National Institute of Dental Research
Division of Intramural Research

Annual Report Summary

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Fiscal Year 1997 Annual Report

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Introduction and Overview

Henning Birkedal-Hansen, Scientific Director

THE DIVISION OF INTRAMURAL RESEARCH
ANNUAL REPORT FOR FISCAL YEAR 1997
INTRODUCTION AND OVERVIEW

The Division of Intramural Research experienced a very productive year, and a year of rapid and profound organizational change. Several months of planning, involving all levels of the organization, culminated in the reorganization and fusion of the Division of Epidemiology and Oral Disease Prevention and the Division of Intramural Research effective November 15, 1996.

In addition to the fusion of the two divisions the reorganization brought a number of highly significant changes. The eleven former Laboratories and Branches were reorganized into seven Branches reaffirming the commitment of the Division and the Institute to the seamless transition from basic, through translational to clinical research in each of the programs.

The Branches resulting from the reorganization were:

- Craniofacial Developmental Biology and Regeneration Branch (CDBRB)
  (Kenneth Yamada, MD, Chief)
- Craniofacial and Skeletal Diseases Branch (CSDB)
  (Pamela Gehron Robey, PhD, Chief)
- Gene Therapy and Therapeutics Branch (GTTB)
  (Bruce J. Baum, DDS, PhD, Chief)
- Oral Health Promotion, Risk Factors and Molecular Epidemiology Branch (OHPRFMEB)
  (Tullio Albertini, DDS, Acting Chief)
- Oral Infection and Immunity Branch (OIIB)
  (Sharon Wahl, PhD, Chief)
- Oral and Pharyngeal Cancer Branch (OPC)
  (J. Silvio Gutkind, PhD, Chief)
- Pain and Neurosensory Mechanisms Branch (PNMB)
  (Raymond A. Dionne, DDS, Acting Chief)

At the organizational level, the hierarchical section-based structure was dissolved and the reporting structure simplified. Emphasis was placed on the individual Senior Investigator who was recognized as an independent scientific and fiscal unit of the program. The details of the DIR reorganization are described in a separate document ("The Division of Intramural Research," November 15, 1996) which was issued to each investigator and shared with everyone in the organization through multiple informational activities before and after the November 15 start-date.

The overriding philosophy of the 1996 reorganization was empowerment of the Senior Investigators. Perhaps the most significant philosophical, structural and administrative change was to break up Laboratory and Branch budgets into individual Senior Investigator budgets. The process required a series of complex negotiations in order to fairly and equitably apportion

existing funding under the leadership of Mr. Ronald Adams, the Principal Administrative Officer. The process is now completed. This process allows us to implement one of the most important provisions of the 1996 reorganization plan and to adopt fully independent, individually negotiated Senior Investigator budgets. Another significant step was the establishment of a significant number of committees to oversee core facilities and advise on policy and administrative matters. These committees serve an important function in providing broad-based input on policy and administrative decisions and in generating discussion about issues that are important to the program.

The main administrative body of the Division continues to be the colloquium of Branch Chiefs who during FY97 addressed a significant number of administrative and programmatic issues and provided important insight and advice for the operation of the program. As a result of the reorganization, Branch Chiefs were appointed to four-year renewable terms to coincide directly with the four-year cycle of the Board of Scientific Counselors review. Drs. Pamela Robey, Bruce Baum, and Kenneth Yamada were appointed Chiefs of CSDB, GTTB, and CDBRB respectively. The reorganization left four vacancies at the Branch Chief's level: Oral Infection and Immunity Branch (Dr. Sharon Wahl, Acting Chief); Oral and Pharyngeal Cancer Branch (Dr. J. Silvio Gutkind, Acting Chief); Pain and Neurosensory Mechanisms Branch (Dr. Raymond A. Dionne, Acting Chief) and the Oral Health Promotion, Risk Factors and Molecular Epidemiology Branch (Dr. Tullio Albertini, Acting Chief followed by Dr. Henning Birkedal-Hansen upon Dr. Albertini's retirement, July 31, 1997). After a competitive search, Dr. Sharon Wahl was appointed Chief, Oral and Infection and Immunity Branch. A second search concluded in the next fiscal year (FY98) resulted in the appointment of Dr. J. Silvio Gutkind as Chief, Oral and Pharyngeal Cancer Branch. Reorganization of the Oral Health Promotion, Risk Factors and Molecular Epidemiology Branch is continuing into the next fiscal year. When the reorganization and alignment of this Branch are completed a search for a Chief will be opened. A search for Chief of the Pain and Neurosensory Branch was put on hold while campus-wide efforts to create a Pain Consortium were being explored. Next steps include convening an outside expert panel in pain, neurosensory mechanisms, and neuroscience to review opportunities for building a multi-institute pain consortium in the intramural program.

Two laboratories were successfully reviewed during the 1997 fiscal year. The CIPCB, now the GTTB, and the BRB, now the CSDB, both were judged highly meritorious in terms of excellence of science and programmatic direction. Consequently the Division and the Institute confirmed its commitment to strong support of each of these programs.

The Animal Care Core Facility changed leadership during the year. Following the departure of Dr. Joseph Bryant after many years of dedicated service to the program, a national search resulted in the selection and appointment of Martin Kriete, DVM, as Chief, Animal Care Core Facility. Dr. Kriete is being charged with further expanding and strengthening our animal research capability and with creating state-of-the-art facilities for animal research. Perhaps the largest growth of activity has been in this area because of the rapid adoption by our scientists of mouse genetic approaches, including gene replacement and transgenic techniques.

The reorganization led to formal establishment of a Clinical Research Core Facility charged with enabling the transition to clinical research by our scientists, with providing consult services to the

Clinical Center and with training of Oral Medicine Fellows. Following a competitive search, Dr. Anne O'Connell was appointed Chief.

