected in non-induced MDA-MB-231 cells (lane 1). As loading controls, all these blots were reprobed with anti-actin antibody (lower panels of A-H). The bands were quantified by densitometric analysis and the values of -fold changes are calculated.

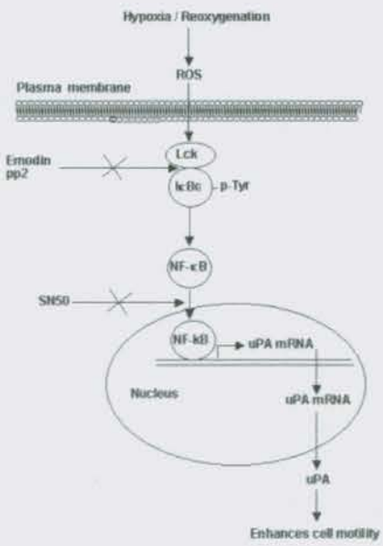

FIG. 4. Molecular mechanism of H/R-induced $p56^{lck}$-dependent NF-κB activation and uPA secretion through tyrosine phosphorylation of IκBa in breast cancer cells. H/R induces cell motility and tyrosine phosphorylation of Lck. H/R stimulates Lck-mediated NF-κB activation through tyrosine phosphorylation of IκBa. H/R induces NF-κB-dependent uPA promoter activity and cell migration through expression of uPA in these cells. Emodin, pp2 and SN-50 specifically disrupt these signaling pathway.

Future Plan

The molecular mechanism of protein tyrosine kinase regulated NF-κB mediated gene expression and cell motility will be further delineated.

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Role of tyrosine kinase, p56lck in regulation of uPA secretion through activation of epidermal growth factor receptor and extracellular signal regulated kinase pathways.

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Pallavi Bapat, Shalini Jain, Sailaja Badi, Sutirtha Datta, Anuradha Bulbule, technician

Background and Abstract

We have recently reported that tyrosine kinase, $p56^{lck}$ regulates cell motility and nuclear factor κB-mediated secretion of uPA through tyrosine phosphorylation of IκBα following hypoxia/reoxygenation (H/R). However, the role of H/R on ERK1/2-mediated uPA secretion and cell motility and the involvement of $p56^{lck}$ and EGF receptor in these processes in breast cancer cells are not well defined. In this study, we report that H/R induces Lck-dependent EGF receptor phosphorylation in breast cancer cells. $p56^{lck}$ in presence of H/R stimulates EGF receptor regulated MEK-dependent ERK1/2 phosphorylation, uPA secretion and cell motility. Taken together, we have demonstrated that $p56^{lck}$ in presence of H/R regulates MEK-1 dependent ERK1/2 phosphorylation, uPA secretion and cell motility through tyrosine phosphorylation of EGF receptor.

Aims:

(a) To examine the effect of hypoxia/reoxygenation (H/R) on $p56^{lck}$ dependent EGF receptor transactivation in low (MCF-7) and highly (MDA-MB-231) invasive breast cancer cells,

(b) To investigate the role of $p56^{lck}$ on EGF receptor dependent MEK/ ERK1/2 phosphorylations in presence of H/R in these cells,

(c) To study whether $p56^{lck}$ regulates EGF receptor/ERK1/2 dependent uPA secretion and cell motility in breast cancer cells.

Work achieved

We provide here evidence that H/R induces Lck kinase activity and Lck-dependent tyrosine phosphorylation of EGF receptor in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. H/R stimulates MEK-1 and ERK1/2 phosphorylations. H/R-induced MEK/ERK1/2 phosphorylations were suppressed by dominant negative form of Lck (DN Lck, K273R) as well as pharmacological inhibitors of Lck and EGF receptor indicating that Lck and EGF receptor are involved in these processes. Transfection of these cells with wild type Lck or Lck F505 but not with Lck F394 induced phosphorylations of EGF receptor followed by MEK-1 and ERK1/2 suggesting that Lck is upstream of EGF receptor and Tyr-394 of Lck is crucial in these processes. H/R also induced uPA secretion and cell motility in these cells. DN Lck and inhibitors of Lck, EGF receptor and MEK-1 suppressed H/R-induced uPA secretion and cell motility. In summary, $p56^{lck}$ in presence of H/R regulates MEK-1 dependent ERK1/2 phosphorylation and uPA secretion through tyrosine phosphorylation of EGF receptor and further demonstrates that all these signaling molecules ultimately control breast cancer cell motility.

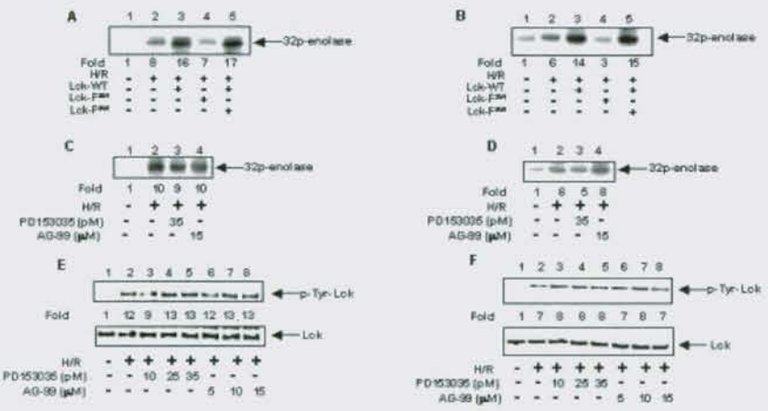


FIG. 1 A and B, hypoxia/reoxygenation (H/R) induces Lck kinase activity in low invasive (MCF-7) and highly invasive (MDA-MB-231) breast cancer cells. Both MCF-7 (panel A) and MDA-MB-231 (panel B) cells were individually transfected with wild type or mutant Lck (F394 or F505) and then induced by H/R for 90 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody. The immunoprecipitated samples were used for Lck kinase assay using rabbit muscle enolase as substrate. Lane1: without H/R; lane 2: induced by H/R; lane 3: transfection with wild type Lck and induced by H/R; lane 4: transfection with Lck F394 and induced by H/R and lane 5: transfection with Lck F505 and induced by H/R. **C and D, H/R-induced Lck kinase activity is independent of EGF receptor activation.** MCF-7 (C) and MDA-MB-231 (D) cells were pretreated with EGF receptor inhibitors and then induced by H/R as described. The cell lysates were immunoprecipitated with anti-Lck antibody and used for Lck kinase assay. Lane1: without H/R; lane 2: induced by H/R; lane 3: treated with PD153035 and induced by H/R and lane 4: treated with AG-99 and induced by H/R. **E and F, H/R-induced tyrosine phosphorylation of Lck is independent of EGF receptor activation.** MCF-7 (E) and MDA-MB-231 (F) cells were pretreated with various concentrations of EGF receptor inhibitors and then induced by H/R. The cell lysates were immunoprecipitated with anti-Lck antibody and the immunoprecipitated samples were analyzed by Western blot using anti-phosphotyrosine antibody (upper panels of E and F, lanes 1-8). The same blots were reprobed with anti-Lck antibody (lower panels of E and F, lanes 1-8).

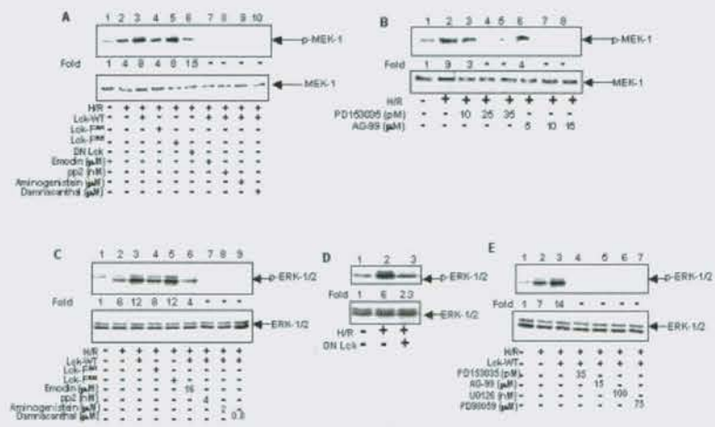


Fig. 2. Roles of $p56^{lck}$ and EGF receptor on H/R induced MEK-1 phosphorylation (Ser-222 and 226). A, MDA-MB-231 cells were transiently transfected with wild type Lck, DN Lck, mutant Lck F394 or Lck F505 and wild type Lck transfected cells were treated with maximum doses of Lck inhibitors and induced by H/R. The levels of phospho-MEK-1 and MEK-1 were detected by Western blot analysis. Cells transfected with wild type Lck or Lck F505 showed maximum phosphorylation of MEK-1 compared with cells transfected with Lck F394 or induced by H/R alone (upper panel A, lanes 1-5). DN Lck inhibited the H/R-induced MEK-1 phosphorylation (lane 6). Lck inhibitors drastically inhibited H/R-induced MEK-1 phosphorylation in wild type Lck-transfected cells (lanes 7-10). **B,** MDA-MB-231 cells were treated with various concentrations of EGF receptor inhibitors, induced by H/R and cell lysates were immunoblotted with anti-phospho MEK-1 antibody. The results indicated that EGF receptor inhibitors suppressed the H/R-induced MEK-1 phosphorylation in these cells (upper panel B, lanes 1-8). The same blots were reprobed with anti-MEK antibody as loading control (lower panels of A and B). **C-E, H/R induced $p56^{lck}$-dependent ERK1/2 phosphorylation is occurred through EGF receptor and MEK-1 mediated pathways.** MDA-MB-231 cells were individually transfected with wild type Lck, DN Lck, mutant Lck F394 or Lck F505 and then induced by H/R. In separate experiments, wild type Lck transfected cells were treated with maximum doses of inhibitors of Lck, EGF receptor or MEK-1 and induced by H/R. The level of phospho ERK1/2 was detected by Western blot using anti-phospho ERK1/2 antibody. The phosphorylation of ERK1/2 was higher in cells transfected with wild type Lck or Lck F505 compared with Lck F394 transfected cells or cells induced by H/R alone (upper panel C, lanes 1-5). Lck inhibitors dramatically blocked the H/R-induced ERK phosphorylation of wild type Lck transfected cells (lanes 6-9). DN Lck suppressed the H/R-induced ERK1/2 phosphorylation (upper panel D, lanes 1-3). Inhibitors of EGF receptor and MEK-1 suppressed the H/R-induced ERK1/2 phosphorylation in wild type Lck transfected cells (upper panel E, lanes 1-7). As loading control, all these blots were reprobed with anti-ERK1/2 antibody (lower panels of C-E). All these bands were quantified by densitometric analysis and the values of -fold changes are calculated.

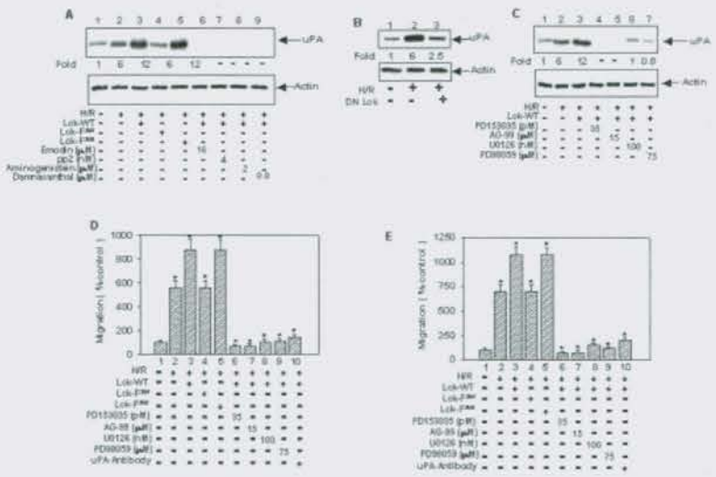


Fig. 3. A-C, H/R enhanced p56^{lck} dependent uPA secretion is occurred through EGF receptor and MEK-1-mediated pathways. MDA-MB-231 cells were transiently transfected with wild type Lck, mutant Lck F394, Lck F505 or DN Lck and wild type Lck transfected cells were treated with inhibitors of Lck, EGF receptor or MEK-1 and then induced by H/R. The expression of uPA was detected by Western blot using anti-uPA antibody. Wild type Lck or Lck F505 enhanced the uPA secretion compared with Lck F394 transfected cells or cells induced by H/R (upper panel A, lanes 2-5). Low level of uPA was detected in MDA-MB-231 cells grown under normoxic condition (lane 1). Lck inhibitors drastically suppressed the H/R-induced uPA secretion in wild type Lck transfected cells (lanes 6-9). Cells transfected with DN Lck suppressed the H/R-induced uPA secretion (upper panel B, lanes 1-3). Inhibitors of EGF receptor or MEK-1 suppressed the H/R-induced uPA secretion in wild type Lck transfected cells (upper panel C, lanes 1-7). All these blots were reprobed with anti-actin antibody (lower panels of A-C). The bands were quantified by densitometric analysis, normalized with respect to actin and -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects. **D and E, EGF receptor, MEK-1 and uPA play crucial roles on H/R-induced Lck-mediated cell migration.** MCF-7 (D) and MDA-MB-231 (E) cells were transiently transfected with wild type Lck, Lck F394 or Lck F505 and wild type Lck transfected cells were treated with inhibitors of EGF receptor or MEK-1 or with anti-uPA antibody and then induced by H/R. These cells were used for migration assay. Cells transfected with either wild type Lck or Lck F505 followed by induction with H/R showed maximum cell migration compared with Lck F394 transfected cells or cells induced by H/R alone (panels of D and E) indicating that Tyr-394 of Lck is crucial for H/R induced cell migration. Inhibitors of EGF receptor or MEK-1 or anti-uPA antibody suppressed the H/R-induced cell migration in wild type Lck transfected MCF-7 (panel D) and MDA-MB-231 (panel E) cells. The results are expressed as means ± S.E. of three determinations. The values were analyzed by Student's t test

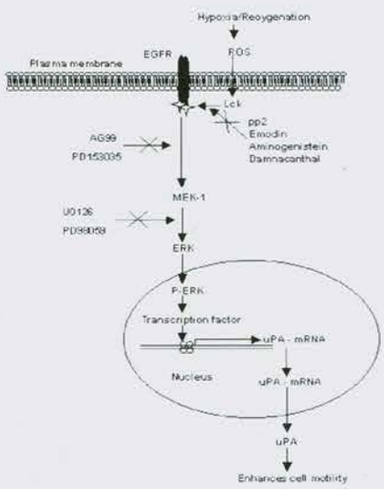
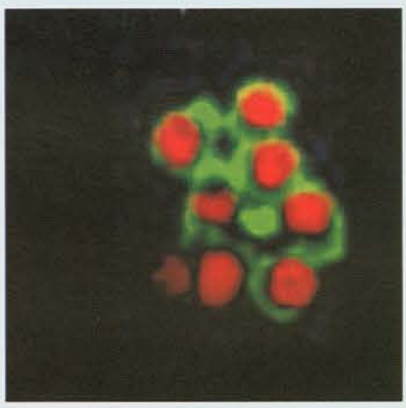


Fig. 4. Molecular mechanism of H/Rinduced Lck-dependent MEK-1-regulated ERK1/2 phosphorylation, uPA secretion and cell motility through tyrosine phosphorylation of EGF receptor in breast cancer cells. H/R induces Lck phosphorylation and its kinase activity through tyrosine phosphorylation of EGF receptor. Lck stimulates MEK-1-dependent ERK1/2 phosphorylation, uPA secretion and cell motility in presence of H/R in these cells. Emodin, pp2, aminogenistein, damnacanthal, AG-99, PD153035, U0126, PD98059 and DN Lck (K273R) specifically disrupts this signaling pathway.

Future work:

The role of p56^{lck} in regulation of MAPK activation and AP-1 transactivation in breast cancer cells will be further studied.

Research Reports



Diabetes

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Divergent approaches for control and management of experimental diabetes

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Abstract and background:

Diabetes mellitus is a multifactorial metabolic syndrome characterized by hyperglycemia due to depletion of beta cell mass resulting into deficient insulin production and/or insulin resistance. Several strategies are in force to control the hyperglycemia which is the root cause for diabetic complications. Elucidation of the regenerative potential in experimental diabetes holds promise as an alternative therapy for diabetes. Islet neogenesis from adult stem cells has tremendous potential for pancreatic regeneration and thereby for the better control and management of diabetes. We have recently shown that intra-islet precursor cells and pancreatic duct epithelial cells from the adult mouse pancreas have the capacity to give rise to new islets in vitro when stimulated appropriately. However little is known about the role of extra-pancreatic stem cells in islet neogenesis and diabetes management. Equally important is the search for novel oral insulin-mimetic substances to alleviate the pains of insulin shots.

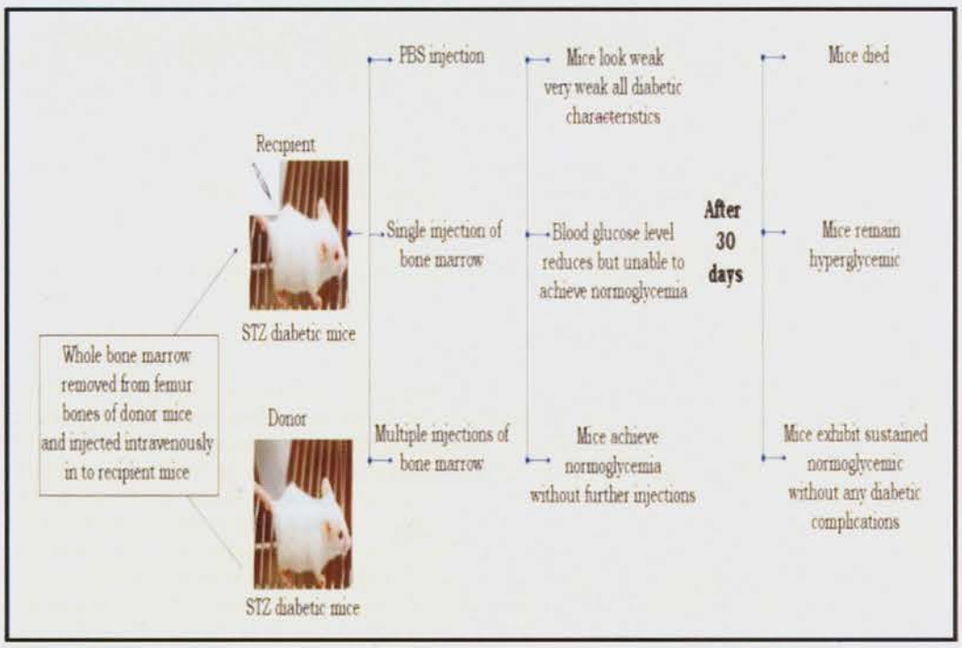
Aims:

1. To induce islet neogenesis from pancreatic as well as extra-pancreatic stem cells.
2. To design, synthesize and screen novel metal-based oral insulin-mimetic agents.