The 1996 reorganization necessitated a fresh look at the distribution and allocation of laboratory and office space. Guided by the leadership of Dr. Jack London, Chair of the DIR Space Committee, and a Special Assistant to the Scientific Director, a detailed process of deliberation culminated with reallocation of space, particularly involving the CSDB, OPC and OIIB. While the lack of space and the need for renovation carries into the next year, the space plan created an interim framework for solution of some serious immediate problems. During FY97, a detailed plan for renovation was explored with the Department of Engineering Services. While attractive, it was finally resolved that the plan which would cost in the range $21-27 million and require evacuation of the entire building for a period of two years and was unrealistic at this time. Alternative plans for renovating both Building 30 and Building 10 are being developed.

In the wake of the NIDR strategic Plan, the DIR Branch Chiefs held a two-day retreat at the Solomons Island Holiday Inn to review Branch by Branch our efforts to implement the scientific and programmatic elements of the NIDR Strategic Plan. The retreat was also attended by the NIDR Director and Deputy Director. The get-together provided an opportunity to review our past progress toward programmatic implementation of the strategic plan and to assess and map out future directions, including strengthening of our clinical research portfolio.

All of the campus experienced a resurgence in the commitment to clinical research. The "Strauss Report" addressed the issue of recruitment and retention of clinical scientists at NIH and made a series of important recommendations. At the national level, Dr. Nathan chaired the "NIH Director's Panel on Clinical Research." The Panel issued a series of important recommendations for improvement and growth in the national capability for clinical research that will impact the intramural program. Moreover, the year saw intensified planning of the new Clinical Center expected to open in 2001. Most of the DIR Branches at this time are involved to a significant degree with the continuum of basic, translational and clinical research. Based on the definition of clinical research established by the Nathan's Committee, the DIR currently commits approximately one third of its resources on clinical research. A similar, or perhaps even larger, fraction of the budget is being committed to translational research. So the Division is close to achieving a balance of one third in each category. It should be emphasized that there is no common agreement inside or outside NIH as to what exact balance to strive for.

During the past year NIDR scientists enjoyed extensive interactions with the extramural community in terms of speaking engagements at scientific meetings and at universities and private industry. Many served as advisors to industry, government and academia and found a myriad of ways to contribute to the advancement of science through participation in professional and scientific organizations, though editorial board memberships and editorships, etc. All of these activities are detailed in the document "Interactions with Scientific Community."

While the administrative and organizational changes of the past year required a significant amount of effort and energy of our people, I am very pleased that our investigators remained very productive scientifically and shared their research findings widely in publications, in

presentations at meetings and in seminars and talks at institutions across the country. The many scientific highlights of FY97 are summarized by Branch in the appended document.

Henning Birkedal-Hansen, DDS, PhD
Scientific Director

The activities of the year have been captured in three other important DIR or NIDR documents

DIR Reorganization plan: "The Division of Intramural Research, November 15, 1996"

"Shaping the Future. The NIDR Strategic Plan"

DIR "Interactions with Scientific Community"

Craniofacial Developmental Biology and Regeneration Branch

Kenneth Yamada, Chief
Hynda Kleinman
Yoshihiko Yamada

CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH
1997

Our Branch continues to generate a variety of exciting research advances and to receive international recognition and honors. Our Branch is also continuing its traditional emphasis on the training of young scientists to become independent leaders in the field, and of extensive service and citizenship activities for NIDR, NIH, and our research fields. This year's reorganization of the NIDR Division of Intramural Research included transformation of the previous 'Laboratory of Developmental Biology' into our Craniofacial Developmental Biology and Regeneration Branch. Our personnel remain the same: Dr Yoshihiko Yamada is Chief of the Molecular Biology Section, Dr. Hynda Kleinman is Chief of the Cell Biology Section, and Dr. Kenneth Yamada is Chief of the Developmental Mechanisms Section. Our mission has been broadened to include new research opportunities.

Our Branch mission now includes objectives spanning the range from basic research through clinical. Our goals focus on creating research breakthroughs (a) to understand the mechanisms of normal and abnormal craniofacial development and function at the genetic, molecular, and cell biological levels, (b) to discover and refine new biologicals and biomimetics relevant to diagnosis, repair, and therapy, and (c) to develop creative, biologically based methods to regenerate craniofacial tissues that are defective or damaged. Our Branch continues to explore important fundamental questions in both development and regeneration, including the molecular and cell biological mechanisms of morphogenesis, structure and function of extracellular matrix and its receptors, tissue organization, signaling from the cell surface to the nucleus for novel gene induction, and cellular differentiation. In addition, we are planning for the development of novel translational and patient-oriented applications based on ongoing innovations in basic research.

Members of CDBRB were invited this year as featured speakers at more than a dozen international meetings and symposia. Some random examples of meeting presentations from this past year included: H. Kleinman, the Salivary Gland Gordon Research Conference and the IBC 4th International Conference on Angiogenesis Targets; Y. Yamada, Ciba Foundation Symposium on Dental Enamel and the International Symposium on Glycoconjugates and Matrix Molecules in Health and Disease; and K. Yamada: Gordon Research Conference on Fibronectin, Integrins, and Related Molecules and the Keystone Symposium on Signal Transduction by Cell Adhesion Receptors.

Members of the Branch also continue to serve on the editorial boards of a number of leading journals including J. Cell Biology (H. Kleinman, and K. Yamada as an Associate Editor), J. Biological Chemistry (H. Kleinman), J. Cellular Physiology (K. Yamada, Editor), J. Cell Science (K. Yamada), Cancer Research (H. Kleinman); H. Kleinman also serves on five other journal boards including J. National Cancer Institute and Angiogenesis, and K. Yamada serves on six other journal boards including J. Craniofacial Genetics and Developmental Biology. Members also serve on the boards of national or international organizations such as the Association for Women in Science (H. Kleinman) and the International Society for Matrix Biology (K. Yamada).

Our Branch distributes its research products extensively by licensing materials, donating them to repositories, and providing numerous gifts to research colleagues. Products generated by members of the Laboratory that are currently being licensed by companies include Matrigel and invasion substrates (Collaborative/Becton Dickinson and Sigma) and monoclonal antibodies against integrins (Becton Dickinson). The Branch has donated the EHS sarcoma to ATCC along with approximately 500 cDNA clones. We will have over 50 new, formal Material Transfer Agreements with non-NIDR researchers this year. Members of the Branch have also received support from outside organizations. Significant support for research on proteoglycans was received from Seikagaku. NASA provided funds to study salivary gland cell differentiation in microgravity. Non-NIH salary support for postdoctoral members of the Laboratory has come a wide variety of sources including the Japan Society for the Promotion of Science (two positions), the International Union Against Cancer, the Dutch Cancer Society, the French CNRS, the German government, and the Japanese Ministry of Education. The Branch also has a Cooperative Research and Development Agreement (CRADA) with the biotechnology company Trevigen, focusing on novel molecular approaches to wound repair.

Researchers in the Craniofacial Developmental Biology and Regeneration Branch have made substantial research progress and some exciting scientific breakthroughs during the past year, and our annual report bibliography lists over 60 publications. A variety of arbitrarily selected research advances are highlighted below. The project reports from each Section provide more comprehensive summaries of the major new findings in our Branch.

New research initiatives in all three sections are identifying and characterizing novel genes important for tooth and craniofacial development. Hundreds of novel genes (i.e., genes never previously described) have been identified by partial sequencing of rodent tooth and embryonic craniofacial unidirectional cDNA libraries, as well as of human salivary gland subtraction libraries. Selected novel genes show interesting and quite distinctive mRNA expression patterns in developing tissues. A new contract is now active to generate antibodies against approximately 100 gene products of interest to provide further characterizations. All clones and the antibodies to be produced will continue to be made freely available to dental scientists and to other qualified investigators to promote research in the area. The approaches being tested in this project will also be of interest to the human genome project, because they focus on the next steps after the identification of a novel gene, i.e. how to determine its function. The first protein to be characterized by the CDBRB using these approaches was ameloblastin, discovered to be a novel tooth-specific, developmentally regulated gene product associated with enamel formation. Ongoing collaborative studies of the Molecular Biology Section show close linkage between the ameloblastin gene and the congenital disorder amelogenesis imperfecta. Other genes can be used similarly as candidates for the identification of genetically linked diseases and disorders of oral and craniofacial tissues.

New transcription factors and mechanisms of gene regulation essential for normal development are being characterized, such as a novel Kruppel zinc finger protein found to be essential for normal tooth development. The Molecular Biology Section has also identified the enhancer of the link protein gene. Sequence motifs homologous to those in the enhancer are found in the enhancers of muscle specific genes, suggesting similar mechanisms regulate expression of both cartilage and muscle genes. The Developmental Mechanisms Section has demonstrated that the zinc

finger protein termed Slug is essential for a first step in the morphogenetic process of epithelial-to-mesenchymal transformation by inducing the loss of desmosomes. Our knowledge about new genes and regulatory mechanisms is being used to examine pathology in animal models and in human diseases. For example, the Molecular Biology Section has discovered age-associated spinal degeneration and disc herniation in heterozygote *cmd* mice defective in aggrecan.

Laminin and laminin peptides were previously implicated by members of this branch in angiogenesis, neurite outgrowth, and tumor growth and metastasis. Laminin peptides involved in these and other biological processes are being evaluated by the Molecular and Cell Biology Sections from a library of over 700 overlapping synthetic peptides spanning the entire laminin molecule (as featured on a cover of FEBS Letters). Twenty of these peptides have been found to affect cell adhesion, growth, angiogenesis, or salivary gland differentiation. Another has been found to be able to switch on the metastatic phenotype. Comparisons of the specific functions, cell type specificity, receptors, and signaling mechanisms of these peptides are in progress. These studies should lead to development of new reagents useful for prevention and therapy.

The interaction of cells with extracellular matrix via integrin receptors induces altered signaling, cytoskeletal organization, growth, and differentiation. A paper published in *J. Cell Biology* by the Developmental Mechanisms Section identifying a hierarchy of these responses was featured as a "Hot Paper" by The Scientist. In further studies, novel mechanisms involving integrin and growth factor receptor co-clustering, receptor phosphorylation, and MAP kinase activation were identified that can help explain the synergism between growth factors and integrins. A new technology was developed to study these signaling processes, and an invention report was filed on the use of leucine zipper domains to control experimentally the localization of any protein in a cell.

The specific molecular and steps in the cellular response to matrix are being characterized in human salivary gland (HSG) cells and in fibroblasts by the Cell Biology Section and by the Developmental Mechanisms Section. When salivary gland cells are placed on extracellular matrix proteins in cell culture, they show large changes in gene expression and protein biosynthesis. Over 30 genes have been identified that are activated after integrin-mediated adhesion of salivary cells to collagen or fibronectin, many of which are completely novel. On a basement membrane extract, salivary gland cells can differentiate and form mini-glands. This process of differentiation can involve a peptide site in laminin affecting cell polarity and differentiation, and its cellular receptor has been found to be syndecan-1. Other genes are being sought that are induced after adhesion to basement membrane substrates. Fibroblastic cells appear to show similar striking changes in gene expression in response to interactions with matrix molecules. Studies in these cell culture systems are building the knowledge base necessary to develop new therapeutic approaches for the repair or replacement of salivary and other tissues. For example, CDBRB is collaborating with the Gene Therapy and Therapeutics Branch toward the ambitious goal of developing an artificial salivary gland.