Work achieved:

1. To induce islet neogenesis from pancreatic as well as extra-pancreatic stem cells:

In our earlier study we have shown that intra islet precursor cell population remains intact even after streptozotocin insult. We further extended our study to search for extra pancreatic stem cells as a candidate for islet neogenesis and/or pancreatic regeneration. We have taken bone marrow stem cells as a representative of extra pancreatic stem cells. In our earlier studies we have shown that whole bone marrow from a normal mouse can induce pancreatic regeneration upon transplantation into experimental diabetic mice. To test the hypothesis that bone marrow stem cell population maintains its stemness under sustained hyperglycemia we compared the major stem cells population of bone marrow that is $CD34^+$ and $CD45^+$ from normal as well as experimental diabetic mice (**Fig. 1**). Work strategy has been diagrammatically represented for the transplantation experiments (**schematic diagram**).



Work Strategy for Bone Marrow Transplantation experiment

We have shown that unfractionated whole bone marrow derived from experimental diabetic mice when transplanted into other diabetic mice can correct the hyperglycemia. Graft received mice showed improvement in weight gain and prolonged survival. We have demonstrated the significance of cell number for transplantation experiments. Single dose of bone marrow (~10^6 cells) transplant helps in blood glucose reduction but not sufficient for normalization of blood glucose level, whereas multiple doses of bone marrow (at least 3 doses each with ~10^6 cells at an interval of 6 days) transplants helps to achieve stable normoglycemia without the need of further injections . Subsequent normoglycemia achieved by recipient mice was evidenced by their blood glucose profile, normal glucose tolerance pattern and also by measurement of serum insulin levels and histological studies exhibiting appearance of many small neo-islets suggestive of pancreatic regeneration.

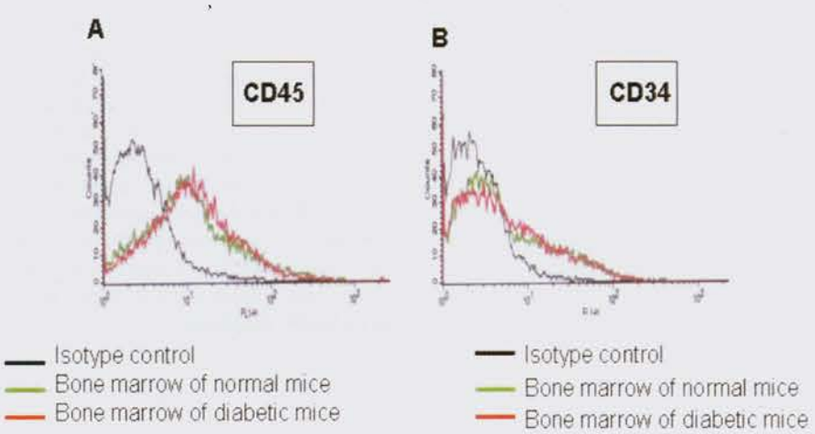


Fig. 1. Figure represents comparative analysis of CD45$^+$ hematopoietic stem cells (A) and CD34$^+$ hematopoietic stem cells (B) of bone marrow derived from normal and streptozotocin diabetic mice. Numbers in parenthesis are representatives of positive cell population.

Major findings in brief:

1 Single injection of diabetic bone marrow reduces blood glucose level and prevent further rise in blood glucose.

2 Multiple injections of diabetic bone marrow rescue experimental diabetes by restoring normoglycemia.

3 The status of bone marrow hematopoietic stem cells derived from experimental diabetic mice remains functionally intact despite sustained hyperglycemic environment.

Future work:

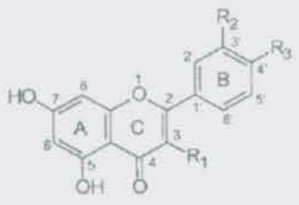
Our present study on bone marrow stem cells suggests the definitive role of bone marrow transplantation in rescue of experimental diabetes. However the exact fate of transplanted bone marrow stem cells remains to be seen. Whether the bone marrow stem cells undergo transdifferentiation (into functional beta cells) or cell fusion or they are differentiated to endothelial cells to induce pancreatic regeneration upon transplantation.

2. To design, synthesize and screen novel metal-based oral insulin-mimetic agents:

Studies in both laboratory animals and humans have now convincingly demonstrated the insulin-mimetic activity of a number of metal-based compounds like organic conjugates of vanadium, zinc, chromium, etc. Generally, pharmacologically beneficial intake of these compounds is higher than optimal intake for nutrients thus raises a problem of toxicity. Hence, drug designing to obtain better pharmaceutical dose below adverse effect levels is a high priority in the ongoing insulin-mimetic research field. We have hypothesized that dietary polyphenolics, common ingredient of our diet, having antioxidant and anti-inflammatory activities can be conjugated with the metals as they can act as suitable ligands to improve bioavailability and stability of the conjugates thus increasing the activity of the compound.

Until now we have synthesized 3 novel vanadium organic complexes (flavone members as ligand) and checked their glucose lowering potential by administering them orally to STZ-induced diabetic mice. Out of the three compounds, two have shown hypoglycemic potential more than their parent molecules. Moreover the one having quercetin as ligand has shown to have glucose-lowering potential twice than that of the bench-mark vanadium compound BMOV (bis (maltolato) oxovanadiumIV) (**Fig. 2**). To further verify whether the complex can mimic insulin in aspects other than hypoglycemic potential, mitogenic potential of the complex was checked since some vanadium compounds have been reported to possess mitogenic potential. We compared and evaluated the effects of vanadium quercetin conjugate with insulin and its parent molecules at various concentrations for its ability to induce proliferation in serum starved CHO cells by [^{3}H] thymidine uptake assay. The quercetin vanadium complex exhibited mitogenic potential comparable to that of insulin at 0.1 µM. It appears from these results that the conjugate not only mimics insulin's hypoglycemic activity but also its mitogenic potential indicating similarity in their signal transduction pathways. This very feature of the conjugate is likely to make this compound useful even in the management of type 2 diabetes.

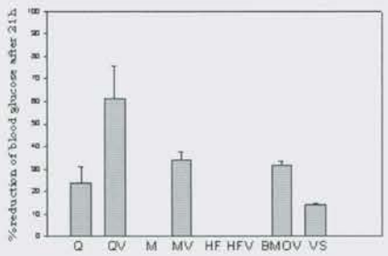
(a) Three flavoniods conjugated with vanadyl sulfate (VS) as organic ligands



LIGAND (L)	R_1	R_2	R_3
Quercetin (Q)	OH	OH	OH
Morin(M)	OH	H	OH
Hydroxy flavone (HF)	H	H	OH

These ligands (L) were conjugated with vanadium (V) to prepare flavonoid vanadium conjugates (LV).

(b) Hypoglycemic potential of the conjugates



Compounds were administered orally at a one-time dose of 0.4 mmol kg^{-1} to diabetic mice. Blood glucose was measured just before and at 21st hour after the dosing.

$$\% \text{ reduction of blood glucose} = \frac{(\text{initial level} - \text{final level})}{\text{initial level}} \times 100$$

Values represent mean ± SD, n = 8.

Fig. 2. Hypoglycemic potential of novel flavonoid vanadium conjugates,their parent molecules and BMOV

Major findings in brief:

1 Two novel oral vanadium based hypoglycemic agents have been synthesized.

2 Quercetin vanadium conjugate is nearly two times more active than the BMOV (compound under clinical trials), which makes it as an attractive and potent molecule for therapeutic formulation.

Future work:

The present study reveals that the vanadium complex of naturally occurring flavonoid, quercetin, is a potential oral insulin mimetic agent of therapeutic value in both type 1 and type 2 diabetes. However the exact molecular mechanism of the compound is currently under investigation. Also we would like to replace vanadium with other metals in order to formulate most suitable drug having best metal-ligand combination. Moreover search for other suitable ligands will be continued.

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Studies in immune reactions in type 1 diabetes

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Mamata Katdare, technician

1) Studies in immune reactions in type 1 diabetes

a) Regeneration of islets from mouse pancreatic ductal culture

Collaborator : R.R.Bhonde.

b) Development of a rat model of insulitis by immunisation with 1-Fluoro-2,4-dinitrobenzene (FDNB) tagged rat insulinoma cells. Assessment through histological and immunological evidences.

Collaborator : Sanjeev Galande

c) Regulation of diabetes by stress : possible role of SMAR1 and p53

Collaborator : Samit Chattopadhyay

2) Characterisation of the growth promoting and differentiation factors in the perivitelline fluid of the developing embryos of the Indian horse shoe crab

Collaborator : Surendra Ghaskadbi, ARI
Anil Chatterjee, NIO, Gao

Research Report

1) Studies in immune reactions in type 1 diabetes

a) Regeneration of islets from mouse pancreatic ductal culture

Mouse pancreatic ductal cultures were established.Neoislets obtained showed similar morphological and functional characteristics as islets isolated from normal mouse pancreas.In this study we have described an improved method for obtaining a constant supply of large number of islets from pancreatic ductal stem cell culture. The newly generated islets undergo functional maturation indicating their suitability for transplantation. These studies will be published in July issue of Journal of Endocrinology.

b) Development of a rat model of insulitis by immunisation with 1-Fluoro-2,4-dinitrobenzene (FDNB) tagged rat insulinoma cells. Assessment through histological and immunological evidences.

Aims:

Type 1 (IDDM) diabetes has two distinct phases; the first phase is insulitis, which finally progresses to overt diabetes. The nature of the triggering and/or of the target autoantigen(s) is elusive and several candidates have been implicated including insulin, GAD, IA -2 and heat-shock proteins. It was shown previously that chemical manipulation such as tagging of cell surface with 1-fluoro 2,4-dinitrobenzene (FDNB) brings about conformational changes on cell surface. In the present study,

immunization of rats with FDNB modified rat insulinoma (RIN) cells produced insulitis. The novelty of the model is explained by further histological and immunological assays.

Work achieved

1. Histological analysis of H&E stained formalin fixed pancreas - It was observed that 80% of the F-RIN immunized animals did show 40% or more mononuclear infiltration in the islets (+1 to +4) compared to pancreases from RIN immunized animals.

2. Antibody levels Circulating IgG2a levels specific to F-RIN cell extract were found to be increased in F-RIN immunized animals indicating Th1- type response in these animals.

3. Pancreatic insulin exhaustion 300 islets each from every RIN and F-RIN immunized rats were challenged with different concentrations of glucose. Islets isolated from F-RIN immunized rats showed significant exhaustion of secretory activity of insulin when challenged with 20 mM glucose and compared with islets isolated from normal and RIN immunized animals.

4. Lymphocyte proliferation assay by ^{3}H-thymidine incorporation - Lymphocytes from F-RIN immunized animals showed significant increase in the proliferation in response to irradiated F-RIN cells as compared to irradiated RIN cells. (Figure 1)

5. Cytotoxicity assay Splenic T cells from F-RIN immunized rats lysed ^{3}H labeled RIN cells as opposed to T cells from RIN immunized animals. Similar results were obtained in STZ-induced diabetic animals. There was upregulation of F-RIN specific CTL response evident in F-RIN immunized rats as well as in STZ-induced diabetic animals. (Figure 2)

6. Development of MAb to F-RIN cell surface antigen F-RIN cell surface monoclonal antibody was developed using standard method. This MAb 71.51.54 found to react with F-RIN cell surface protein but not with RIN cell surface protein as observed by confocal microscopy. This MAb recognised a 33 kDa antigen in RIN and F-RIN cell extract in western blot. (Figure 3).

7. Flow cytometry RIN immunized sera did not show much reactivity to RIN cells but showed high reactivity to F-RIN cells, whereas F-RIN immunized sera reacted to both RIN as well as F-RIN cells. This suggests FDNB treatment of RIN cells results in exposure of new antigenic sites.

8. Recognition of newer antigens by F-RIN immunized sera by immunoblotting RIN and F-RIN cell extracts were electrophoresced on SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and exposed to RIN and F-RIN cells immunized rabbit sera. A 33 kDa protein was present in F-RIN and RIN cell extracts recognized by only F-RIN immunized sera and not by RIN immunized sera.

9. 2-D gel electrophoresis RIN and F-RIN cell extracts were analysed by using Etton IPGphor (Amersham) instrument. Gel spots corresponding to 31 and 33 κDa proteins are analyzed for peptide mass based identification using mass spectrometry and found to be 1. Elongation factor 1 beta2 2. Tropomysin 5.

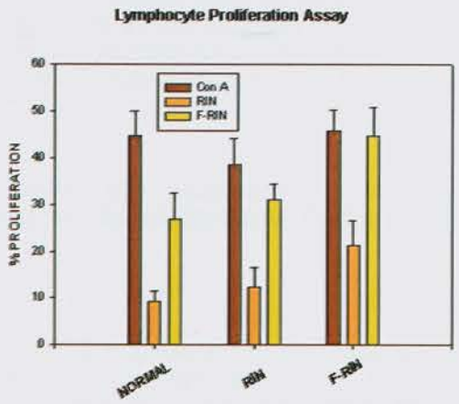

Fig. 1. % Proliferation of splenic T cells from Normal, RIN, F-RIN Immunised rats with response to irradiated RIN & F-RIN cells as an antigen. Con A used as positive control

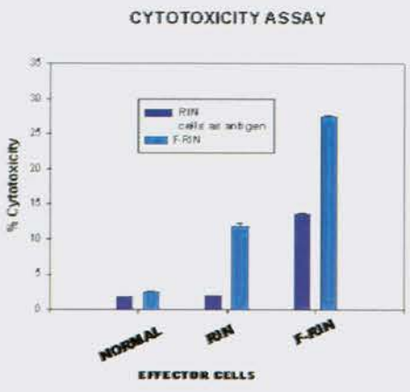

Fig. 2. % Cytotoxicity to 3H-labeled target RIN cells by splenic T cells from normal, RIN, F-RIN immunised rats

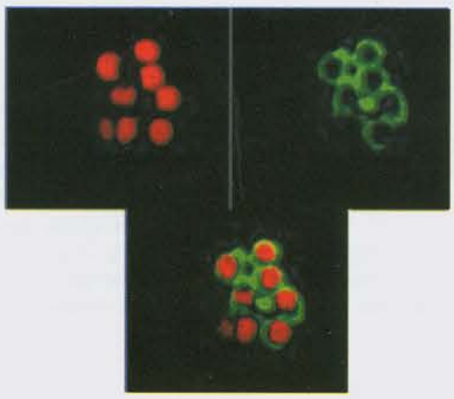

Fig. 3. MAb F71.51.54 raised against F-RIN cells found to react with surface antigens of F-RIN cells (Green Fluorescence). Nucleus stained red with propidium iodide.

c) Regulation of diabetes by stress : possible role of SMAR1 and p53

Diabetes is a form of disease in which the blood glucose homeostasis gets altered through the deficiency of insulin (also known as Insulin dependent diabetes mellitus) or due to inappropriate secretion/resistance of body cells to insulin uptake (also known as Non-insulin dependent diabetes mellitus). The present study is based upon the governing molecular mechanisms that lead to beta cell destruction. Despite the abundant data obtained in two animal models of spontaneous autoimmune diabetes, the NOD mouse and the BB Wistar rat, the initial beta cell specific factors and the molecular mechanisms triggering the autoimmune process have yet to be identified. SMAR1 is a member of MAR binding proteins that exists in 2 isoforms. It has been shown that SMAR1 overexpression in cells causes cell cycle arrest that is mediated through the direct physical interaction of SMAR1 with p53. The p53 is a tumor suppressor protein that is involved in maintaining genomic stability. The overexpression of this protein also causes cell cycle arrest or apoptosis depending on the strength of stress. The present work is conducted to elucidate the role of two molecules namely, SMAR1 and p53 in the pathogenesis of type-1 diabetes with respect to a major autoantigen GAD65.

Aims:

1. To study whether streptozotocin a major diabetogenic compound causes changes in the RINm5F cells in terms of expression of SMAR1, p53 and GAD65.

2. To study whether SMAR1 upregulation in RINm5F cells effects the GAD65 expression.

Work achieved

STZ causes changes in the expression of SMAR1, p53 and GAD65

In in-vitro studies RINm5F cells were treated with different concentrations of STZ (1.25mM, 2.5mM and 5mM) for a period of 24 hours. Total protein and RNA was prepared and the samples were subjected to SMAR1, p53 and GAD65 analysis. At RT-PCR level all the three molecules showed relative increase in expression. Under STZ stress the upper form of SMAR1 showed increased expression compared to lower form showing that there occurs some differential regulation. A mechanism governing alternative splicing machinery may be operating at this stage. At protein level also p53 and GAD65 expression showed similar expressions of both the molecules.

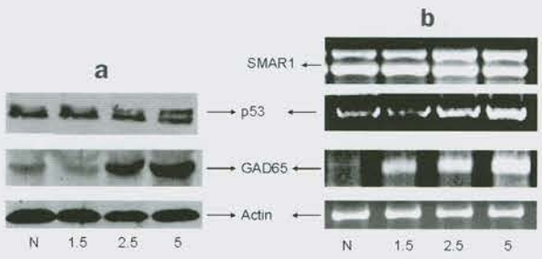

Fig. 1. a. shows the western blot for p53 and GAD65 in RINm5F cells at 1.5Mm, 2.5mM and 5mM of STZ after 24 hours. **b.** shows the RT-PCR for SMAR1, p53 and GAD65 in RINm5F cells at 1.5mM, 2.5mM and 5mM of STZ after 24 hours

SMAR1 transiently transfected RINm5F cells overexpress GAD65

RINM5F cells were transiently transfected with either upper or lower form of SMAR1 for a period of 48 hours. Total RNA was prepared and analyzed for p53 and GAD65 expression. Our results showed that both isoforms cause overexpression of p53 and GAD65 leading to a possibility that SMAR1 may be responsible for regulating the GAD65 protein and consequently induce autoimmune response.