The Cell Biology Section has discovered that two small thymic peptides, thymosin $\beta 4$ and thymosin $\alpha 1$ promote endothelial cell migration and the process of angiogenesis. The peptides were active in nanogram concentrations, and thymosin $\alpha 1$ was also found to accelerate wound

repair in a rat skin punch biopsy model. This molecule may be valuable for promoting human wound healing.

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Craniofacial and Skeletal Diseases Branch

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CRANIOFACIAL AND SKELETAL DISEASES BRANCH
1997

During the past fiscal year, the Branch has continued to reorganize and focus its efforts on determining the processes by which skeletal elements are formed during embryonic and postnatal development, and during aging, and how these processes are altered in disease states that affect the skeleton. This new focus is fostered through the coordinated efforts of senior investigators working in the areas of cell and molecular biology, and protein and mineral chemistry. The Branch underwent review by the Board of Scientific Councilors, who wholeheartedly endorsed the new directions, and urged continued focus on specific areas within the mission of the Branch.

Organizational changes in the Branch were predicated by the departure of Dr. Masaki Yanagashita, Chief of the Glycobiology Program and Dr. Teresa Morales, Group Leader of the Cartilage Metabolism Project. Studies in these areas were concluded and resources associated with these programs were used to expand the recently established Skeletal Clinical Studies Program. Although a national search for a chief of this Program identified two highly qualified candidates, the position remains unfilled and will be readvertised in the next fiscal year. Nonetheless, efforts in translational and clinical research are progressing rapidly and received enthusiastic support by the Board of Scientific Councilors. In addition, a tenure track position was created for studies in Skeletal Developmental Biology. Again, the ensuing search identified two highly qualified candidates, however, neither has accepted the position, and it has been decided by Branch senior investigators that the current projects will be completed by existing staff and that the resources set aside for this program will be redirected into development of a molecular pathophysiology program at some point in the future.

Scientifically, the Branch has made major advances in several areas, as is summarized below.

Cell Biochemistry Program (Skeletal Biology Section)

The Cell Biochemistry Program has focused on characterizing osteogenic cells, including marrow stromal fibroblasts (MSFs), a heterogeneous population of clonogenic cells that give rise to osteoblasts/chondrocytes, adipocytes and hematopoiesis supportive stroma. Studies were designed to test the hypothesis that products produced by non-adherent (hematopoietic) cells in bone marrow cultures are responsible for stimulating the proliferation of CFU-F (colony forming unit-fibroblastic) to form MSF colonies. By using blocking antibodies in combination with medium conditioned by non-adherent marrow cells, it was found that PDGF, TGF-beta, bFGF and EGF are required to stimulate colony formation. However, it was also found that MSFs from different animal species have different growth factor requirements. Mouse MSFs require both serum and products synthesized by non-adherent cells and that colony formation can be stimulated in part by bFGF alone, whereas human MSFs require only serum, and no single factor stimulates colony formation. In addition to determining the growth factor requirements, the osteogenic capacity of individual clones of human MSFs was determined for the first time by using a newly developed *in vivo* transplantation system. It was found that 58% of the clones

studied supported bone formation, but only half of these clones supported bone formation and hematopoiesis. The differences between non-bone forming clones, clones that form bone and those that form bone and support hematopoiesis are currently under investigation.

Molecular Biology Program (Molecular Biology of Bones and Teeth Unit)

The matrix protein of bones and teeth play key roles in the structure and functions of these tissues. The objective was to study their function and regulation using a combination of in vitro and in vivo analysis. The genes studied are biglycan (BGN) and bone sialoprotein (BSP) both of which are highly expressed in bones and teeth. The first approach was to use isolated cells to study gene control at the nuclear level. Such experiments employed cDNA and genomic DNA isolation and cloning as well as extensive DNA gene mapping and sequencing. To understand the mechanisms that control RNA production, the promoters for these genes were charcaterized by transfecting them into cultured skeletal cells. DNase protection mobility shift assay, UV cross-linking and southwestern blotting were also used to understand the nature of the DNA protein interactions that control BSP and BGN gene expression. These studies indicated the transcription factors YY1 and c-krox were key in the control of these genes respectively. To study matrix protein function, experiments are underway to create transgenic mice that are null or "knockout" (not able to make) for specific matrix genes. The approach is to replace specific genes by a targeting method that relies on homologous recombination in embryonic stem cells. In addition, in parallel, mice that have a "gain of function" (make more of a specific gene) using conventional transgenics are being generated. It is theorized that the combination of targeted "gene knockout" and "gain of function" transgenic animals will provide new insight into the role of matrix proteins in the development and aging of skeletal tissue.

Protein Chemistry Program (Matrix Biochemistry Unit)

The fundamental question of how cells of bones and teeth assemble and mineralize their respective matrices in such a coordinated and superbly biofunctional way is still largely unanswered. The Protein Chemistry Unit has been performing structure-function analyses on several of the non-collagenous proteins. One approach has been to determine the amino acid sequences within a protein that are involved in the interactions of matrix components or between cells and the surrounding matrix. Decorin is a small proteoglycan thought to be involved in the control of collagen fibril assembly. Using *in situ* mutagenesis, it was determined that a single glutamate (Glu-180) within decorin is critical for its interaction with type I collagen thereby localizing the probable point of interaction of this TGF-beta-binding protein with the matrix. Within the integrin-binding glycoprotein, bone sialoprotein (BSP), studies have mapped both its strong apatite-binding domains and the peptide domains required for RGD-independent cell attachment properties. Furthermore, collaborative studies have also shown that BSP is an intriguing marker for those cancers that not only frequently present with microcalcifications within the primary lesions, but also have a high propensity of metastasizing to bone. In addition, recent studies determining conditions that cause temporal shifts in matrix mineralization using common hormones indicate that this approach will allow for further identification of the elements that control the mineralization of bones and dentin.