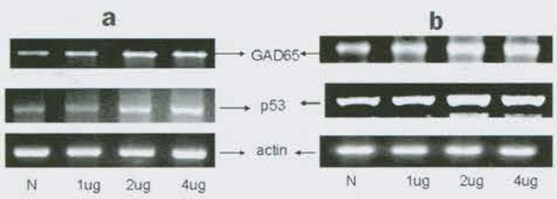

Fig. 2. a shows the GAD65 and p53 levels in RINm5F cells transfected with different concentrations of upper form of the SMAR1. **b** shows the GAD65 and p53 levels in RINm5F cellstransfected with different concentrations of lower form of SMAR1

Future plan

1. To study whether GAD65 expression in RINm5F cells is p53 dependent or independent
2. To find any SMAR1 or p53 response elements in GAD65 promoter.

2) Characterization of the growth promoting and differentiation factors in the perivitelline fluid of the developing embryos of the Indian horse shoe crab

Objective

In our preliminary experiments, gastrulating chick embryos were allowed to develop in the presence of various dilutions of PVF for several hours and the differentiation of specific organs and systems were monitored. The PVF treatment resulted in stimulation of differentiation of specific organs such as brain and heart. Also it was observed that PVF has cell proliferation activity. All these effects clearly indicate that the PVF contains molecules that stimulate growth, probably through increased mitosis as well as molecules that influence differentiation of specific organs. The following studies are initiated.

PVF (Protein) Fractionation

The crude PVF extract from different batches was fractionated on superose 12 columns by FPLC. Total of eight peaks were obtained. These fractions were eluted more or less at same positions when the fractionation carried out for 6 times. Out of these fractions, 7th fraction was used in chick embryo culture in Agharkar Institute, this fraction has showed increased cardiac developmental activity in terms of size of the heart. Further studies are in progress.

Cell proliferation

Cell proliferation studies using different cell lines at various concentration of PVF protein were carried out. There was no considerable change in growth pattern observed in the presence of PVF in the case of RINm5f and HepG2 cell line. However in the case of AR42J, an acinar cell line, a concentration dependent increase in the cell number was observed.

Future Work:

1) Development of cardiomyocytes from mouse stromal cells and to assess the development of cardiac activity in presence of various fractions of perivitelline fluid.

Research Reports

Insect Molecular Biology

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Cloning and characterization of Hexokinase genes.

Cyril Jayakumar, Vijay V. Musande, P. K. Umasankar, Sachin V. Surve

Collaborator:
Yogesh S. Shouche

Background:

Hexokinase is a rate limiting enzyme in glucose metabolism. It plays important role in energy metabolism in flying insects and parasites. We have cloned two complete coding sequences for Hexokinase isoenzymes from *Drosophila melanogaster.* The differential expression of the two cloned enzyme indicated that the nature of 5'upstream non-coding regions may have different cis-acting elements necessary for their expression. This prompted us to clone and characterize the promoter elements. The promoter of Hex-A locus was found to be a TATA less and has typical Inr element and DPE element. Analysis of DM1 promoter showed presence of TCATT and TCAAT sequences. Analysis of several house keeping genes from drosophila showed presence of TCATT and TCAAT sequences at the vicinity of reported Inr and DPE elements of these genes.

Aim:

To analyze the cloned 5' upstream sequences of Hexokinase isoenzymes for testing its ability for transcription initiation.

Work achieved:

1. Implication of the possible role of TCAWT sequences in expression of housekeeping genes in fruit-fly was ascertained. Various mutant promoter sequences have been constructed by site directed mutagenesis method. The mutant promoters were cloned upstream of reporter gene and were tested for their transcriptional activity in vivo in drosophila cell line. It was found that mutations in TCAWT sequence reduce the transcriptional potential of the promoter region as seen by the reporter gene expression. Thereby indicating importance of TCAWT like sequences in TATA-less promoters of drosophila.

2. Complete coding sequence for hexokinase gene is cloned from parasite *Leishmania major.* By homology cloning, the gene was also cloned from four other species of Leishmania. The homology analysis suggests that parasite enzyme is very similar to plant hexokinases. The genome analysis indicated that there are two copies of hexokinase loci per haploid genome of parasite. Both the copies of the gene were found to be on a single chromosome.

3. Using total RNA, RT-PCR was performed to clone complete coding sequence of hexokinase gene from *Aedes aegyptii*, another dipteran than fruit-fly. Genomic analysis indicated that unlike fruit-fly, mosquito has only one gene sequence, coding for all the hexokinase isoenzymes. The mosquito gene also has introns, a feature not found in case of drosophila.

Future work:

Experiments will be done to study the hexokinase isoenzymes in mosquito and targeting of Leishmanial hexokinase to glycosomes.

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Cloning and characterization of genes for heat shock proteins from *Aeromonas.*

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Collaborator:
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Background:

Aeromonas is ubiquitous bacterial genus widely found in different ecological conditions. Many species of Aeromonas are involved in clinical infections in fish, animals and humans. As the bacterium survives in different environmental conditions and most of them are adverse in nature, the key question asked is how the bacteria survive and do heat shock proteins play important role in survival and pathogenecity of Aeromonas. Degenerate primers were designed for amplification of complete operon of Hsp10 and 60 and Hsp 70. Using these primers complete genes for Hsp10, 60 and 70 have been amplified and completely sequenced from Aeromonas culicicola.

Aim:

To clone and characterize genes for heat shock protein and chaperone from Aeromonas.

Work achieved:

1 The gene for Hsp60 protein from *A. hydrophila* has been cloned in pET vector for expression of protein in large quantity. But the expression was found to be absent even though the construct was found to be perfect as judged by DNA sequence. The gene for Hsp10 was found to express the protein in sufficient quantity. The protein will be used as antigen to obtain polyclonal antibodies.

2 Inverse PCR was performed to clone the promoter region of the GroE operon. The cloned PCR products are being sequenced to perform the promoter characterization.

Future work:

Experiments will be done to monitor the expression at RNA and protein level of various heat shock proteins under different environmental conditions.

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Molecular taxonomy and diversity studies using rRNA gene sequence and other tools.

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Collaborators:
M. S. Patole

Abstract and background:

The concept of using macromolecular sequences as indicators of evolution was first introduced by Linus Pauling. Since then various macromolecular sequences have been used to explore the phylogenetic diversity and evolutionary relatedness of organisms. Ribosomal RNAs have been widely used for this purpose as they are essential components of all living cells that are functionally and evolutionarily conserved. This has made them a valuable tool in molecular taxonomy, phylogeny and diversity studies. However, it suffers from certain shortcomings like differences in the copy number and limited resolving power. In the past few years many additional gene sequences are being increasingly used for diversity and evolutionary studies. We have been using ribosomal RNA and other molecular chronometers for the diversity studies including those on "uncultivable" microbes. Knowledge of this will provide information about metabolic diversity of these microbes further enabling their exploration for biotechnological purposes.

We isolated a novel bacterial strain, *Aeromonas culicicola*, from the midgut of the mosquito, *Culex quinquefasciatus*. A remarkable increase in the cell count of this bacterial strain was observed in a blood fed mosquito. We, thereby, initiated studies on gene regulatory circuits involved in this strain that enable it to show such characters. We are also studying the virulence factors of this strain and its pathogenic nature.

Aims:

To understand the "uncultured" microbial diversity with long term aim of utilizing it for the biotechnological purpose.

To study the diversity and evolutionary relationships in different organisms.

To study the genetic regulatory circuits and virulence factors in *Aeromonas culicicola*

Work achieved:

Studies on understanding of "unculturable microbial flora"

PCR amplification of a "molecular chronometer" gene from total DNA extracted from environmental sample, followed by cloning and sequencing is the most commonly used strategy for studies of microbial community structure. We have adopted this strategy for some systems of either academic or applied interest.

a) Microbial Diversity of Anaerobic Digesters:

To assess the diversity of microorganisms in anaerobic digester ecosystem, libraries of eubacterial and archeal specific PCR amplified 16S rRNA gene were prepared and the clones are being analyzed. We have also employed taxa specific genes like methyl co-enzymeM reductase (mcrA) of methanogens and particulate methane monooxygenease gene (pmoA) of methanotrophs for molecular characterization of functional and phylogenetic diversity of these microorganisms. The use of fast

evolving metabolic gene for studying the functional diversity of methanogens and methanotrophs will also help in bridging the gap between function and phylogeny. Studies of sequence information from microbial diversity of such ecosystems will also provide guidelines for designing new and improved culturing methods for microbes that will resemble their natural niches.

b) Eubacterial and Archeal diversity of Lonar Lake.

This study was undertaken with an aim to look for the evidence of life in outer space by studying the microbial flora of an impact crater lake known as Lonar crater and is situated in Buldana district of Maharashtra. The study involved the isolation of DNA from four sediment samples collected from different sites from the bed of the lake using standard DNA isolation protocol. The 16S rRNA gene was amplified from the isolated DNA using two different sets of primers that were specific for two domains of bacteria, eubacteria and archeabacteria. Two 16S rRNA gene libraries were prepared, each specific for one domain. Diversity of the clone libraries was accessed by amplified ribosomal DNA restriction analysis (ARDRA). Different RFLP groups were made and one representative clone from each group was selected for sequencing.

Molecular Taxonomy & Genetic Diversity

A number of molecular markers are used to delineate taxonomic affiliations and estimation of diversity of various organisms. We have used these markers to understand taxonomy of some important organisms and also to assess diversity. The range of organisms studied ranges from viruses to mammals and some important examples are given here.

a) Methanobrevibacter Taxonomy:

The phylogeny of the genus Methanobrevibacter and the taxonomic affiliation of the isolates and clones representing the genus Methanobrevibacter, was revised on the basis of 16S rRNA gene sequence similarity (S) and genomic DNA reassociation (D) values and group specific signature nucleotide positions showing specific nucleotide substitutions in the 16S rRNA gene sequences were determined. Our analysis based on 786 bp aligned region from 54 representative sequences of the 120 sequences available for the genus revealed seven multi-member groups namely, Ruminantium, Smithii, Woesei, Curvatus, Arboriphilicus, Filiformis, and the Termite gut symbionts along with three separate lineages represented by Mbr. wolinii, Mbr. acididurans, and termite gut flagellate symbiont LHD12 (Fig. 1). The cophenetic correlation coefficient (0.913), a test for the ultrametric properties of the 16S rRNA gene sequences used for the analysis, indicated the high degree of goodness of fit of the tree topology. A significant relationship was found between S and D with the correlation coefficient (r) for logD and logS, and for [ln(-lnD) and ln(-lnS)] being 0.73 and 0.796 respectively. Our analysis suggested that D would be less than 70 % at least 99 % of the times when S= 0.984, and with 70% D as the species "cutoff", any 16S rRNA gene sequence showing <98% sequence similarity can be considered as a separate species for this genus (Fig. 2). We propose to include the termite gut flagellate symbiont LHD12, the methanogenic endosymbionts of the ciliate Nyctotherus ovalis, and rat feces isolate RT reported earlier, as separate species of the genus Methanobrevibacter.

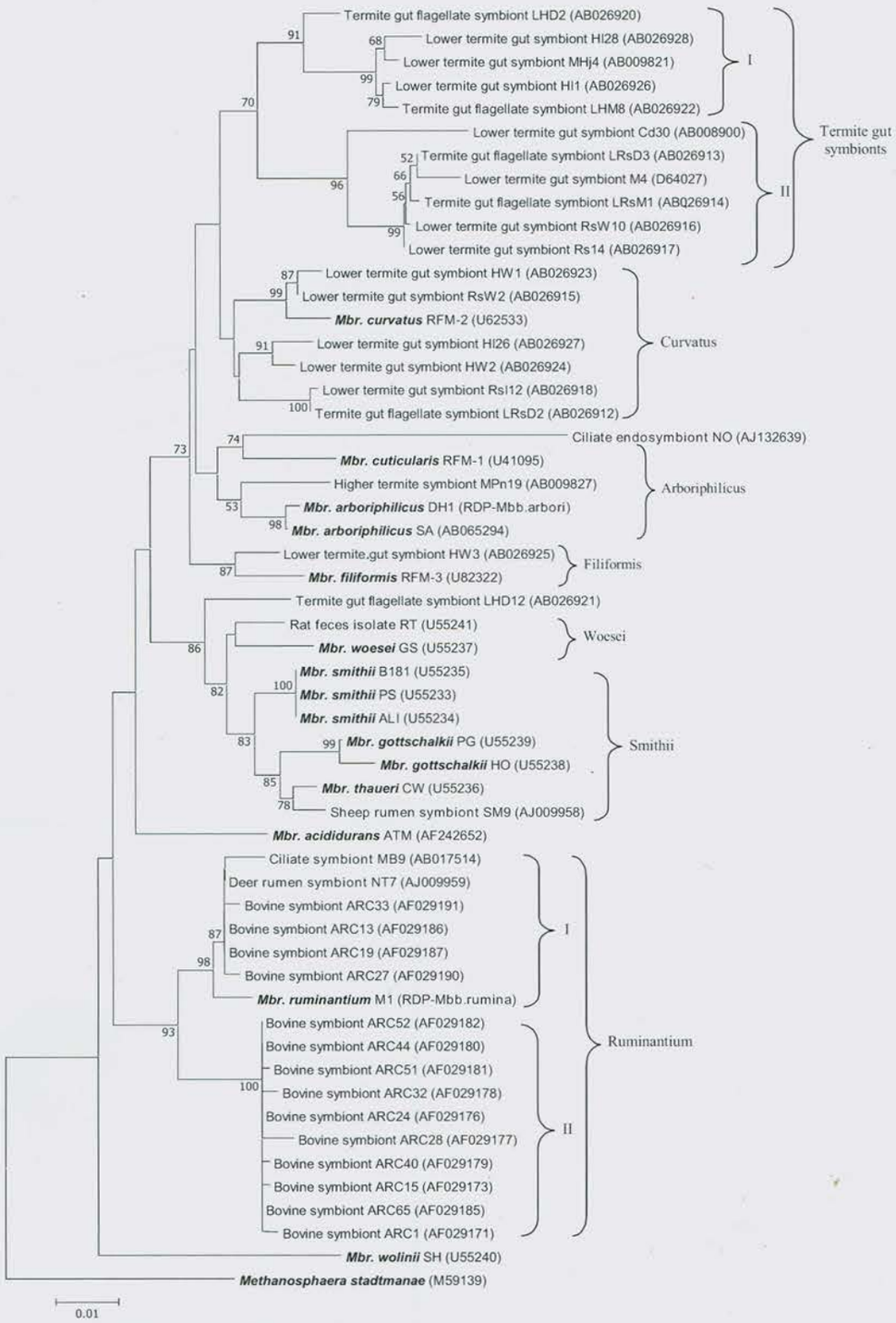


Fig.1. Phylogenetic tree of the members of the genus *Methanobrevibacter* based on the 16S rRNA gene. The tree was generated using CLUSTAL W program [30] for sequence alignment (786 bp) and by the neighbor joining method using Kimura 2 parameter distances in MEGA 2.1 software. Only positions 286-1120 (E. coli numbering) were considered with Msp. stadtmanae as the outgroup. Numbers at nodes indicate percent bootstrap values above 50 (1000 replicates). Bar indicates Jukes-Cantor evolutionary distance. Bold letters indicate isolated strains whereas normal font indicates clones.

b) Genome diversity of *Helicobacter pylori* from Indian patients (DBT funded project):

Comparative genomic diversity of H.pylori was studied directly from 135 Urease-positive biopsies by PCR based analysis of virulence genes like CagA, VacA, iceA and neutral DNA markers like 16SrRNA and IS605 from Western (B.J. Medical college, Pune, Maharashtra) and Northern India (M.N.M.C. Medical College, Allahabad, Uttar Pradesh). In addition, more than 350 biopsies were screened for the presence of H. pylori and 10 isolates were obtained. These were studied by *H. pylori* specific primers for virulence and housekeeping genes by Multiple Locus Sequence typing approach (MLST).

c) Genetic Diversity of Bacillus anthracis **isolates from India (LSRB funded project):**

PCR was done by primers specific for rpoB gene, GyraseB-GyraseA intergenic spacer region [ISR], vrrB and vrrC genes. We sequenced 16S-23S rRNA ISR of 20 isolates, partial protective antigen gene of 20 isolates, 8 Gyrase B gene of 10 isolates and Bacillus anthracis specific Gyrase B gene 5 isolates.

c) Molecular systematics and population studies on Indian Amphibians including caecilians, Lizards, Fresh Water Fishes, and Uropeltid snakes:

PCR amplification and sequencing of mt12S, mt16S, CytB, CO I and ND 4 is being studied for the implications in systematics. More than 80 different species of Amphibians (from Western Ghats and North-East India), and 75 Lizard species from Andaman-Nicobar islands, 20 Uropeltid Snake specimens, 96 Fresh water Fish specimen are being characterized.

Genetic Regulatory circuits and virulence factors in *Aeromonas culicicola*

a) Quorum sensing in *Aeromonas culicicola*:

The isolate, Aeromonas culicicola was reported to show increased count in blood fed mosquito midgut. Preliminary studies with biosensor strain *Chromobacterium violaceum* VCO26 indicated that the strain produces homoserine lactone based signalling compounds, a component of the two component "Quorum sensing" (QS) regulatory circuit (Fig. 3). PCR amplification of the complete QS regulon from the strain using degenerate primers designed on the basis of available sequences for other species of the genus *Aeromonas*, resulted in amplification of the regulator part of the regulon. Continuous efforts towards cloning the inducer part using gene targeting involving southern hybridization analysis and Inverse PCR amplification using inverse primers (deduced on the basis of AcuR region) were successful. We now have the complete AcuIR regulon sequence. Interesting facts and diverse nature of the system have come up after the sequence analysis. Furthermore, we have also purified the Homoserine lactone from culture supernatant using solvent extraction with dichloromethane.

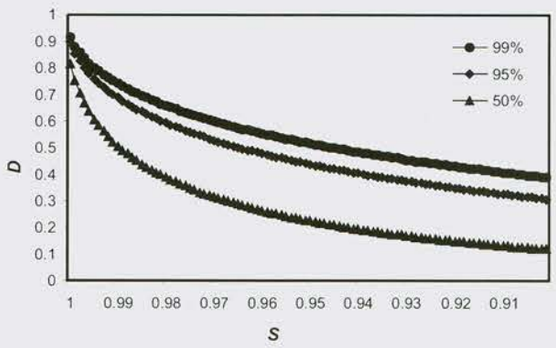


Fig.2. Relationship between the 16S rRNA gene sequence similarity (S) and the extent of DNA hybridization (D) for the genus Methanobrevibacter. Probability of D for S from 0.90 to 1.00. The line was determined by linear regression of the values. The extent of DNA hybridization was measured by the membrane filter method and the data used for DNA hybridization was as reported earlier [8, 21, 22]. The Distribution of D was calculated from the equation ln(-lnD) = 0.5077 [ln(-lnS)] + 1.8999 and the SD of the residuals in D, which was 0.3498

Fig. 3. Plate indicating the purple pigment production in response to presence of homoserine lactones produced by A. *culicicola* MTCC 3249^T as detected on an LB agar plate seeded with *Chromobacterium violaceum* CVO26. The other strain, i.e., *E. coli* JM 109 was used as a negative control.

b) Molecular characterization of hemolysin [Aerolysin] **, super oxide dismutase** [sodA **and** sodB] **genes.**

Virulence is a multifactorial trait, and depends on the coordinated expression of many bacterial products. Hence, it may be expected that the regulatory circuits that control the relevant genetic determinants, are some how interconnected. There are few reports of complete characterization of the gene related to virulence in *Aeromonas* spp. and a lot more study is needed for complete understanding. With respect to this, we are studying the role of hemolysin (Aerolysin), superoxide dismutase (sodA and sodB) genes from *A. culicicola* as virulence factors. We have cloned the genes and efforts are on to make isogenic mutants and their relative studies in available animal models. Work is in progress to study how these genes are regulated.

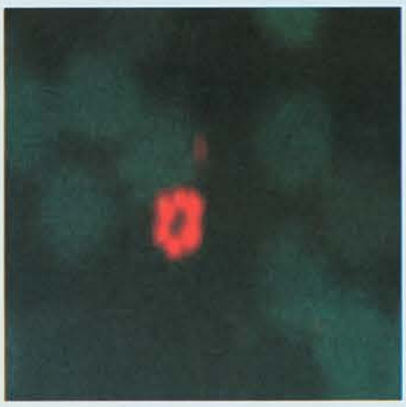
Future work:

The chemical nature of the signaling compound involved in quorum sensing in *Aeromonas culicicola* will be studied using MS and NMR. Further analysis of the QS regulon involves protein expression studies of the autoinducer synthase gene. Attempts will also be made to determine the threshold cell density at which the strain initiates the QS regulon.

Further studies on additional Helicobacter pylori isolates are in progress with collaborative institutions like CDFD, Hyderabad and SGPGI, Lucknow.

Genetic diversity *B. anthracis* will be further studied by Single Nucleotide Polymorphism markers, AFLP and MLVR .

Research Reports



Infection and Immunity

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Studies on malaria parasite biology: Characterization of erythrocytic stages of *Plasmodium falciparum* malaria parasites-spontaneously growing outside the host RBC

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Collaborators:
Promod Khandekar, Bioinformatics Centre , University of Poona, Pune.

Abstract and background:

Continuous culture of the erythrocytic cycle of *P. falciparum* within the human erythrocytes has facilitated a large body of fundamental investigations. It has revealed complex interrelationship between the intracellular developing parasite and the host-RBC. In pursuit of further understanding these relationships of host cell and parasites, parasites were grown for a short period in the matrigel supplemented with erythrocytic extract. Recently, it has been demonstrated the transformation of sporozoites of *P.berghei* obtained from Anopheles stephensi mosquitoes into early exoerythrocytic stages in vitro without intracellular residence in the host hepatocytes and the transformation is achieved by just elevation of temperature to 37°C and supplimentation of serum. It has been reported that, in mutant parasite transcriptional failure of genes leads to loss of gametocyte formation in *P. falciparum*. The invasion of merozoite into host cell is a multi-step and complicated phenomena and being intraerythocytic is crucial for the parasite survival *in vivo*, the stage which is responsible for malaria disease.

Here we report for the first time spontaneous development of parasites out side the human host erythrocytes. During prolonged in vitro cultures of FAN5HS isolate, we have observed merozoites attachment to the erythrocytes but failed to invade the host cells, instead of disintegration, further these merozoites transformed into ring stages, then into pigment (hemozoin) containing trophozoites and finally developed into schizonts. The merozoites which formed in these schizonts seen growing out side the human erythrocytes. We have isolated these parasites and cultured in presence of fresh RBC and characterized by molecular tools. The proportion of these parasites ranged from 10% to 45 % of the total parasitemia (intraerythrocytic stages) in the different intervals of the cultures.

Aims:

1 Characterization of parasites growing extracellular spontaneously.
2 Identification factors/ molecules involved in the inhibition of merozoites into the host cell.
3 Studies on biology of these parasites.

Work achieved:

Culture of malaria parasites: *Plasmodium falciparum* malaria parasites (FAN 5HS passage No. 45, and 3D7) were cultured as described earlier. The culture medium was supplemented with 10% human serum.

Culture of parasites out side the host cell **:** Parasites which were growing outside the human host erythrocytes were separated by percoll gradient. The separated parasites were washed and resuspended in fresh culture medium and cultured in the presence of fresh O+ ve RBC, 200 U /ml catalase / SOD, 50 % O+ve serum and 50µl/ml RBC extract was added to the medium. The parasite also shows viability by rhodamine 123 dye when cultured in the presence of total RBC extract (I00 µl/ml) without intact RBCs. The parasite were cultured in 24-well plates and incubated in desiccators at

37°C. The PI staining of rings shows morphological similarities with the intraerythrocytic rings (Fig.1A) with dot like chromatin and circular cytoplasm, and Similarly the young and mature trophozoites with blackish-brown hemozoin pigment also comparable with the intraerythrocytic stages of the trophozoite stage of the parasites.(Fig.1B&C). We have seen morphological disintegration of about 15% of these trophozoites during the transformation into (development) schizonts. This may be due to the rupture of parasite plasma membrane which is not surrounded or reinforced by parasitiphorous vacuole membranes. Unlike intraerythrocytic schizonts which forms 18 to 32 merozoites, these schizonts were seen with only 6-8 merozoites (Fig.1D).Further, these parasites do not invade in any group of RBC (A+, B+, AB+, or O+).

Assessment of parasite growth: Parasite growth was assessed every 96 h. by microscopic observation, parasites growing out side the erythrocytes (no. of rings, trophozoites and schizonts were counted per 100 RBCs, and 10 -100X fields were counted (Fig. 2).

Viability of parasite: Parasites viability was assessed as described earlier. Every 24 h. an aliquot of the parasite cultures were treated by Rhodamine 123 (10 µg/ml) for about 3h. washed with medium and resuspended in medium and a drop of culture placed on a glass slide and viewed under microscope. Incorporation of the dye into parasites indicated viability of the parasites.

In corporation of (^{3}H) Hypoxanthine and (^{3}H) Ethanol-amine: The parasite cultures were exposed to (^{3}H) hypoxanthine or (^{3}H) ethanol amine (1µCi/well/, 200µl medium/well) in 96-well tissue culture plate and incubated for 18h. The cultures were harvested on glass filter papers and cpm counts were recorded in liquid scintillation counter. Along with this experiment, incorporation of these molecules were also tested on intraerythocytic 3D7 cultures. The radioactive labeled hypoxanthine and ethanol-amine are readily incorporated into parasite DNA during its development This is being used as standard method to assess the growth of the parasites *in vitro*. We have seen the incorporation of this molecule in the intraerythrocytic culture of 3D7 parasites.

However, the parasites growing outside the host RBC do not up take either hypoxanthine or ethanol-amine, though the parasite are viable (by rhodamine incorporation) and growth pattern recorded by microscopic observation. It has been reported by many researchers, that intraerythocytic parasites forms membrane network (TVM-tubovesicular membranes) outside the parasite-but in the cytosol of the infected RBC for nutrient import from outside the RBC. It has also reported that TVM arrested /blockade by PPMP (dl-threo-1-phenyl-2-palmitoylamino-3-morpholino-1propanol which blocks resident parasite sensitive sphinogomyelin synthase or SSS) of this net work reduced the accumulation of exogenous hypoxanthine in the parasites (but do not inhibit parasite's machinery for nucleic acid synthesis). Similarly, it has been reported that blocked of TVM net work by PPMP, delivery of exogenous Lucifer Yellow, nucleosides, and amino-acids to the parasites were inhibited without inhibiting secretion of plasmodial proteins and the authors suggests that TVM is a transport work that allows nutrients efficient access to the parasite and could be used to deliver antimalarial drugs directly into parasite. The lack of incorporation of hypoxanthine and ethanolamine into parasites growing out side the host erythrocytes may be due to the absence of the TVM transport organelle. Further, it has been reported that trophozoites stages of the parasites growing extra-cellularly has no parasitiphorous membrane, but only parasite plasma membrane.

Fig. A. Lanes 1,3,5,7&9:Extracellular Parasites FAN 5 HS. Lanes 2,4,6,8&10 Intracellular Parasites FAN 5 HS. **Lanes :** 1&2 RESA (900 bp), 3&4 MSP-2 (750 bp), 5 & 6 MSA-1 (528 bp) 7&8 MSP-1 (300-350 bp), 9& 10 VAR (350 bp)

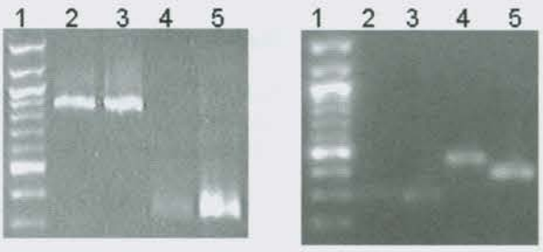

Fig. B.: lane 1 100bp Ladder, 2 & 4 Intra erythrocytic parasites (FAN 5 HS) 3 & 4 Extra cellular Parasites (FAN 5 HS).Lane 2 & 3 RESA, 900 bp, lane 4 & 5 Var, 350 bp

Fig. C. lane 1 100bp Ladder, 2 & 4 Intra erythrocytic parasites (FAN 5 HS) 3 & 4 Extra cellular Parasites (FAN 5 HS). Lane 2 & 3 MSP-1(300 350 bp), lane 4 & 5 MSA-1 (528 bp)

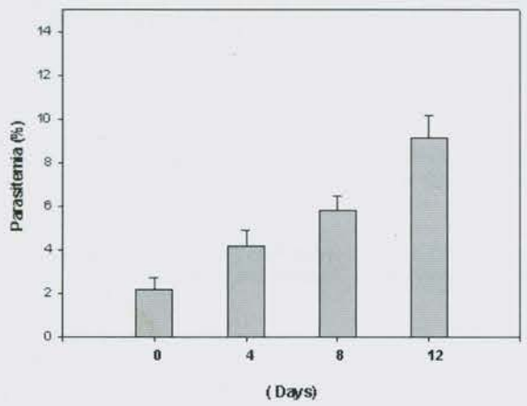

Fig. 2. Growth Pattern of extracellular parasites

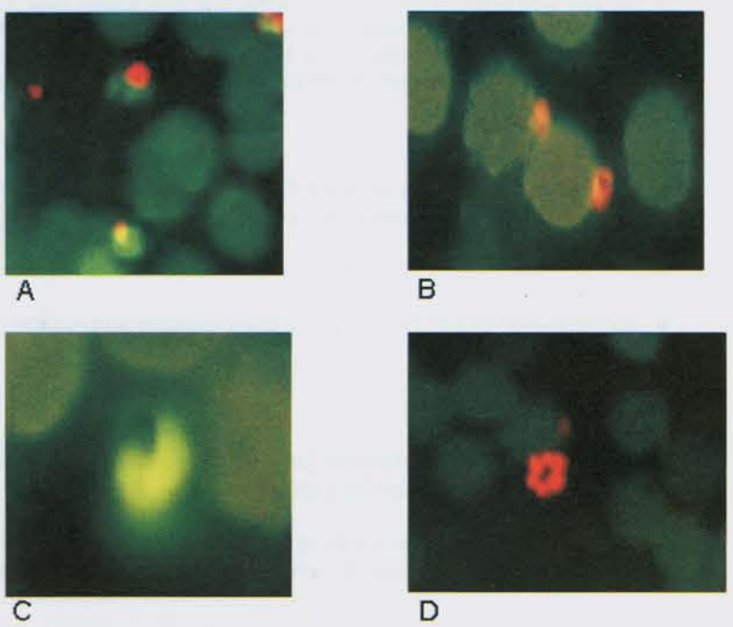

Fig 1. A Ring stage parasites with chromatin and cytoplasm. B Young Trophozoites with blackish Heamozoin Pigment C. Mature Trophozite. D Schizonts with 6 Merozoites with heamozoin pigment in centre

Genomic DNA and RNA were extracted from extracellular and intraerythrocytic parasite cultures. PCR and RT-PCR were performed using P. falciparum specific primers (Fig. A,B &C). The culture system provides better understanding of the parasite biology.

Future work:

Studies on merozoites formation (schizogony) in the intraerythrocytic parasites which give to defective merozoites which failed to invade the host cells. Identification of defective factors (genes/proteins) in the merozoites (so far, many molecules are reported which plays important role in the invasion of merozoite into host RBC). Biological studies of these parasites. Characterization of the parasites with P. falciparum specific antibodies (IFA).

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Molecular and cellular basis of HIV pathogenesis

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M. K. Gurjar (NCL, Pune), S. P. Joshi (NCL, Pune), K. N. Ganesh (NCL, Pune)

Abstract and Background:

Human immunodeficiency virus is the causative agent of acquired immunodeficiency syndrome (AIDS), which is defined by a reduction in the number of CD4+ T cells (less than 200 cells/ µl) and the onset of opportunistic infections. The incidence of HIV infection has reached alarmingly high levels worldwide including India. The therapeutic strategies being used at present can reduce the viral load remarkably but is not the ultimate answer to AIDS patients. Our group has been working on three different aspects of HIV related to viral pathogenesis, immune response and drug discovery. The primary objective is to gain more understanding of the virus and its interaction with the host cell, which may lead us to new antiviral strategies.

Aims:

- Role of viral regulatory proteins Tat and Nef in HIV pathogenesis, and differential gene expression studies in HIV-1 infected cells.
- Immune response to HIV infection towards generation of DNA vaccine.
- Identification of anti-HIV activity in plant extracts and marine animals.

Work achieved

Role of viral regulatory proteins Tat and Nef in HIV pathogenesis, and differential gene expression studies in HIV-1 infected cells.

The Human Immunodeficiency Virus Type 1 encodes a 27 KDa protein, Nef, which has come a long way from being termed as a negative factor to being one of the most important proteins of HIV-1. However, its role in HIV-1 replication and gene expression remains to be clearly elucidated. In our endeavor to identify Nef interacting host cell proteins, we have screened a human cDNA library using yeast two hybrid system, which has resulted in identification of several interacting proteins. The functional relevance of these newly identified interactions are currently under study. HIV-1 Tat protein reprograms cellular gene expression of infected as well as uninfected cells apart from its primary function of trans-activating HIV-1 long terminal repeat (LTR) promoter by binding to a nascent RNA stem loop structure known as transactivator response region (TAR). Several studies have shown convincing evidences that Tat can transactivate HIV-1 gene expression in absence of TAR, the molecular mechanism of which remains to be understood. We have shown a direct interaction of Tat with nuclear factor kappa B (NF-κB) enhancer, a global regulatory sequence for many cellular genes both in vitro and in vivo using Electrophoretic mobility shift assay, SELEX based library screening, reporter assay and chromatin immunoprecipitation assay (Figs.1 & 2). This interaction not only provides a novel molecular basis to explain TAR independent transactivation in HIV-1, but also point towards the potential mechanism of Tat mediated modulation of cellular genes.

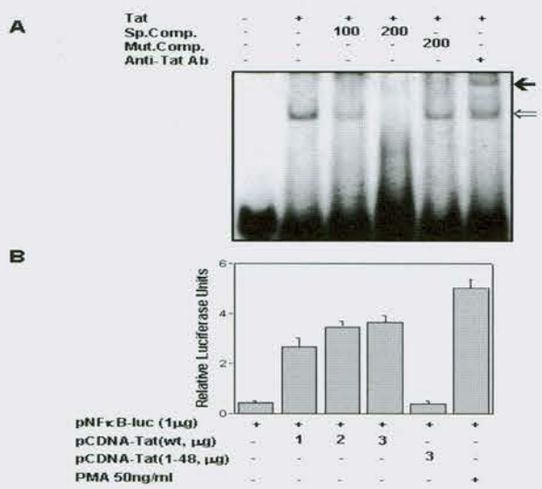

Fig.1. HIV-1 Tat interacts specifically with NF-κB enhancer sequences and activates enhancer driven gene expression. **A)** Gel shift assay using NF-κB consensus oligonucleotide as labeled probe and purified Tat protein. Cold specific and mutated oligonucleotides were used as competitors. Lane1: Free probe; Lane 2: Tat protein; Lane 3: 100 fold excess of cold specific oligonucleotide; Lane 4: 200 fold excess of cold specific oligonucleotide; Lane 5: 200 fold excess mutated oligonucleotide; Lane 6: Super shift with anti-Tat antibody. **B)** HEK 293T cells were transfected with pNFB-luc (1 g) reporter vector together with either pCDNA-Tat or pCDNA-Tat(1-48), or stimulated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA). Luciferase assays were performed using Luclite™assay kit on TopCount microplate counter (Packard Bioscience, USA).

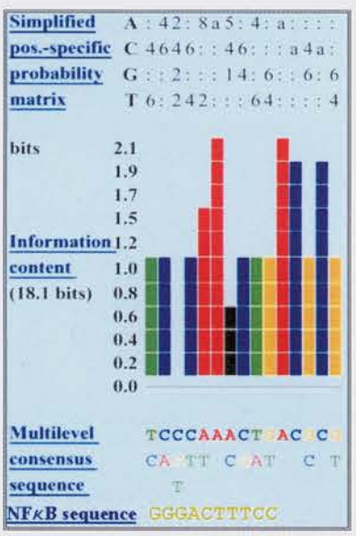

Fig. 2. Tat interaction with DNA: position specific matrix generated by motif discovery (MEME) software analysis. DNA sequences obtained by oligonucleotide library screening with Tat protein using SELEX protocol were analyzed for identifying consensus-binding motif for Tat. The matrix shows bit score for occurrence of particular nucleotide in the motif, total bits score as calculated and consensus obtained. The sequence derived is complementary to NF-κB enhancer like sequence.