Developmental Biology Project

A cysteine to tyrosine substitution in Cartilage-Derived Morphogenetic Protein-1 (CDMP-1) has been identified as the causative mutation of a clinically severe disorder, Grebe type chondrodysplasia (OMIM number 200700). The underlying mechanism of the mutation has been studied and it was found that the defective CDMP-1 is not processed or secreted, and prevents the secretion of other, related, Bone Morphogenetic Proteins (BMPs) through the formation of heterodimers. This type of mutation, called a dominant negative, is the first example of such a mutation in the TGF-ß superfamily. Its proposed mechanism of action provides the first compelling evidence for heterodimer formation between related BMPs *in vivo*. A Collaborative Agreement (CRADA) with an industrial partner was established to provide recombinant CDMPs in order to address the biological functions of CDMPs and compare their biological profile with other BMPs. Data indicates that the CDMPs are functionally related to the BMPs and are capable of inducing cartilage and bone formation in a subcutaneous site in rats *in vivo*. Interestingly, there is a preferential stimulation of chondrogenesis as opposed to osteogenesis when compared with other BMPs. The underlying mechanism for this preferential chondrogenic activity is probably linked to their binding affinity for specific BMP receptor complexes. An unexpected series of exciting events has evolved with regard to the functional studies of the frzb gene. It was found that the frizzled proteins are a family of receptors for the Wnt family of proto-oncogenes. Wnt proteins are signaling molecules which participate in a wide variety of developmental and neoplastic processes. Given the high similarity of the frizzled-like domain of Frzb with the presumed ligand binding domain of the frizzled receptors, it was hypothesized that Frzb would also interact with Wnts and this was demonstrated by a series of immunoprecipitation experiments using co-transfected mammalian cells. The critical domains for the Frzb/Wnt interaction were demonstrated and showed that this direct interaction results in the inactivation of Xwnt8 *in vivo* when co-injected in Xenopus. The discovery that Frzb can act as a secreted antagonist of Wnt activity represents a major leap forward in research into Wnt signaling. To further analyze the role of Frzb in craniofacial and skeletal development we are conducting tissue-specific gene targeting studies in mice using homologous recombination and the Cre-loxp system. The targeted deletion of Frzb at different stages of development should provide important information on its physiological role.

Skeletal Clinical Studies

A major goal of the Clinical Studies program is to elucidate the role of osteogenic cells in the generation of a variety of skeletal dysplasias. It was found that the metabolic activity of bone-forming cells is altered by a number of known mutations. In the first instance, in Osteogenesis imperfecta, studies indicated that the presence of a type I collagen mutation has pleiotropic effects on cells--altering not only collagen levels but also the levels of noncollagenous extracellular components, inducing error-checking machinery within the cell, and altering cellular proliferation. In McCune-Albright Syndrome which presents with severe fibrous dysplasia, there are missense mutations of the G protein, Gsα, leading to overproduction of cAMP. It was found that this protein is dramatically upregulated as marrow stromal fibroblasts (MSFs) mature into osteoblasts, and the effects of the mutations are manifested by abnormal cell-cell (hyperosteocytic bone), cell-matrix interactions (cellular retraction), and the formation of an abnormal bone matrix (high in anti-adhesive proteins, versican and osteonectin, low in adhesive

proteins, osteopontin and bone sialoprotein). In addition to studying the role of osteogenic cells in skeletal diseases, another goal is to examine the ability of *ex vivo* expanded marrow stromal fibroblasts, MSFs, which contain osteogenic precursors, to regenerate normal bone tissue. The results have now identified the *in vitro* culture conditions as well as the transplantation vehicles that support complete bone regeneration by direct transplantation in a variety of experimental systems. Further studies are aimed at developing clinical protocols for bone regeneration in patients with a variety of skeletal defects.

Mineral Chemistry and Structure Section

The purpose of studies in this section is to study the physical, chemical, and ultrastructural properties of calcium phosphate salts, and to clarify the kinetic and thermodynamic processes and the interactions with substances of biological interest that uniquely enable calcium phosphate salts to carry out their specialized role *in vivo.* The properties of calcium phosphate salts are being studied with a variety of ultrastructural and physical-chemical techniques such as spectroscopic methods, x-ray diffraction, surface area analyses, chromatographic and standard analytical chemistry procedures. Currently, a principal effort is to study the physicochemical effects of biologically important ions such as fluoride, carbonate, organic anions such as citrate, and magnesium have on the texture, *i.e.*, size/shape, of apatite crystals grown *in vitro* under constant physiological-like solution conditions. The aim is to distinguish the direct effect these solution substances have on the texture of biological apatites from that brought about by the metabolic and matrix changes these substances induce *in vivo.* In addition, amorphous calcium phosphate (ACP), an important intermediate in the formation of apatite, is being investigated for possible use as a dental material. When either used alone, or in combination with other dental materials, especially polymeric resins, ACP has a wide range of possible applications such as in restorative composites, cavity liners and bases, luting and pulp capping agents, prophylactic and endodontic sealants, and as a component in periodontic packs and impression pastes. The hypothesis is that as a component in appropriate resin-based composites, sealants and adhesives, ACP may be useful as a remineralization agent. In this regard, ACP-embedded, methacrylate resins, which release calcium and phosphate ions at levels that exceed the thermodynamic minimum necessary for apatite formation, restored *in vitro* up to 71% of the mineral lost from caries artificially induced in extracted bovine incisor enamel. ACP cosynthesized with glass-forming fillers enhanced the mechanical properties of the resin-based composites without compromising their remineralization potential. These methods are utilized in dental materials research to determine and monitor reactions during syntheses of various inorganic and organic compounds used for composites, coupling agents, cements and other dental materials.