Despite significant advancement in our understanding of the pathogenesis of AIDS, the mechanisms by which HIV-1 infection induces $CD4^+$ T cell depletion is not clearly understood although apoptosis has been shown to be one major mechanism. The objective of the present work is to identify differentially expressed molecules in cells undergoing apoptotic cell death as compared to non-apoptotic cells in the HIV infected T- cell population and to elucidate the interaction of those molecules in the signaling cascade leading to cell death. Using a reporter T cell line, CEM-GFP and NL4.3 virus, we have been able to purify the apoptotic cells from the non-apoptotic cells and differential gene expression studies have been performed by differential display technique and microarray analysis. Several differentially regulated genes are currently being characterized. Our data points towards impairment of mitochondrial functions in HIV infection and apoptosis.

Immune response to HIV infection towards generation of DNA vaccine.

HIV-1 is a uniquely difficult target to develop immunological intervention against it. The high rate of replication, mutation and recombination of HIV enable the virus to evolve rapidly in the host and so outsmart immune response evoked by natural immunity or a vaccine. It has been shown that $CD8^+$ T cells are active in containing acute HIV infection, but their functions are compromised in chronic infection. It has been postulated that this inability is related to the failure of these cells to mature into fully differentiated effector cells. The molecular basis for impaired function is multi-factorial, due to incomplete T-cell signaling and activation, reduced perforin expression, and inefficient trafficking of HIV-specific $CD8^+$ T cells to lymphoid sites of infection. Our DNA immunization studies using gp120 from subtype C isolate prevalent in India has revealed some startling facts about the elicitation and maturation of CTL, which is currently under active investigation.

Identification of anti-HIV activity in plant extracts and marine animals.

The current therapeutic strategy involving the use of reverse transcriptase and protease inhibitors in combination (HAART) has proven to be useful in controlling the virus but is unable to eradicate the virus from the patients. Extensive work is being done throughout the globe to identify new ant-HIV therapeutic strategies. One of the strategies has been to identify anti-HIV compounds in natural resources. We have performed screening of anti-HIV activity in marine bivalves of Indian coastline and

also in plants and trees of medicinal importance in western region. Our studies have revealed the presence of anti-HIV activity in some marine bivalves, which are being currently fractionated to identify and characterize the active components.

Future work:

The future work involves characterizations of the Nef interacting clones and identifying their functional relevance in HIV lifecycle. The interaction of Tat with NF-κB DNA is being further characterized using biochemical assays. We are also interested in looking at the chromatin modulation by Tat protein in HIV infected cells and studies are in progress to find a physiological relevance for Tat-NF-κB interaction. Identification of differentially expressed genes and their relevance to HIV induced cell death will be continued, with a focus on mitochondrial functions in infected cells. We intend to also continue our studies on the elicitation and maturation of CTL in response to gp120 immunization in mice. Finally, the identity of the active molecule involved in anti-HIV activity from marine bivalve is currently under investigation

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Role of T cells and non-T cells in the resistance or susceptibility to Leishmania infection.

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Meenakshi Jadhav, technician

Collaborators:
Boppana Ramanamurthy, Debashis Mitra

Abstract and background:

Macrophages play host to *Leishmania*, a parasite that causes leishmaniasis in 0.5 million people annually. Macrophage expressed CD40, a costimulatory molecule, induces interleukin-12 (IL-12)-and IFN-g-dependent host-protective immune response to Leishmania and other intracellular pathogens. Paradoxically, interleukin-10 (IL-10), another CD40-induced cytokine in macrophages, promotes *Leishmania* infection. How CD40 signals to regulate the secretion of these two counter-effective cytokines remains unknown. We demonstrate that weak CD40 signals induce extracellular stress-related kinase (ERK)-1/2-dependent IL-10 expression whereas stronger signals induce p38-mitogen activate protein kinase (p38MAPK)-dependent IL-12 production. p38MAPK and ERK-1/2 counter-regulate each other. *Leishmania* skews the CD40 signaling towards ERK-1/2, inducing IL-10 that inhibits CD40-induced p38MAPK activation, iNOS2 and IL-12 expression. ERK-1/2 inhibition or IL-10 neutralization restores CD40-induced p38MAPK activation and parasite killing in macrophages and in BALB/c mouse, a susceptible host, suggesting a novel drug-designing principle. These data unfold a novel immune evasion strategy, where a parasite differentially modulates the CD40 engaged reciprocally functioning signaling modules, and a novel strategy for defining drug target(s).

Aims:

We tested whether or not the anti-CD40 antibody-mediated cross-linking of CD40, which was proposed to perform host-protective anti-leishmanial function, induced both IL-10 and IL-12 by differential activation of p38MAPK and ERK-1/2.

Work achieved

We observed that cross-linking of CD40 using an anti-CD40 antibody at concentrations more than 4μg/ml increased p38MAPK phosphorylation but decreased ERK-1/2 phosphorylation and that CD40 cross-linking at low doses induced IL-10 but at higher doses induced IL-12 in uninfected macrophages. In *Leishmania*-infected macrophages, CD40-induced p38MAPK phosphorylation was decreased with a concomitant increase in ERK-1/2 phosphorylation. The treatment of Leishmania-infected macrophages with an intermediate dose (3 μg/ml) of anti-CD40 antibody that induced both IL-10 and IL-12 resulted in more IL-10 and less IL-12 production, as compared with the uninfected macrophages. The IL-12:IL-10 ratio was inversely proportional to the parasite: macrophage ratio suggesting that *Leishmania* infection promotes IL-10 production.

While exogenous IL-10 diminished CD40-induced IL-12 and iNOS2 expression, IL-10 neutralization restored CD40-induced expression of IL-12 and iNOS2 in *Leishmania*-infected macrophages perhaps by recovering CD40-inudced p38MAPK phosphorylation. Corroborating to these observations, IL-10 neutralization restored

the CD40-induced anti-leishmanial function of macrophages significantly. Since IL-10 production is increased during Leishmania infection, we assessed the effect of PD098059 on CD40-induced iNOS2 expression in presence of IL-10. PD098059 prevented the IL-10 inhibition of CD40-signaled iNOS2 induction in uninfected macrophages suggesting that ERK-1/2 inhibition reinstates the CD40-induced leishmanicidal function in infected macrophages. Indeed, treatment of Leishmania-infected macrophages with anti-CD40 antibody and PD098059 together induced a significantly higher leishmanicidal function than that observed with anti-CD40 antibody alone or with PD098059 alone, perhaps by enhancing iNOS2 and reducing IL-10 expression. Therefore, knowing the reciprocal wings of signaling arising from the cell surface receptor can serve as a new principle for identifying a definitive drug target (Figure 1). The observed reciprocity of CD40 signaling, regulating both inflammatory (IL-12) and anti-inflammatory (IL-10) cytokines, implies even a broader application in the prevention of autoimmune diseases and graft rejection.

Future work:

We are currently investigating the mechanism of such reciprocal CD40 signaling in macrophages. The other objective of further research is to find out how Leishmania modulates CD40 signaling.

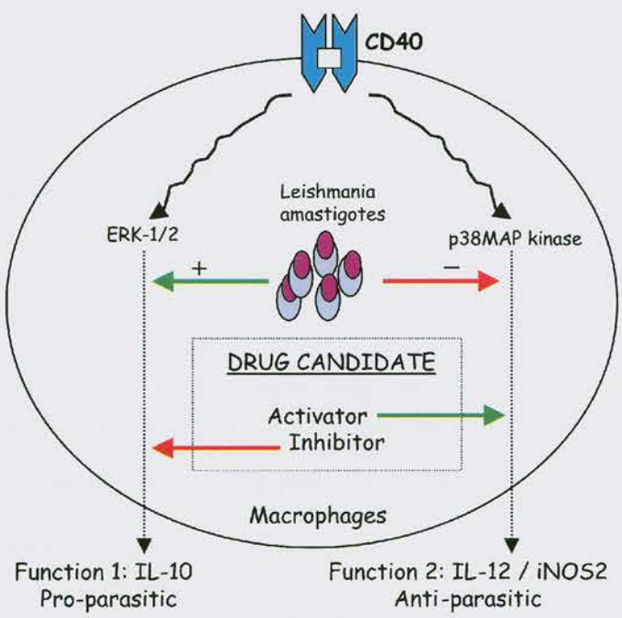

Fig. 1. The model explains a novel immune strategy of the parasite and a new strategy of defining anti-parasitic drug target. CD40 elicits either p38MAPK-mediated iNOS2- and IL-12-dependent anti-parasitic or ERK-1/2-mediated IL-10-dependent pro-parasitic immune response. Leishmania skews CD40 signaling towards ERK-1/2 inducing IL-10 that enhances its own production and inhibits CD40-induced p38MAPK-mediated IL-12 and iNOS2 expression and parasite killing. We exemplify how selective inhibition of a host-signaling pathway, which is augmented by the parasite for its own survival, results in host-protection. Since the drug does not act on the parasite, it does not exert any selection pressure on the parasite to develop drug-resistance.

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Role of viral complement control proteins in immune evasion

Jayati Mullick, John Bernet, Akhilesh Singh, Archana Kadam,
Yogesh Panse, technician, Vivekanand Yadav, technician

Collaborator:
Pradeep B. Parab

Background:

The complement system is an integral participant in the innate mechanisms of immunity and thus has the burden of performing surveillance in the host, and protect it from all the pathogenic non-self targets including viruses. Although viruses are small and have relatively simple structure, both acute and latent viruses can be efficiently recognized and neutralized by the complement system. Recent studies have clearly pointed out that the complement system is not only important in controlling the generalized hematogenic spread of viruses, but is also important in controlling viral infections through the mucosal route and through the peripheral solid organs. Thus, to combat host responses and succeed as pathogens, viruses must develop principles to elude the host complement system. The genome sequencing of vaccinia virus (VV), herpesvirus saimiri (HVS), and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) have shown that these viruses encode for complement control protein homologs (vCCPHs). We believe that these viruses encode CCPHs to mask themselves against host's complement attack and are essential for their successful in vivo survival and propagation. Our current emphasis is on understanding the molecular mechanism(s) underlying the interaction between host's complement proteins and CCPHs of VV, HVS and KSHV. Our long term goal is to understand how complement controls viral infections and how vCCPHs help subverting it.

Aims:

1. How viral complement control proteins inactivate complement?
2. Which are the determinants of viral complement control proteins important in complement inactivation?
3. How vCCPHs control complement-mediated inactivation of viruses in vivo?

Work achieved:

Our work focuses on the vCCPHs of vaccinia virus, Kaposi's sarcoma-associated herpesvirus and herpesvirus saimiri. These proteins are composed of four tandemly repeating bead-like structures termed short consensus repeats (SCRs) or complement control protein (CCP) domains. Previously we have characterized the mechanism of complement inactivation of vaccinia virus CCPH (VCP) and KSHV CCPH (named kaposica). Our data indicated that like host complement control proteins these proteins also possess factor I cofactor activities for complement proteins C3b and C4b, and also possess decay-accelerating activities for the classical as well as alternative pathway C3 convertases. In short, these proteins work at the level of C3 convertases. It is pertinent to point out here that although these viruses encode small proteins, they possess all the complement regulatory activities of host complement proteins. The only human complement regulator that has all these activities is CR1, which is a 30 CCP domain containing protein. Thus, small sizes of viral proteins reflect the limited size of their genomes, but this does not limit their activities.

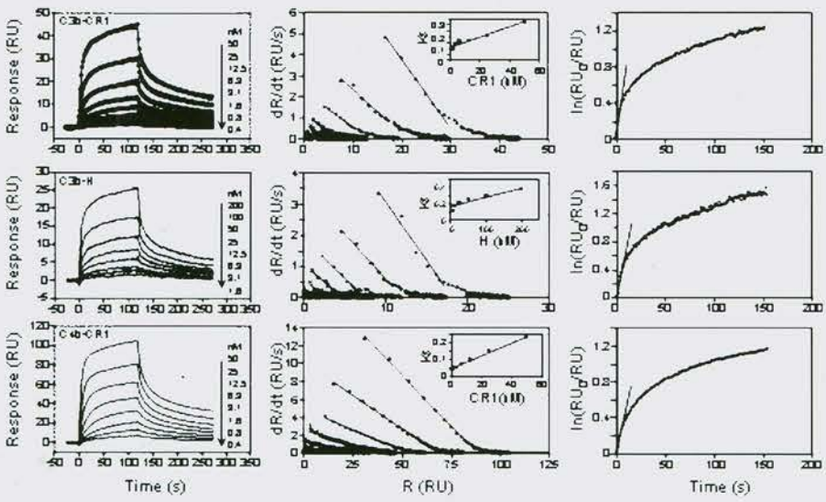


Fig. 3. Binding of complement receptor 1 (sCR1) and factor H to immobilized C3b and C4b. Left panels, overlay plots for binding of sCR1, and factor H to immobilized C3b, and sCR1 to immobilized C4b. Middle panels, Linear transformation of the association phase data of the respective sensogram data shown on the left. The straight lines are linear least squares fits to the data. Inset, values of k_s (determined from the slope of the fits of the association data) replotted against analyte concentration. Right panels, Linear transformation of the highest concentration of the dissociation phase data of the respective analyte shown on the left.

We are also making efforts to determine the functional determinants of viral complement control proteins. Currently we are using two different approaches to address this issue. The first strategy involves generation of mutants by site-directed mutagenesis and the second approach involves use of monoclonal antibodies.

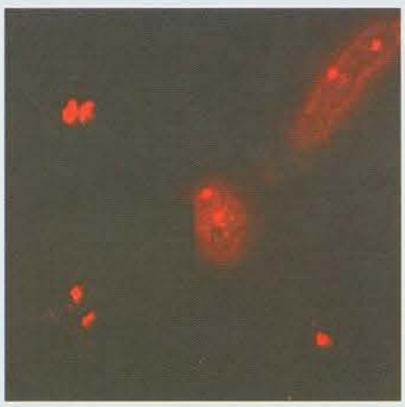
Future work:

1. Detailed functional characterization of kaposica and HVS CCPH.

2. Functional characterization of mutants of VCP and kaposica.

3. Characterization of in vivo role of VCP.

Research Reports



Chromatin Architecture and Gene Regulation

LSM 510
HBO 50

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Structure-function analysis of tumor suppressor protein SMAR1 and its role in T cell receptor gene rearrangement

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Kamini Singh, Pavithra L., Surajit Sinha, Devraj Mogare, technician

Collaborators:
Debashis Mitra, Bhaskar Saha

Abstract and background

We are interested in understanding various functions of a MAR (Matrix Associated Region) binding protein SMAR1. Earlier we have shown that SMAR1, located in human chromosome 16q24, acts as a tumor suppressor. The tumor suppressor functions of SMAR1 is mediated through its direct interaction, phosphorylation and activation of p53. Since, p53 is known to be the guardian of genome, structure-function analysis of SMAR1 is important to further identify mode of its action in modulating p53. Sinces SMAR1 modulates chromatin at various MAR sequences and thus regulates cell cycle, we are also interested in other aspects like cell signaling, stress mediated upregulation, regulation of SMAR1 in various cancers, and diabetic conditions. Apart from its p53 mediated cell cycle regulation, SMAR1 expression also has been shown to be tightly regulated during the development of T cells within thymus. We have shown that SMAR1 transgenic mice exhibit architectural abnormalities in lymphoid organs associated with changes the chromatin, near T cell receptor enhancer that results in perturbation of V(D)J recombination of receptor gene segments (**Fig. 1B and 4**).

Aims:

1. To identify minimal domain of SMAR1 that allows p53 interaction and activation.
2. To analyze chromatin modulation and transcriptional repression by SMAR1 at the TCRβ locus: Recruitment of repressor protein Cux at MARβ locus.
3. Control of V(D)J recombination in SMAR1 transgenic mice.

Work achieved

Identification of an RS domain that controls p53 activation

Various stresses and DNA damaging agents trigger transcriptional activity of p53 by post-translational modifications making it a global regulatory switch that controls cell proliferation and apoptosis. SMAR1 interacts with p53 and arrest the cells at G2/M phase. Now we have delineated the minimal domain of SMAR1 that is responsible for p53 interaction, activation and stabilization within the nucleus. We also have demonstrated that this arginine-serine rich (RS) domain triggers various cell cycle modulating proteins resulting in cell cycle block. Furthermore, phenotypic knock-down experiments using siRNA showed that SMAR1 is required for activation and nuclear retention of p53. Interestingly, a small RS domain of SMAR1 fused with the PTD domain of HIV-1 Tat, shows strong p53 activation which in turn resulted in regression of tumors when injected in nude mice (**Fig. 1**). Thus, our current results emphasize that the p53 modulating activity of SMAR1 resides within the RS domain and might therapeutically important for regressing tumors. In short, SMAR1 might replace mdm2, directly interact with p53 and finally allow phosphorylation at Serine-15 (**Fig. 3**).

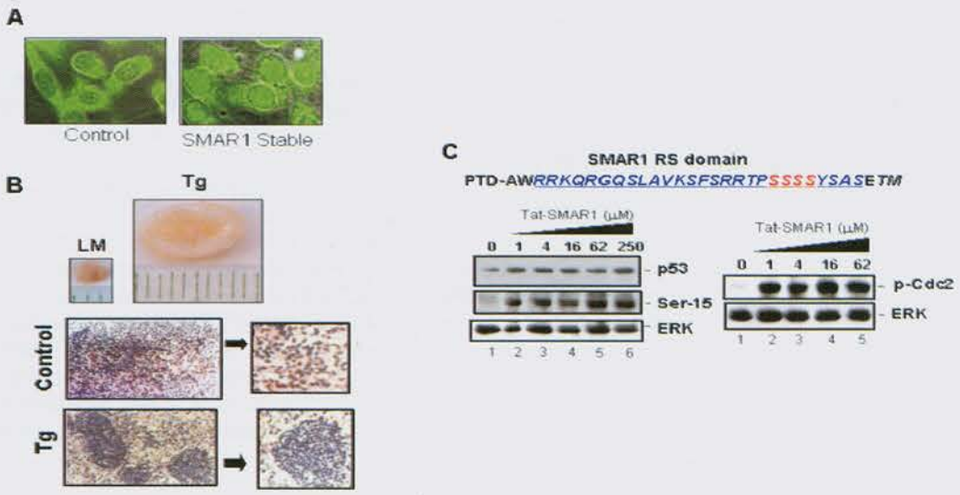

Fig. 1. A. Overexpression of SMAR1 allows longer retention of p53 within nucleus. Left and right panels shows staining of p53 in control and SMAR1 expressing stable cells. The 293 cells were processed for confocal analysis after staining with p53 antibody. B. Organomegaly showing larger lymph nodes in SMAR1 transgenic (Tg) mice compare to control littermate mice. The histological sections shows abnormal architecture of LN in SMAR1 transgenic mice. C. Sequence showing the fusion of PTD domain of HIV-1 Tat with the RS domain of SMAR1. Western blot experiments showing phosphorylation of p53 by the RS peptide of SMAR1. Right panel shows upregulation of phospho-cdc2 upon induction by SMAR1 peptide.