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Gene Therapy and Therapeutics Branch

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GENE THERAPY AND THERAPEUTICS BRANCH
1997

Fiscal year 1997 was a year of considerable significance for this branch for two major reasons. First, after 14 years under our former name, Clinical Investigations and Patient Care Branch, we were renamed the Gene Therapy and Therapeutics Branch (GTTB) to reflect the approach we are taking to move biology into the clinic. Second, we were reviewed by the NIDR Board of Scientific Counselors in December, 1996. The review was highly successful and strongly endorsed the bench to clinic, integrated way in which we conduct our science.

The GTTB has 5 principal investigators (PIs), each with a unique research focus, who cooperate to address problems related to salivary gland dysfunction. Indu Ambudkar is Chief of the Secretory Physiology Section (SPS), and is generally studying signal transduction mechanisms operative in salivary glands with a primary interest in Ca^{2+} entry pathways of non-excitable cells. Jim Turner is Chief of the Membrane Biology Section (MBS), and is generally addressing ion transport pathways leading to transcellular salt gradient formation in salivary acini, with a main focus on the secretory isoform of the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter. Bruce Baum is Chief of the Gene Transfer Section (GTS), and has primarily used adenoviral-mediated gene transfer in strategies to repair irradiation-damaged salivary glands and to direct the secretion of transgene products for systemic use. Brian O'Connell is Chief of the Gene Regulation and Expression Unit, (GREU), and is studying the cellular factors and genomic elements modulating salivary cell gene expression in addition to developing a gene transfer strategy to manage azole-resistant mucosal candidiasis. Phil Fox is Chief of the Clinical Investigations Section (CIS), which is focused on the management of patients with primary Sjogren's syndrome (SS).

While we are now called by a different name, we still approach our science in the same fashion. We are a group which believes significant advances in clinical care will only come from understanding biological mechanism. We focus on salivary glands, secretory epithelial tissues with a complex, highly regulated biology. As a group, the 5 PIs coordinate their efforts so as to permit the efficient movement of relevant biological progress to patient care.

Neurotransmitters are the primary regulatory agents for salivary secretion. Neurotransmitter regulation of fluid secretion in salivary glands is dependent on the activation of plasma membrane Ca^{2+} entry pathways that allow the controlled influx of extracellular Ca^{2+} into acinar cells. Surprisingly, the Ca^{2+} influx pathways in salivary glands and other non-excitable cells are not characterized. This represents a current major physiological void in our understanding of such tissues. Because of this knowledge gap, the SPS has devoted considerable effort to elucidating the main Ca^{2+} entry pathways in salivary glands. These have initially taken a more classical biochemical approach and it has led to remarkable progress in the last year. Starting with purified basolateral membranes from either rat parotid or submandibular glands, the SPS solubilized, in octylglucoside, a protein of ~135-160 kDa which exhibits a La^{3+} and Zn^{2+} sensitive cation channel activity when fused to lipid bilayers. These same proteoliposomes exhibit considerable high affinity Ca^{2+} influx activity (K_{Ca} = 242 mM). To compliment these biochemical studies, the SPS has during this year also initiated new efforts using both patch-

clamp electrophysiological technology and molecular biology. The initial studies with these two new approaches are quite promising.

Once a salivary acinar cell is signaled to secrete fluid, i.e. through Ca^{2+} mobilization, it is the cell's job to generate a transcellular salt (NaCl) gradient. This involves the activation of several specific ion channels and transporters. The Na^{+}-K^{+}-$2Cl^{-}$ cotransporter is considered to be a key component of this process. It is thought to be the major Cl^{-} entry pathway in acinar cells and the principal factor in driving Cl^{-} secretion (and ultimately fluid secretion) from these cells. The MBS has over the years focused considerable energy in better understanding this cotransporter. Studies during this past year have been directed at understanding the phosphorylation site associated with upregulation of cotransporter activity in response to secretory stimuli, especially following activation of the b-adrenergic receptor. As a first step, the MBS has begun to localize the site of phosphorylation through peptide mapping approaches. Much of the MBS' future efforts will be geared towards understanding the structure: function relationships that underlie cotransporter activation. To enhance these efforts, the MBS Chief, Jim Turner, undertook a sabbatical at Johns Hopkins University during this year with an emphasis on acquiring new experimental skills in structural biology.

Research in the GTS and GREU continued to be directed at clinically-relevant applications of adenovirus-mediated gene transfer to salivary glands. Two specific clinical situations have been targeted by the GTS; the repair of irradiation-damaged salivary glands and growth hormone deficiency. For both projects, during this year GTTB scientists showed proof of principle results with in vivo rat studies. For example, the GTS made a replication-deficient recombinant adenovirus encoding the (human) water channel aquaporin 1. When this vector was administered to adult rats that had been irradiated 4 mos previously, which resulted in a 65% reduction in salivary fluid flow, normal levels of salivary flow were seen in 3 days. In the second project we constructed an adenovirus encoding human growth hormone and showed that it could be secreted from rat salivary gland cells into the bloodstream at supra-therapeutic levels (~16ng/ml). This led to an increase in serum IGF-1 concentrations (~30%) as well as significant changes in other general clinical chemical parameters (triglycerides, BUN/creatinine) indicating anabolic events were occurring. These results suggest that salivary glands may provide a convenient gene transfer site for the systemic delivery of growth hormone and, possibly, other hormones and bioactive peptides.