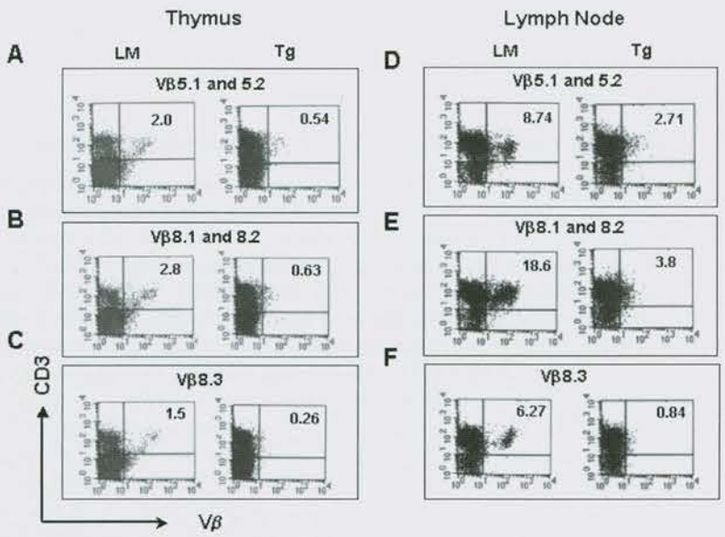

Fig. 2. A model showing mode of action of SMAR1. SMAR1 allow direct interaction and phosphorylation of p53 at serine-15 residue. We hypothesize that SMAR1 replaces mdm2 and binds to p53 allowing phosphorylation. This also allows p53 to be stable and retain in the nucleus as shown in figure 1. The downstream elements of p53 are activated leading to cell cycle arrest.

SMAR1 controls V(D)J recombination of T cells

To analyze the functions of SMAR1, transgenic mice were generated that express SMAR1 in a tissue independent manner. Apart from organomegalies, SMAR1 expressing mice show severely altered frequency of the T cells expressing commonly used V segments Associated with diminished transcription, rearrangements of V5.1/5.2, V8.1/8.2/8.3 loci are also reduced (**Fig. 2 & 4**). T cells from SMAR1 transgenic mice remain immature, as thymocytes exhibit a partial block at the early DN stage of T cell development. Interestingly, SMAR1 transgenic mice exhibit hypercellularity both in lymph nodes and spleen along with prominent architectural defects in these organs (**Fig. 1B**). Thus, apart from its role in the control of cell cycle, V(D)J recombination and T cell development, SMAR1 is also involved in the development of lymphoid organs.

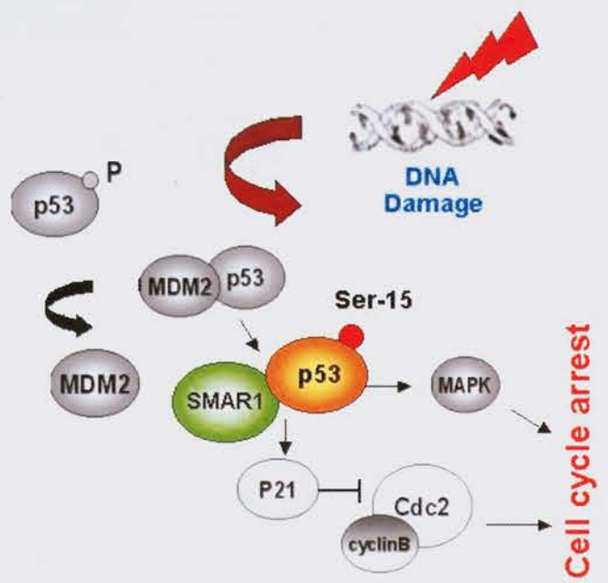

Fig. 3. Defective V(D)J recombination in SMAR1 transgenic mice. The cells from thymocyte or lymph nodes were stained with Vb's. As shown, the T cell repertoire in transgenic mice were defective in expressing Vb5.1/5.2, 8.1/8.2 and 8.3.both in thymus and Lymph nodes (LN) indicating the role of SMAR1 in V(D)J recombination.

SMAR1, a strong repressor that recruits Cux/ CDP

Chromatin modulation at various cis acting elements is critical for V(D)J recombination during T and B cell development. MARβ, a matrix associated region (MARβ) located upstream of the TCR enhancer (E), serves a crucial role in silencing E-mediated TCR activation. MAR is the target for MARβ binding proteins - SMAR1, Cux/CDP and SATB1, the latter two known to function as transcriptional repressors in a context-dependent manner. By DNaseI hypersensitivity assays, we have shown that chromatin accessibility at MARβ is maximal at the double positive (DP) stage of T cell development where TCR rearrangement occurs. By DNaseI hypersensitivity assays, we have also shown that overexpression of SMAR1 and Cux/CDP modulate the chromatin structure at MARβ (**Fig. 4**). We further demonstrated that the silencer function of MARβ is mediated independently by SMAR1 and Cux/CDP as judged by their ability to repress Eβ dependent reporter gene expression. Moreover, the repressor activity of SMAR1 is strongly enhanced in the presence of Cux/CDP. The repressor domain of SMAR1 is separate from the MAR binding domain and contains a nuclear localization signal (NLS) and an Arginine-Serine (RS) rich domain, characteristic of pre-mRNA splicing regulators. Our data suggests that at the DP stage of T cell development, a time commensurate with V(D)J recombination, cis-acting MAR elements recruit the strong negative regulators Cux and SMAR1 to control Eβ enhancer-mediated recombination and transcription (**Fig. 4**).

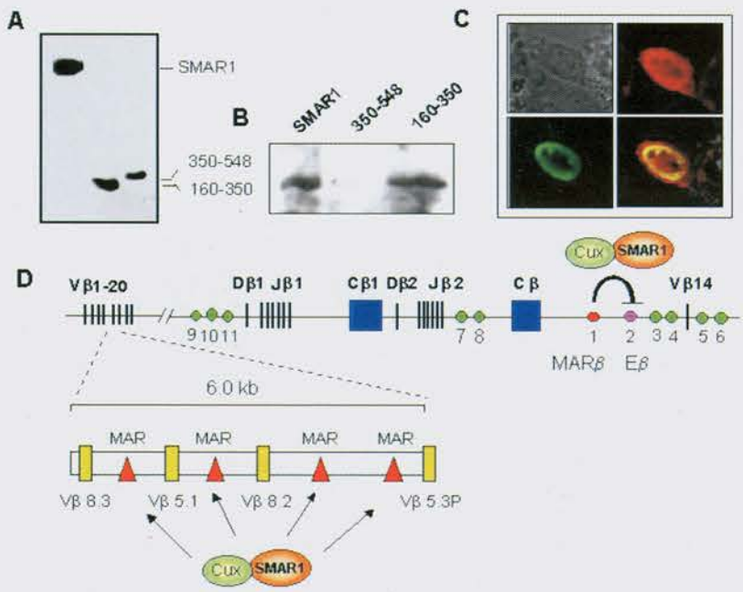

Fig. 4. SMAR1 directly interacts with Cux/CDP. A. Various truncations of SMAR1 were tested for interaction with Cux. The FLAG tagged proteins were purified and processed for IP experiments. B. A region from 160-350 of SMAR1 is important for Cux interaction. C. Confocal analysis showing direct interaction of a region from 288-350 with Cux/CDP in the nucleus forming a perinuclear ring. D. A model showing the interaction of SMAR1 with the repressor protein Cux and the control of V(D)J recombination. The eleven DNase1 hypersensitive sites are shown by circles. The red and green circles represent MARb and Eb enhancer regions. Lower horizontal bar shows various Vb gene segments flanked by MARs. A complex of SMAR1 and Cux might bind to these MARs and perturb V(D)J recombination from Vb's. In the 2nd pathway, the complex might bind to the MARb sequence and interfere with Eb enhancer halting V to DJ recombination.

Future work:

1. To elucidate SMAR1-RS peptide binding to p53 and MDM2 displacement in vitro and in vivo.
2. SMAR1 promoter cloning and analysis.
3. Regulation of Cyclin D1 promoter by SMAR1 in breast cancer cell lines.
4. Identification and characterization of SMAR1 interacting proteins.

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Study of the mechanism(s) involved in the regulation of the MAR-binding activity of SATB1

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Abstract and background:

Special AT-rich sequence binding protein 1 (SATB1) is a cell type restricted chromatin organizer. SATB1 organizes T cell chromatin into domains via periodic anchoring of base-unpairing regions (BURs) to the nuclear matrix. In thymocyte nuclei, SATB1 has a cage-like 'network' distribution circumscribing heterochromatin and selectively tethers BURs onto its network resulting in coordinated regulation of distant genes. SATB1 regulates large chromatin domains by acting as a 'landing platform' for several chromatin-remodelling enzymes in T cells. Interestingly, the dimerization domain of SATB1 is homologous with many other PDZ domains. As a PDZ- and homeo domain-containing protein SATB1 may provide a framework that mediates assembly of specific protein complexes onto a discrete set of BURs. We set out to search for proteins that interact with SATB1 via its PDZ signalling domain and to investigate the outcomes of such interaction on the function of SATB1. To understand the mechanism of gene regulation by SATB1, we also isolated many genomic binding sites of SATB1. Understanding how SATB1 and its interaction partners coordinate their activities at these loci would shed light on the role of SATB1 in the regulation of cell type specific gene expression.

Aims:

1. To identify proteins interacting with SATB1 through its PDZ-like domain.
2. To study the mechanism(s) by which SATB1 regulates transcription by interacting with other cellular proteins through its PDZ domain.

Work achieved:

SATB1 interacting proteins : PDZ domains are modular protein-binding domains that have at least two distinct mechanisms for binding. They can bind at the carboxyl termini of proteins to specific sequences or they can bind with PDZ domains of other proteins and potentially regulate intracellular signals. These domains are generally found in the trans-membrane receptors or ion channels. In signal transduction pathways surface receptors, ion channels and internal signaling molecules combined with kinases act together in a sequential cascade and transmit signals to nuclear receptors which in turn leads to activation of gene(s). Many of these receptors contain PDZ domain(s). As a PDZ- and homeo domain-containing protein SATB1 may provide a framework that mediates assembly of specific protein complexes onto a discrete set of BURs. SATB1 may therefore act as an architectural protein on the nuclear matrix. The consequence of such protein-protein interaction(s) may lead to the activation or repression of SATB1 activity. Examination of the each associated protein may provide insights into the specificity and activity of SATB1 in T-cell development and function. We used the PDZ-like domain (amino acids 90-204) as baits for two-hybrid screening. After screening of the human T cell leukemia library in pACT2.1 vector, we obtained several positive clones and identified four candidate gene products among these as interaction partners of SATB1 (Fig. 1). We are in the process of characterizing these at a molecular level.

Fig. 1. Isolation of PDZ-interacting proteins by yeast two hybrid analysis: The PDZ-like domain of human SATB1 was fused with the GAL4 activation domain (AD) in pGAD424 vector. A Human T-cell leukemia library cloned as fusion with the GAL4 DNA-binding domain (DBD) in the pAS2.1 vector was used for screening. The AD and DBD fusion constructs were cotransformed in yeast strain AH109 and assayed for protein-protein interaction using standard protocols, with HIS3 and LacZ as the reporter genes. The picture depicts number of b-gal-positive clones with variable degree of activity.

Structure-function analysis of SATB1: For elucidation of the structure-function relationship of SATB1 we first standardized overexpression of SATB1 and purification. We have succeeded in purification of full-length as well as truncated forms of SATB1 including three of its functional domains. The PDZ-like domain (Fig. 2) and the DNA-binding domain of SATB1 have been purified in milligram quantities and will be used for biophysical studies. We have designed a novel expression vector for efficient purification of fusion proteins, and using this we have purified a number of SATB1 truncations as well as other proteins implicated in chromatin function. We have also performed protein footprinting analysis and observed that specific conformational changes are induced in SATB1 upon binding to its cognate DNA sequences.

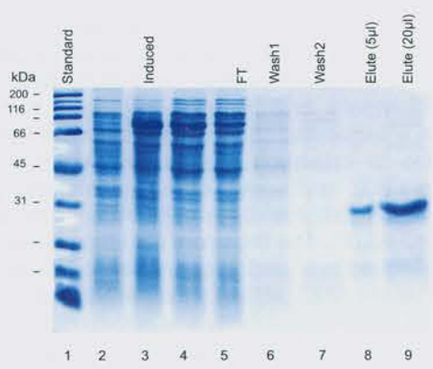

Fig. 2. Purification of the PDZ-like domain of SATB1. Briefly, the induced protein was expressed as a fusion with intein and tag. After binding to affinity matrix the fusion protein is incubated with reducing agent, thereby inducing the internal splicing. The spliced PDZ-like domain is free from the fusion tag and is seen as pure product in the eluate (lanes 8 and 9).

Isolation of genomic SATB1 binding sites: We isolated a large number of in vivo genomic targets of SATB1 in T cells using chromatin immunoprecipitation followed by cloning. Interestingly, many of the isolated DNA sequences shared similarities. Multiple alignments of these sequences revealed high degree of sequence similarity that prompted us to search for defined sequence patterns. We show that occurrences of hexameric sequence patterns in these sequences revealed statistically significant differences over randomly generated sequences. Interestingly, motif search analysis yielded a set of 16-50 bp consensus motifs that appear to be present preferentially in the sequences of SATB1-binding sites but not in the random sequences. We have unraveled, for the first time, primary sequence signature(s) that are embedded into cell type specific chromatin architecture. We propose that the primary sequence features such as the consensus motifs and repeating hexameric patterns project a unique chromatin context in vivo. We have generated some unique bioinformatics tools for primary sequence analysis and will be utilized to analyze sequence features further.

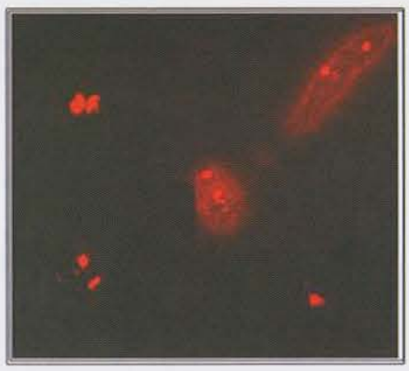

Fig. 3. Amplified FISH for one in vivo genomic SATB1-binding target sequence. Briefly, nuclear matrices were prepared in situ, and biotin labeled DNA probe was used for hybridization. The signal was amplified using Tyramide signal amplification method. The individual spot corresponds to each locus, therefore generating two spots per nucleus.

Future work:

1. To investigate the role of the PDZ-mediated interactions on regulation of transcription by SATB1 in T cells.

2. Study the SATB1-mediated organization of chromatin at its genomic binding sites.

Experimental Animal Facility (2003-2004)

The Team:

B. Ramanamurthy, K. N. Kohale, M. D. Sheikh, A. Inamdar,
P.T. Shelke, Vaishali Bajare, Anand Bidlan

The Experimental Animal Facility is an infrastructural service department of the Institute. It is a barrier-maintained facility for the breeding, maintenance and supply of high quality and standardized laboratory animals viz. inbred mice, rats, rabbits etc. for the ongoing research projects of the Institute. The following is the list of various laboratory animals maintained at the facility:

MICE
Strain: BALB/cJ
C57BL/6J
DBA/2J
FVB/NJ
NOD/LtJ
SWISS
BALB/c*
Nude Mice
Gene knock-out mice

RATS
Strain: WISTAR
LEWIS

RABBITS: NEWZEALAND WHITE

MASTOMYS: MASTOMYS COUCHA

Defined barrier practices are followed scrupulously without any exception or allowance, with access to a select few personnel, to minimize the risk of microbial infection to the animals housed in the facility.

The breeding program for the propagation of the three different inbred lines viz. BALB/c, C57BL/6, and DBA/2 is structured in a two-tier format, i.e. the Foundation colonies (FC) and the Production colonies (PC). The remainder inbred and mutant (gene knock-out) mice are maintained as foundation colonies. Strict full-sib pairing only propagates the animals in the FC. These colonies are the nuclear colonies for the long-term propagation of an inbred strain.

A single male mouse with spontaneous congenital cataract and micropthalmia was detected in a production colony of BALB/c strain of mice. Breeding studies undertaken suggested that the defect is inheritable and that this mutation is caused by a gene defect inherited in an autosomal recessive manner. The morphological observations suggested that the defect is manifested at the 14th postnatal day when the eyes are open. The other phenotypic features of this congenital defect include clear cornea, central lens opacity and intact lens capsule. Ophthalmoscopic examination of the mutant eyes also confirmed the central lenticular opacity with clear zone at periphery and the disturbance of the typical iris pattern together, with the maldevelopment of the pupillary muscles. The other features noticed were both males and females were fully active, viable and fertile in homozygous condition and no other gross external or visceral abnormalities were seen except the eye defect.

Histology of the mutant lenses during embryonic stage of eye development revealed abnormal morphology, vacuolation and degeneration of the lens fiber cells.
Efforts have been initiated to separate lens crystallin proteins on SDS-PAGE and high resolutions 2D electrophoresis to look for new species or up regulation or down regulation of the existing proteins. Attempts shall also be made to identify the genes responsible for this defect and its relationship with the phenotype observed, with the help of molecular biological tools.

The mutant colony is currently at F24 level of inbreeding.

The facility has imported a total of 73 mice comprising of15 different gene knock-out mice/mutant mice in 2002-2003. Attempts were made to establish and breed these mice for supply and use in the ongoing R&D projects. These mice are housed in Individually Ventilated Isolator systems. An aseptic/sterile routine has been standardized for the housing, breeding and handling of these mice. Already these mice have been supplied in reasonable numbers for use in ongoing research projects.

The complete technical support and advice has been extended regularly to Scientists/ Research Scholars in the various aspects of animal experimentation namely, handling of laboratory animals, collection of blood and other samples, immunizations, surgical procedures etc. and the procurement of animals.

As a part of the health-monitoring program around 46 mice samples were subjected for parasitological screening and around 14 samples for clinical biochemistry.

Histopathology: Over 150 stained and unstained slides have been processed and prepared for histopathological examination (The service was provided for some research groups of the Institute.

The breeding of laboratory animals has been planned to meet the needs of Scientists/Research Scholars for various animal experiments. The details of the animals bred in the facility, procured from various sources, and supplied for various R & D activities are given below.

1-04-2003 – 31-03-2004

SR.NO	STRAINS/SPECIES	ANIMALS PROCURED	ANIMALS BRED	ANIMALS SUPPLIED
	RATS			
	Wistar	----	150	69
	Lewis	----	37	8
	MICE			
	BALB/c	250	3969	4169
	C57BL/6	50	2441	1112
	SWISS	45	1290	827
	DBA/2	----	----	10
	Nude (nu/nu)	196	26	160
	BALB/c*	----	506	467
	Mutant Mice(including Jax line of inbred mice)	23	2677	1370
	MASTOMYS COUCHA		20	----
	RABBIT(NZW)		24	13

* **BALB/c with cataract and microphthalmia mutation.**

Fluorescene Activated Cell Sorter (FACS)

During the period approximately 6220 samples were acquired and analysed. The samples were of surface staining, DNA staining, analysis of GFP fluroscence, calcium uptake.

The Confocal Laser Scanning Microscope (CLSM)

The confocal laser scanning microscope (CLSM) is a state of the art laser scanning microscope (Zeiss LSM 510) having four lasers UV, 488 nm, 543 nm and 633 nm wave length. Presence of four lasers enhanced the choice of various fluorophores during real time observation. This year our facility has acquired more than 4500 images from NCCS users and approximately 500 images for users from various other institutes.

Library

The NCCS Library has collection in the frontier areas of biotechnology having relevance to NCCS research activities. The Library holds approximately **five thousand two hundred bound journals**, **eighteen hundred books**, and subscribe to **fifty- eight scientific journals** and **twenty four other periodicals**.

In the development of its collections, the Library's priority is to support NCCS research activities. The Library collection is expanded in consultation with NCCS scientists. The Library's print collections are growing by approximately **750 volumes** per year. During the period of 2003-2004 , the Library has added **eighty six books** and **672 volumes of journals** to its collection. A limited full text online material is provided for faster access to research information.

Additional documentation facilities include local area network for library activities and PubMed database access, a number of CD-ROM database including, full text and factual database. The Library continues to be a part of the Pune Library Network and Medical Library Association of India.

Computer Centre

Highlights of the work done by Computer Centre in 2003-2004

- **Development of new NCCS website**

We have designed and developed new NCCS website and have also been involved in management and maintenance.

- **Installation and Configuration of 4 new Servers and Server Rack**

We have installed and configured four Compaq servers for DNS, E-Mail, Proxy and Web hosting services running on RedHat Linux Enterprise server 2.1. The data and user accounts have been successfully transferred from the old system to these new systems and web mail services have been started for NCCS users. Moreover Interscan Virus Wall software has been installed and configured for checking e-mail, http and ftp traffic for viruses or malicious codes.

- **Procurement and installation of new Software:**

The following softwares have been installed in computer Centre for NCCS use:

i) **Interscan Viruswall** with 100 users License for checking mail, http and ftp traffic for viruses sourced or destined to our LAN.
ii) **Macromedia Studio MX (single copy)** for dynamic website designing of NCCS.
iii) **Norton System Works (single copy)** for PC diagnostics
iv) **RedHat Linux ES 2.1 (single copy)** for upgrading current OS.
v) **Reference Manager** for management of scientific publications.

- **Development of Software Modules for Experimental Animal Facility:**

We have developed software modules for our animal house. In this connection two students from Computer Science Dept. from Pune University have undergone training from July Dec. 2003. The software module displays the animal house data like scientist information, project information; Animal used information, animal requisition information, etc. online.

- **Computer Support Services provided:**

To increase the quality of scientific presentation of NCCS staff and students attending national and international conferences/seminars, computer centre is helping for DTP work, CD writing, scanning images and transparency printing on color LaserJet printer.

- **Computer System & LAN Management, Maintenance and Upgradation:**

Computer Centre is providing technical support to more than 80 computers and 45 printers which includes installing Operating System, Software's and Drivers, Anti-Virus package, Installation and configuration of new computers, CD-ROM / RAM / Network card installation and connecting to LAN, Server operating system upgradation and maintenance.

Accepted Publications 03-04

1. Sarrias MR, Sahu A, Lambris JD. 2004. Characterization of the interactions of complement receptor 2 with its ligands iC3b, C3d and EBV glycoprotein gp350/220. Biacore Journal, 4: 14-17 (Invited article).

2. Bernet J, Mullick J, Panse Y, Parab PB and Sahu A. 2004. Kinetic analysis of the interactions between vaccinia virus complement control protein and human complement proteins C3b and C4b. J. Virol., In press.

3. Bapat SA and Mishra GC. 2004. Stem Cell Pharmacogenomics Current Topics in Medicinal Chemistry Vol. 4, In Press.

4. Salunke DB, Hazra BJ, Pore VS, Bhat MK, Nahar PB and Deshpande MV. 2004. New Steroidal Dimers with Antifungal and Antiproliferative Activity. Journal of Medicinal Chemistry, 47: 1591-1594.

5. Singh S and Bhat MK. 2004. Carboplatin induces apoptotic cell death through downregulation of constitutive activity of nuclear factor-B in human HPV-18 E6 positive HEp-2 cells. Biochemical and Biophysical Research Communications 318: 346-353.

6. Risbud MV, Bhargava S, Bhonde RR. 2003. In vivo biocompatibility evaluation of cellulose macrocapsules for islet immunoisolation: Implications of low molecular weight cut-off. J Biomed Mater Res, 1; 66A(1):86-92.

7. Shukla R, Barve V, Padhye S and Bhonde R. 2004. Synthesis, structural characterization and evaluation of insulin-mimetic potential of bis (quercetinato) oxovanadium (IV)-conjugate. Bio-organic Medicinal Chemistry Letters (Accepted)

8. Purbey PK, Jayakumar PC, Deepalakshmi PD, Patole MS and Galande S. 2004. An improved GST fusion vector with caspase 6 cleavage site for on column removal of fusion tag during purification. Biotechniques, Accepted pending minor revision (submitted).

9. Kale VP. 2004. Differential activation of MAPK signaling pathways by TGFâ1 forms the molecular mechanism behind its dose dependent bi-directional effects on hematopoiesis. Stem Cells and Development, 13:27-38.

10. Kale VP and Vaidya AA. 2004. Molecular mechanisms behind the dose dependent differential activation of MAPK pathways induced by TGFâ1 in hematopoietic cells. Accepted as lead article in "Stem Cells and Development" October issue.

11. Das R, Mahabeleshwar GH and Kundu GC 2003. Osteopontin stimulates cell motility and NF-kB mediated secretion of urokinase type plasminogen activator through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells. J. Biol. Chem. 278 (31), 28593-28606.

12. Mahabeleshwar GH and Kundu GC. 2004. Tyrosine kinase, $p56^{lck}$ regulates cell motility and NF-kB mediated secretion of urokinase type plasminogen activator through tyrosine phosphorylation of IkBa following hypoxia/ reoxygenation. J. Biol. Chem. 278 (52), 52598-52612, 2003.

13. Mahabeleshwar GH, Das R and Kundu GC. 2004. Tyrosine kinase, $p56^{lck}$-induced cell motility and urokinase type plasminogen activator secretion involves activation of epidermal growth factor receptor/extracellular signal regulated kinase pathways. J. Biol. Chem. 279 (11), 9733-9742.

14. Philip S, Bulbule A and Kundu GC. 2004. Matrix metalloproteinase-2: mechanism and regulation of NF-kB-mediated activation and its role in cell motility and ECM-invasion. Glycoconjugates and Drug Research, In press.

15. Das R, Mahabeleshwar GH and Kundu GC. 2004. Osteopontin Induces AP-1-mediated Secretion of Urokinase Type Plasminogen Activator through c-Src Dependent EGF Receptor Transactivation in Breast Cancer Cells. J. Biol. Chem. 279 (12), 11051-11064.

16. Sasnoor LM, Kale VP and Limaye LS. 2003. Supplementation of conventional freezing medium with a combination of catalase and trehalose results in better protection of surface molecules and functionality of haematopoietic cells. J of Haematotherapy and stem cell research, 12(5):553-64.

17. Kapoor M, Reddy CC, Krishnasastry MV, Surolia N, Surolia A. 2004. Slow-tight binding inhibition of enoyl-acyl carrier protein reductase from Plasmodium falciparum by triclosan. Biochem. J. [Epub ahead of print]

18. Ettickan B, Lenka N, Prabu SK, Fang JK, Wilkinson F, Atchison M, Giallongo A, Avadhani NG. 2004. Regulation of murine cytochrome c oxidase Vb gene expression during myogenesis: YY1 and a CArG binding protein reciprocally regulate the transcription activity by physical interaction with BERF-1/ZBP-89 factor. J. Biol. Chem., In press.

19. Dandekar DH, Ganesh KN, and Mitra D. 2004. HIV-1 Tat directly binds to NFêB enhancer sequence: role in viral and cellular gene expression. Nucleic Acids Res.32:1270-8.

20. Kohale, K.N., Ingle, A. D., Asha Kelkar and P.B. Parab, 2004. Dense Cataract and Microphthalmia- New Spontaneous Mutation in BALB/c mice. Comparative Medicine, Vol.54, No.3, p-275-279.

21. Katdare MR, Bhonde RR and Parab PB. 2004. Analysis of morphological and functional maturation of neoislets generated in vitro from pancreatic ductal cells and their suitability for islet banking and transplantation. J.Endocrinology, In press.

22. Mathur RK, Awasthi A, Wadhone P, Ramanamurthy B, Saha B.2004. Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses. Nat Med.,10 (5):540-4.

23. Saha B, Tonkal AM, Croft S, Roy S. 2004. Mast cells at the host-pathogen interface: host-protection versus immune evasion in leishmaniasis. Clin Exp Immunol.137(1):19-23.

24. Latha M, Pari L, Sitasawad S and Bhonde R. 2004. Insulin-secretagogue activity and cytoprotective role of the traditional antidiabetic plant Scoparia dulcis (Sweet Broomweed). Life Sciences, In Press.

25. Raut CG, Paramasivan R, Gokhale MD, Thakare JP, Padbidri VS, Sapkal GN, Mishra AC, Shouche YS, Jayakumar PC and Mourya DT. 2003. A focal outbreak of Japanese encephalitis among horses in Pune district, India. Journal of Communicable Diseases, 35: 40-42

26. Jaykumar PC, Musande V, Shouche YS, and Patole MS. 2004. The Selenophosphatase gene from Leishmania major. DNA Sequence, 15: 66-70.

27. Dighe AS, Jangid K, Gonzalez JM, Pidiyar VJ, Patole MS, Ranade DR and Shouche YS. 2004. Comparison of 16S rRNA gene sequences of genus ethanobrevibacter. BMC Microbiology, 4: 20.

28. Pidiyar VJ, Jangid K, Patole MS and Shouche YS. 2004. Studies on cultured and uncultured microbiota of wild Culex quinquefasciatus mosquito midgut based on 16S rRNA gene analysis. American Journal of Tropical Medicine and Hygiene,70: 597-603.
29. Jangid K, Rastogi G, Patole MS and Shouche YS.2004. Methanobrevibacter: Is it a potential pathogen. Current Science, 86: 1475-1476.

30. Kauser F, Khan AA, Hussain MA, Carroll IM, Ahmad N, Tiwari S, Shouche YS, Das B, Jyothirmayee CS, Alam M, Ali M, Habibullah S, Sierra CM, Megraud F, Sechi LA and Ahmed N. 2004. The cag pathogenicity island (cag-PAI) of Helicobacter pylori is disrupted in majority of patient isolates from different human populations. Journal of Clinical Microbiology, In press.

31. Kohale K. N. (2003). Red urine in New Zealand white Rabbits- a case report, Indian Veterinary Journal, Vol. 80 No. 12, 1310-1311.

32. Raut C.G; Gengaje B.B; Nipunage S.V; Rajarshi M.P; Vaidya S.R; Rane, S.R*; Pol S.S; Kohale K.N. (June, 2003). Capillaria hepatica infection in bandicoot rat *(Bandicota indica)*. Journal of Veterinary Parasitology Vol. 17 (1), 71-72.

33. Rastogi G, Bharde A, Dharne M, Pandav VS, Ghumatkar SV, Shinde BM, Patole MS, and Shouche YS. 2004. Species determination and authentication of meat samples by mitochondrial 12S rRNA gene sequence analysis and conformation sensitive gel electrophoresis. Current Science, In press.

34. Kulkarni, A., Pavithra L, Rampalli, S., Mogare, D., Babu, K. Shiekh, G., Ghosh, S. and Chattopadhyay, S. (2004) HIV-1 integration sites are flanked by potential MARs that alone can act as promoters. Biochemical and Biophysical Research Communication, 322; 7672-77.

35. Deshpande, P. and Shastry, P. (2004) Modulation of Cytokine Profiles by Malaria Pigment-Hemozoin and Role of IL-10 in Suppression of Proliferative Responses of Mitogen Stimulated Human PBMC, Cytokine (In press).

36. Pany, S., Vijayvargia, R. Krishnasastry, M. V. (2004) Caveolin-1 binding motif of alpha-hemolysin: its role in stability and pore formation. Biochem. Biophys. Res. Comm. 322, 29-36.

37. Kaul-Ghanekar R, Jalota A, Pavithra L and Chattopadhyay S.(2004.) SMAR1 and Cux/CDP modulate chromatin and act as negative regulators of the TCR enhancer (E) NAR, 32, 4862 - 4862

Bulletins:

1. B. Ramanamurthy & K.N. Kohale, 2004. Animal Biotechnology-Past, Present & Future. MAEER"s MIT Pune Journal, Special issue on Biotechnology Vol.X (Nos1 to 4)

2. Kohale K. N. (2003). Red urine in New Zealand white Rabbits- a case report, Indian Veterinary Journal, Vol. 80 No. 12, 1310-1311.

2003 - 2004

Patents Granted :

1. Wani M. R., Parab P. B. and Chatterji A. (2003) Pharmaceutical composition useful for inhibition of osteoclast formation and a process for the extraction of mussel hydrolysate from Indian green mussel (Perna viridis). US patent # 09/944, 497. Joint patent National Centre for Cell Science, Pune and National Institute of Oceanography, Goa.

2. K. V. S. Rao, Mohan. R. Wani, V. Manivel, P. S. Parameswaran, V. K. Singh, R. V. Anand, E. Desa, G. C. Mishra, A. Chatterji. Novel molecules to develop drug for the treatment of osteoporosis. US Patent # 10/747,671.

Patents Submitted/filed

2 Parab P. B. and Chatterji A. (2002).Radioprotective activity from an extract from Indian green mussel (Perna viridis) Patent submitted (2002)
Joint patent National Centre for Cell Science, Pune and National Institute of Oceanography, Goa.

3. Parab P. B. and Chatterji A. (2002) Process for the identification of insulin producing b cells proliferating factor from the perivitelline fluid of fertilized eggs of the Indian horseshoe crab. Patent submitted (2002)
Joint patent National Centre for Cell Science, Pune and National Institute of Oceanography, Goa.

4. D.V.R. Prasad, P. B. Parab and G. C. Mishra (2003) Monoclonal antibody against costimulatory molecule (m150) and the process for preparation.
Patent submitted (2003) Provisional patent registration No. 30,480 dated 14.08.2003. National Centre for Cell Science, Pune

5. Ghaskadbi S.M., Parab P. B., Chatterji A., Patwardhan V. and Parameshwaran (2004) Cardiac development promoting activity in perivitelline fluid of embryos of Indian horseshoe crab. Patent application under preparation (2004)

6. Manoj Bhat et al. One US patent filed jointly with NCL " Bile acid derived steroidal dimmers with amphiphilic topology having antiproliferative activity"

Memberships/Awards/Fellowships:

Arvind Sahu
Member of the International Complement Society (1999-onwards)

R. R. Bhonde
Task Force member : Aquaculture and Marine Biotechnology, DBT (2002-2006)
Secretary-Treasurer : Indian Society for Developmental Biologists (ISDB),(2003-2006)

Samit Chattopadhyay
Member, Molecular Immunology Forum (2003 onwards)
Life member, Indian Society of Cell Biology (2003 onwards)
Board member, Indian Society for Developmental Biologists

Sanjeev Galande
Second best poster award at the Indo-Australian conference in Biotechnology, IISc, Bangalore, for poster entitled- "Displacement of SATB1-bound HDAC1 corepressor by HIV-1 transactivator induces expression of Interleukin-2 and its receptor in T cells" by Kumar et al.
International Senior research Fellowship of the Wellcome Trust, UK, 2004 - 2009.

Jayant M. Chiplonkar
Life Member, Indian Society of Cell Biology.

Gopal C. Kundu
National Bioscience Award for Career Development, Department of Biotechnology, New Delhi, 2003
Prof. R. C. Shah Memorial Lecture Award, The Indian Science Congress Association, 2004

Debashis Mitra
AITRP-Fogarty Fellowship
28th January to 5th May 2004
Departments of Microbiology and Molecular Genetics
Albert Einstein College of Medicine
New York, USA

Nibedita Lenka
91st Indian Science Congress Award in recognition of Scientific achievements (2004).

P. B. Parab
Member of European Association of Studies in Diabetes.2004-2006 Offered Welcome Travel Award in Sept.-Oct.2003 to visit Southmead Hospital, Bristol, UK.

Bhaskar Saha
Society for Biological Chemists, India. Life Member.

Mohan Wani
Nominated by President, Govt. of India for attending SAARC Training Course on Bioassays and Pharmacological Techniques for the Drug Development at Karachi, Pakistan during 10-15 October, 2003.

Extramural funding received by NCCS scientists:

Arvind Sahu
Wellcome Trust Overseas Senior Fellow (2001-2006)

Samit Chattopadhyay
Received funding from Department of Biotechnology, Govt. of India on "p53 interacting protein SMAR1 modulates p53 function by direct Phosphorylation : Discovery of a new signal transducing protein and its potential use in anti-cancer therapy". Duration: 2003-2006.
Received funding from Department of Biotechnology, Govt. of India on "Extracellular Tat mediated inhibition of HIV-1 replication: Generation of novel siRNA expressing vectors that will inhibit HIV replication". Duration: 2003-2006.

Dr. Gopal C. Kundu
Received funding from Department of Biotechnology, Govt. of India on "Role of novel factor in suppression of breast cancer cells migration and metastasis".Received funding from Department of Science and Technology, Govt. of India on "Role of osteopontin on matrix metaloproteinase-2 expression, cell migration and ECM-invasion in melanoma cells".