The GREU has spent considerable energy this year addressing the immunopathologic response which occurs subsequent to recombinant adenoviral-mediated gene transfer to the salivary glands. The approach taken was to attempt to induce host immunological tolerance to adenovirus via the oral administration of antigen. Rats were fed UV-inactivated virus following various regimens, and subsequently live virus was administered to rats through their parotid glands. Conditions were established by which oral tolerance could be induced. This facilitated effective second and third administrations of the vector to the glands without the reduction in transgene expression seen without induction of tolerance. Additionally, the GTTB continued its efforts to bring a gene transfer-based approach to treat azole-resistant oropharyngeal-esophageal candidiasis into the clinic. This uses a recombinant adenovirus encoding the anticandidal peptide, histatin 3. During this year, studies were begun with an often used animal model of this

disease (immunosuppressed rabbits). Initial results show that rabbit glands are capable of directing the expression and secretion of high levels of histatin 3 into the saliva.

During this year, the CIS continued its landmark studies on the natural history and pathogenesis of primary SS. Two particularly valuable clinical studies were conducted this year. In one, the utility of salivary interleukin-6 (IL-6) as a convenient marker for involvement of salivary glands in SS was assessed. By comparing healthy controls, and patients with either primary SS or primary biliary cirrhosis (without salivary involvement), support for IL-6 as a convenient diagnostic and prognostic marker was obtained. In the second study, CIS scientists explored immunopathiologic mechanisms involved in SS by examining differential cytokine gene expression in subpopulations of cells found in the minor salivary glands (acinar, ductal, lymphoid) of patients and controls. The approach used was a cell-specific microdissection technique coupled with RT-PCR and Southern hybridization. Results showed that all cell types from patients are active participants in the autoimmune process, with the lymphoid focus apparently playing a role in modulating cytokine gene expression by epithelial cells.

Overall, FY97 has been a highly productive year for the GTTB. We continue to make progress bringing innovative clinical management tools, grounded in solid basic science, to benefit patients with salivary gland disorders.

GENE THERAPY AND THERAPEUTICS BRANCH
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Oral Health Promotion, Risk Factors, and Molecular Epidemiology Branch

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ORAL HEALTH PROMOTION, RISK FACTORS, AND MOLECULAR EPIDEMIOLOGY BRANCH 1997

The Oral Health Promotion, Risk Factors, and Molecular Epidemiology Branch (OHPRFMEB) seeks to improve and promote dental, oral and craniofacial health through epidemiology and health promotion research. The Branch employs an integrated biological and social model which uses molecular, genetic, analytical, biostatistical and behavioral science research methods to study the distribution, diagnosis, etiology, progression and prevention of dental, oral and craniofacial diseases and disorders.

Research in OHPRFMEB can be viewed as a continuum from basic fundamental research through clinical investigations, epidemiological studies, and national surveys to demonstration and science transfer. Central to the Branch are research questions about the relationships between oral and systemic health; the identification of genetic, biological, social, and environmental factors that contribute to oral disease initiation and progression; the elucidation of the natural history and clinical course of oral and craniofacial diseases; the discovery and quantification of factors which convey protective properties against disease; and methods of intervention at the individual, group and community levels which may lead to the prevention of disease and the promotion of health. The Branch develops new methods for large-scale genotyping and statistical genetics, new approaches for assessing oral health status in large-scale studies, and new statistical methodologies for clinical data, especially those involving complex data structures. The Branch is composed of three components managed by Senior Investigators who provide leadership in one defined scientific area focusing on molecular genetic epidemiology; analytical epidemiology; or oral health promotion research. Interactions and collaborations among investigators strengthen the multidisciplinary approach essential to contemporary research.

Molecular Genetic Epidemiology Section

Research is targeted toward applying the molecular and statistical tools of genetic epidemiology to discover the basic causes of oral and craniofacial disorders. This involves disentangling the complex interaction among environmental risk factors (*e.g.*, smoking, alcohol use, *etc.*) and susceptibility due to one or more genes. Current studies are focused on cleft lip and palate, Kartagener syndrome, early onset periodontitis, oral cancers and nasopharyngeal carcinoma. Most research is focused on studies of human subjects but animal models are also utilized where appropriate. Specific molecular genetic laboratory activities, have continued long term efforts aimed at developing new methods for achieving the accuracy, efficiency, and productivity necessary for fully automating large-scale genotyping. Over 400 fluorescently labeled markers, located at approximately 10 cm intervals, are being assessed to quantify and ultimately increase the percentage of genotypes accurately scored by the GENOTYPER software versus those requiring manual editing.

Non-syndromic clefting of the lip and palate in humans has a highly complex etiology, with both multiple genetic loci and exposure to teratogens influencing susceptibility. Previous studies

using mouse models have examined only very small portions of the genome. Data are analyzed for a genome-wide search for susceptibility genes for teratogen-induced clefting in the AXB and BXA set of recombinant inbred mouse strains. Results are compared using phenytoin that induces cleft lip and 6-aminonicotinamide that induces cleft palate. A large-scale family study of nasopharyngeal carcinoma in Taiwan was implemented and biological samples and clinical data are undergoing processing for gene mapping and segregation analyses. Studies of oral cancer are under way using samples obtained in Taiwan, Greece, Puerto Rico and from the Oral Pathology archive of the National Naval Dental Center. Samples are being collected from these collaborative studies for gene mutation analyses (p53), human papilloma virus detection and molecular candidate gene association analyses.