Nibedita Lenka
Research Grant from Department of Biotechnology,
Government of India (2003-2006).
National Institute of Health (NIH), USA travel award (2003).

Yogesh S. Shouche
Genetic diversity of Helicobacter pylori isolates from patients of Indian origin, funded by Department of Biotechnology.
Studies on Genetic Relatedness of Bacillus anthracis Strains of Indian Origin, funded by Life Sciences Research Board.
Expressed Sequence Tag analysis of mosquito genome, funded by Department of Biotechnology.
Looking for evidence for life in outer space: Studies on meteor crators, funded by Indian Space Research Organization.
Investigation of the microflora of insets of Western Ghats for potentially useful bioactive molecules, funded by Department of Biotechnology.
Molecular characterization of microbial and invertebrate diversity of Indian West Coast, funded by Department of Biotechnology.

Mohan Wani
Cellular and molecular mechanisms of action of IL-3 on osteoclast differentiation and Activation.
Duration 2004-2206. Funding Agency: Department of Biotechnology
Isolation, purification and characterization of active component(s) from the extract of Indian green mussel (Perna viridis) that shows the inhibitory action on osteoclast differentiation and bone resorption. (In collaboration with Dr. Kanury Rao, ICGEB, New Delhi and Dr. Anil Chatterji, NIO, Goa)
Duration: 2003-2005. Funding Agency: Council of Scientific and Industrial Research (CSIR).

Sanjeev Galande
Wellcome Trust Overseas Senior Fellow (2004-2009).

Debashis Mitra
DBT project, BT/PRO/897/AAQ/03/099/00.
Identification and characterization of anti-HIV compounds in Indian marine bivalves.
ICMR project, 4/3-2/2000, NM/BMS/TRM.
New Natural products as HIV-1 reverse transcriptase inhibitor from the genus Calophyllum.
CSIR project, 31/NIO/124/2001-RPBD
Isolation of active compound in the extract of mussels to develop drugs.

Seminars / Invited talks given by NCCS Scientists at other places

R. R. Bhonde

1. Applications of animal tissue culture in marine biotechnology
   May 20-21, 2003.
   Goa Unoversity, Panjim, Goa.
2. Recent advances in diabetes research
   June 27, 2003.
   Under COURT 2003 (Campaign On University Research and Training)
   Shivaji University, Kolhapur.
3. Stem cells and regenerative medicine.
   Dec 12-13, 2003.
   Under 50 years of DNA, Seminar organized by Panchwati College, Nasik.
4. Magic of cells.
   Dec 23, 2003.
   Wadia College, Pune.
5. Recent advances in diabetes research
   Dec 29, 2003
   Fergusson College, Pune.
6. In vitro models as an alternative to animal experiments.
   Jan 10, 2004
   Under Ranbaxy Science Foundations 13th Round Table Conference on Ethics in Animal Experimentation at India Habitat Centre, New Delhi.
7. Stem cells in islet neogenesis
   Feb 2-4, 2004
   Indo-US workshop on Tissue Engineering and Stem cell Technology (TEST-2004)
8. In vitro models and Technology transfer.
   Feb 21, 2004
   TIE presentation at Symbiosis Centre, Pune
9. Stem cells and tissue engineering
   Feb 27, 2004
   Vidya Pratishthan School of Biotechnology, Baramati
10. Bioartificial organs
    March 6, 2004
    Radio talk
11. Islet Neogenesis from adult stem cells
    March 8-9, 2004
    L V Prasad Eye Institute, Hyderabad.

Samit Chattopadhyay

1. SMAR1, A novel chromatin modulator involved in p53 activation, cell cycle regulation and T cell development. January 7-10, 2004. Invited Speaker: XXVII All India Cell Biology Conference & International Symposium, Zoology Department, Pune University, Pune.
2. Generating transgenic and knock-out mice to understand tumorigenesis and T cell development. October 13-17, 2003. Workshop on production of transgenic mice. NCCS, Pune.
3. MARs and MAR Binding Proteins in HIV Transcription and Tumorigenesis. December 4-6, 2003. 7th Transcription assembly, JNCASR, Bangalore,
4. SMAR1, a novel regulator of T cell development that controls V(D)J recombination through chromatin modulation. February 13-15th, 2004, Molecular Immunology Forum. Center for Biotechnology, JNU, New Delhi.
5. A novel MAR binding protion SMRI regulators T cell development. Feburay, 2004 IMTECH, Chandigarh

Sanjeev Galande

1. "Designing DNA Constructs for Making Transgenic Mice: Principles, Methodologies and State of the Technology", October 13-17, 2003 at the Workshop on Production of Transgenic Mice. NCCS, Pune.
2. "Displacement of SATB1-bound HDAC1 corepressor by HIV-1 transactivator induces expression of Interleukin-2 and its receptor in T cells" delivered at
   i. Transcription Assembly VII meeting, JNCASR, Bangalore, Dec. 4-6, 2003, and
   ii. XXVII All India Cell Biology Conference & International Symposium, Frontiers in Biomedical Research & Technologies, Dept. of Zoology & Institute of Bioinformatics and Biotechnology, University of Pune, Jan. 7-10, 2004.

Rajesh J. Solanki & Shivaji S. Jadhav

1. Presented a paper entitled "Current trends in network and Information security" at the 22nd Annual convention and conference of Society for Information Science, organized by IIT Madras at Chennai between 21-23 Jan. 2004. Rajesh J. Solanki & Shivaji S. Jadhav
2. Delivered a invited guest lecture on "Bioinformatics: An Overview" at PICT Bharti Vidypeeth, Pune on 8th Mar. 2004.Rajesh J. Solanki

Gopal C. Kundu

1. Role of osteopontin in regulation of NF-kB-mediated pro-MMP-2 activation/uPA secretion, cell motility and tumor growth. 73rd Annual Session of the National Academy of Sciences, India, October 10-12, 2003, Gujarat University and Physical Research Laboratory, Ahmedabad.
2. Signal transduction pathway that leads to the activation of transcription factor, NF-kB and regulates cell motility. 10th meeting of Trendys, November 28-29, 2003, Centre for DNA Fingerprinting & Diagnostics, Hyderabad.
3. Osteopontin regulates cell motility, promatrix metalloproteinase-2 activation and urokinase type plasminogen activator expression through activation of transcription factor, NF-kB. Molecular Aspects of Cellular Signaling, December 3-4, 2003, University of Hyderabad, Hyderabad.
4. Role of ECM-protein in regulation of cancer cell motility, NF-kB-mediated pro-MMP-2 activation and uPA expression, Guha Research Conference, December 25-29, 2003, Siliguri, West Bengal.
5. Transcription factor NF-kB: mechanism of activation and its role in cancer. The Indian Science Congress Association, January 3-7, 2004, Panjab University, Chandigarh.
6. Mechanism regulating the activation of transcription factor, NF-kB and its role in cell signaling. XXVII All India Cell Biology Conference & International Symposium, January 7-10, 2004, University of Pune, Pune.

Lalita S.Limaye

Invited speaker in "Biotalk symposium on Stem Cell Technology"
Low temperature biology of haematopoietic stem cells.
16/11/03
IMA hall , Nagpur . Organized by Biotech Society and Hislop School of Biotechnology.

Nibedita Lenka

1 N. Lenka. Embryonic Stem Cells: The Elegant in vitro Paradigm in Understanding Prospective Embryonic Development. 91st Indian Science Congress, Punjab University, Chandigarh, January 3-7, 2004 (Invited Plenary Speaker).
2 N. Lenka. Harnessing the potential of Embyonic stem cells: Cardiomyogenesis and Neurogenesis. Stem Cell Seminar, LV Prasad Eye Institute, Hyderabad, March 8-9, 2004 (Invited Speaker).
3 Stem Cell Differentiation and Potential. Department of Genetics, Osmania University, Hyderabad, March 9, 2004.

Yogesh S. Shouche

1 Genomic Insights into Life Style diseases: Lessons from Environmental & Insect Genomics and Biodiversity. Talk given at Indo USA Genomics meeting at Bangalore, October 2003.
2 rRNA sequences in phylogeny, taxonomy and diagnostics at Centre for Biotechnology, University of Allahabad, November 2003
3 Trends in Taxonomy at Indian Science Congress, Chadigarh, January 2004.
4 Molecular Phylogenetics, answer to modern taxonomy at National Institute of Oceanography, Goa. March 2004.
5 Impact of molecular biology on environmental biotechnology at Agharkar Research Institute, Pune March 2004.
6 Popular Science Talks
7 "Genes, Genomes and Genomics" at Nagpur University as part of Science day celebrations
8 "Human Genome " at Panchavati College, Nashik
9 "DNA Fingerprinting" at College Kolhapur as part of Science Day celebrations
10 "Extraterrstrial Life" at All India Radio, Pune
11 "Premature aging" at All India Radio, Pune

Mohan Wani

Invited to deliver a talk on "Recent Advances in Biotechnology and Biomedical Science: Implications in Veterinary Medicine" in Technical Seminar organized by Indian Society for Veterinary Surgery at Jalgaon, December 13-14, 2004.

P.B. Parab

Islet cell regeneration from mouse pancreatic ductal cultures.
24th Sept. 2003., Dept. of Diabetes and Metabolism, Southmead Hospital.

Bhaskar Saha

1 Reciprocity of CD40 signaling in macrophages. Federation of Asian and Oceanian Biochemists and Molecular Biologists, Indian Institute of Science, Bangalore. December 7 11, 2003.
2 CD40 signaling in macrophages: Immunotherapy versus Immune evasion. INSA-DFG (Indo-German) meeting on Leishmaniasis. Indian Institute of Chemical Biology, Calcutta. December 14 -16, 2003.
3 Deep Roots, Open Skies: New Biology in India. National Institute of Immunology, New Delhi. February 25, 2004.

Poster presentations:

R. R. Bhonde

'Role of diabetic bone marrow in induction of pancreatic regeneration'
Meenal Banerjee, RR Bhonde.

'A novel insulin mimetic: Flavone vanadium conjugate- A step towards oral insulin'
Ruchi Shukla, Vivek Barve, Subhash Padhye, Ramesh Bhonde.

'Curcumin pretreatment ameliorates the risk of experimental diabetes'
Meghana Kanitkar, Ravi Shukla, RR Bhonde.

Sanjeev Galande

"Displacement of SATB1-bound HDAC1 corepressor by HIV-1 transactivator induces expression of Interleukin-2 and its receptor in T cells". Pavan Kumar P., Prabhat Kumar Purbey, Dyavar S. Ravi, Debashis Mitra, and Sanjeev Galande, presented at

1. "Indo-Australian Conference in Biotechnology", IISc, Bangalore, Feb. 9-11, 2004, and
2. "Gene Regulation and Signaling in the Immune System", Cold Spring Harbor Laboratory, New York, USA, April 28-May 2, 2004.

Lalita S.Limaye

Addition of membrane stabilizers and antioxidants in the conventional freezing medium to improve the quality of the frozen graft with special reference to in vitro and in vivo homing
Lalita M.Sasnoor,Vaijayanti P.Kale and LalitaS.Limaye
Poster presented by Lalita Sasnoor.

Dr. Anjali Shiras

Are the multipotent brain tumor stem cells (BTSC's) involved in glioma development? Molecular characterization of a novel brain tumor stem cell-line derived from human glioma. International Neuroscience Meeting entitled " Brain in the postgenomic era " held at Hyderabad 6th -8th May 2004.

Seminars given at NCCS by visiting scientists:

Prof. Aaron Lewis

Nanonics Imaging Systems 16th July 2003

K. P. Mohankumar

OH NO Calcium in Parkinson's disease 5th Sept. 2003
IICB, Kolkata

Gobardhan Das

Inflammatory Bowel disease requires the interplay between Innate andAdaptive Immune Signals 5th Dec. 2003
Aventis Pharmaceuticals, USA

Shahid Jameel

Molecular Biology and pathogenesis of hepatitis E virus 20th January
ICGEB, New Delhi, India

O. P. Jangir

RajasthanHomeotic regeneration of median "Third eye" from pineal gland in the amphibian tadpoles and development and regeneration of lens in amphibian tadpoles, adult frogs, Swiss albino mice, guinea pigs, rabbit and pigs. 21st Jan.2004
Govt. Dungar College,

Manisha Raje Bhosale

Molecular Mechanisms elucidating role of Neuronal Calcium Sensor and Vesicular Associated Membrane proteins in neurosecretion. 22nd Jan. 2004
University of Manchester, UK

Senthil Muthuswamy

Genesis of carcinoma: A cell biologist's perspective 24th March, 2004
Cold Spring Harbour Laboratory, USA

Vishwas Agashe

Molecular Chaperones: Protein folders and more 26th March, 2004
Max Plank Institute for Boichemistry, Germany

Rajesh Gokhale

Biochemical Crosstalk between Fatty Acid Synthases and Polyketide Synthases in Mycobacteria. 20th May 2004
NII, New Delhi

Murly Shastry

Nano-Bio Interface 26th May 2004
NCL, Pune

Workshops Organized at NCCS:

Organized a National "Workshop on Production of Transgenic Mice" from 13 October 2003 to 17 October 2003. A total of 18 personnel (9 from external organizations and 9 internal) were trained in the basic techniques of transgenic mice generation. The faculty comprised of both external and internal experts.

Conferences/workshops attended:

Arvind Sahu

1. Jayati Mullick, "Kaposi's sarcoma-associated herpesvirus open reading frame 4 protein (kaposica) targets C3 and C4 to inactivate complement"
   Name: 9th European Meeting on Complement in Human Diseases
   Duration: September 6-9, 2003
   Venue: Trieste, Italy.

S. A. Bapat

1 XVI Cell Biology Conference
7^{th} 10^{th} January, 2004.
University of Pune

2 VII National Workshop and Conference of the Indian Society of Prenatal Diagnosis and Therapy (ISPAT)
20^{th} 25^{th} January, 2004, Ahmedabad.

1 Paper presented at ISPAT Bapat SA, Mali SM, Prabhakara K and Gambhir P Development and characterization of in vitro models useful in investigating the Rubinstein Taybi Syndrome.

R. R. Bhonde

27^{th} All India Cell Biology Conference
Jan 7-10, 2004.
Department of Zoology, University of Pune, Pune.

Sanjeev Galande

1. "Workshop on Production of Transgenic Mice" October 13-17, 2003. NCCS, Pune.
2 "Transcription Assembly VII" meeting, JNCASR, Bangalore, Dec. 4-6, 2003
3 "Indo-Australian Conference in Biotechnology", IISc, Bangalore, Feb. 9-11, 2004
4 "Gene Regulation and Signaling in the Immune System", Cold Spring Harbor Laboratory, New York, USA, April 28-May 2, 2004.
5 "NIH Grantsmanship Workshop" organized by the Office of AIDS research, NIH, Bethesda, USA, May 6-7, 2004.

Jayant M. Chiplonkar

1 First International meeting on epithelial-mesenchymal transition, 5-8 October 2003 at Rydges Reef Resort, Port Douglas, Australia. Presented a Poster "Analysis of Epithelial mesenchymal transition in SiHa, cervical carcinoma cells" By Madhura R. Vipra and Jayant M. Chiplonkar. Madhura Vipra was awarded a travel scholarship by the organizers of the conference to attend the conference and present this poster. She was also awarded INSA grant for the same.

2 Madhura Vipra was awarded Ph. D. (Biotechnology) from Pune University, March 2004, under my guidance. Her thesis was "Analysis of epithelial mesenchymal transition using in vitro models."

Vaijayanti P. Kale

1. International symposium on "Molecular medicine and cancer: Contemporary issues". Organized by Jaslok Hospital and Research Center at Taj Lands End, Mumbai on 1st Feb 2004.
2. Presented an overview of my research work in "StemSem" seminar organized by LVPERI, Hyderabad on 8th and 9th March, 2004.

Lalita S.Limaye

5th International SASS conference on "Emerging Concepts in Leukemia and Lymphoma"
13/12/03 – 16/12/03
Hotel Taj ,New Delhi, organized by AIIMS.

Debashis Mitra

1. "HIV-1 Tat protein is also a DNA binding transcriptional activator" Invited talk at Transcription Assembly VII meeting, 4-6th December 2003, JNCASR, Bangalore, India.

2. Global gene expression analysis in HIV induced T cell apoptosis" 26th March 2004.
Center for AIDS Research
Albert Einstein College of Medicine
Bronx, New York, USA.

Musti V. Krishnasastry

Possible membrane penetration mechanism by a-hemolysin.
GRC 2003, Dec 24 - Dec 27, New Jalpaiguri, West Bengal.

Nibedita Lenka

4th Indo-US Cytometry Workshop at ACTREC, Mumbai (Feb. 2- 6, 2004).

P. B. Parab

Attended XXVII All India Cell Biology Conference and International Symposium from Jan.7-10, 2004. Pune University, Pune.

Bhaskar Saha

International Symposium on Leishmaniasis. Chaired a Session on the Epidemiology of Leishmaniasis. Indian Institute of Science, Bangalore. March 10, 2004.

Yogesh S. Shouche

8th ADNAT Convention and Symposium on Functional Genomics. Hyderabad, India. (February 2004).

Sandhya Sitasawad

"XXVII All India Cell Biology Conference & International Symposium, Frontiers in Biomedical Research & Technologies". From 7-10th January, 2004 at Zoology Department and IBB, University of Pune, Pune.

Mohan Wani

1. IL-3 acts directly on osteoclast precursors and inhibits RANKL induced osteoclast differentiation. Paper presented in 25th Annual Meeting of the American Society for Bone and Mineral Research, September 19-23, 2003 at Minneapolis, Minnesota, USA.

2. I was member of organizing committee of XXVII All India Cell Biology Conference and International Symposium, January 7-10, 2004, Department of Zoology and IBB, University of Pune, Pune 411 007.

B. Ramanamurthy:

Delivered a talk as an invited speaker in the VII CPCSEA Zonal Workshop held on the 21st /22nd April 2003 at the National Institute for Research on Reproduction (NIRRH) Mumbai.

K. N. Kohale

Participated training course entitled, 'Molecular Biology and Biotechnology Techniques in Animal Research', at Indian Veterinary Research Institute, Izatnagar, Bareilley, from 30th August to 27th September, 2003.

Samit Chattopadhyay

Transcription Assembly Meeting, JN CASR , Bangalore, Dec. 4-6, 2003

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