Analytical Epidemiology Section

Branch scientists conduct epidemiologic research on the distribution and characteristics of oral, dental, and craniofacial diseases in human populations. It also conducts studies of the patterns and determinants of the natural history or clinical course of these diseases. Branch projects include: (1) oral and pharyngeal malignant neoplasms; (2) surveys of the oral health status of the U.S. population and trends in oral health status; and (3) longitudinal or special studies of the determinants of oral and craniofacial diseases. Oral cancer research focuses on risk factors for oral cancer and premalignant oral lesions (behavioral, socioeconomic, medical, viral, and genetic) through case-control and other human population studies. Two key studies are underway: one on the etiology of oral and pharyngeal cancer in Puerto Rico and another on behavioral, clinical and genetic determinants of oral premalignant lesions among DVA hospital dental clinic patients. Other major efforts focus on patterns and determinants of initial stages of treatment for oral, pharyngeal, and laryngeal cancer, outcomes of oral cancer treatment and costs of head and neck cancer care.

Branch Scientists develop, monitor, and analyze data from nationally-representative surveys of the oral health status of Americans. Efforts have focused on the distribution and determinants of oral health status in the U.S. population and trends in oral diseases, especially conditions related to dental caries, periodontal diseases and tooth loss. Several special studies are underway. One includes an oral health component of the Baltimore Longitudinal Study of Aging, which has as its purpose the study of systemic factors responsible for oral health and the aging process. Another major study addresses the occurrence and behavioral, virologic, and immunologic determinants of oral manifestations of HIV. Periodontal conditions and oral soft tissue and mucosal lesions are a special focus of this study, which is based on a unique population of HIV positive military personnel. A third special study addresses the multiplex determinants of tooth loss in patients in dental practices in North Carolina and Connecticut.

Health Promotion Research Section

The Branch's oral health promotion research is the primary organizational focus with the Institute for developing, applying, evaluating, and disseminating advances from the socio-dental sciences with regard to methods, regimens and procedures for oral health promotion. These activities: (1) are responsive to the current and future needs of individuals and communities across the lifespan; (2) take into account emerging trends in the demographic, ecological, social,

economic, and political aspects of American society and its regional and international environments; and (3) provide leadership among the pluralism of worldviews that attempt to define the underlying themes, values, and beliefs of the cultural matrix in which oral health promotion operates.

Current research projects focus on: (1) neglected oral diseases (*e.g.*, primary caries in infants and young children, oral cancers, and the oral manifestations of persons with HIV-infection or AIDS); (2) neglected oral conditions (*e.g.* orofacial injuries and their psycho-social sequelae among school-age children and adolescents); (3) socioeconomically deprived individuals and sub-populations (*e.g.*, the poor in metropolitan and non-metropolitan communities, and racial and ethnic minorities); (4) the effects of socioeconomic status and differentials in oral health care on the oral health status of racial and ethnic minorities; (5) the oral health of women; (6) the oral health needs and risks of the elderly (e.g., through studies of the institutionalized frail elderly, particularly in home health nursing homes, and elders living in the community; and (7) special age cohorts, such as persons born between 1946 and 1964 (the "Baby Boomers") as they approach retirement and beyond.

The Health Promotion Research Section also addresses a number of conceptual, measurement, research design, analysis and interpretive issues which cut across a broad spectrum of its substantive endeavors. These include: (1) the adequate and effective conceptualization of oral health both as a multidimensional construct which integrates disease, disability and well-being perspectives of oral health and as a dynamic construct which has relevance both across the lifespan as well as at any one point within the lifespan by providing a quantitative estimate of the probability of transiting from one state of oral health to another; (2) the use and integration of traditional psychometric measurement approaches with newer sociometric ones, particularly those based on explicit measurement models, or which include new methods of statistical computation focused on identifying subsets of individuals with certain clusters of oral and oral-health related characteristics; (3) the development of a research agenda which balances traditional research designs, such as the randomized clinical trial and cross-sectional and longitudinal survey designs, and newer applications of participatory action research and ethnographic case study methods to the issues traditionally approached by "demonstration" studies; (4) a clearer distinction between descriptive, predictive, and explanatory models of analysis; and (5) more explicit attention to the formulation and use of analytical models in the development of theory as a purpose and focus of data analysis and as a tool for the codification of research findings as a basis for the development of guidelines for dental public health research and practice.

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Oral Infection and Immunity Branch

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ORAL INFECTION AND IMMUNITY BRANCH
1997

The Oral Infection and Immunity Branch (OIIB) was created from three preexisting laboratories in November 1996. The Laboratory of Immunology, Laboratory of Oral Medicine and Laboratory of Microbial Ecology, all with longstanding traditions of excellence, were merged to enable new opportunities, new interactions and new directions in infectious disease research. In April, 1997, Dr. Sharon M. Wahl was appointed Branch Chief. The new Oral Infection and Immunity Branch plans, fosters and carries out research relating to the causes, diagnosis, treatment and prevention of infectious and inflammatory diseases. Efforts to understand the functional and molecular organization of infectious organisms, and research into the cellular, biochemical and molecular components of inflammatory and immune responses provide the basis for dissecting the interactions between pathogens and the host immune system. These multifaceted approaches provide opportunities for microbiologists, virologists, immunologists, cell biologists, molecular biologists and clinicians to work together to understand physiologic mechanisms of host defense, how these pathways may become dysregulated to become pathogenic, and how to intervene for the benefit of the host.

Throughout the past year, members of the Branch have been widely recognized for their research accomplishments, dedication and leadership capabilities. Recognition came in the form of group awards, individual awards, FARE and NIDR travel awards, publications in prestigious peer-reviewed journals, invitations to speak at national and international symposia and to write review articles. Notable was the NIDR sponsored OIIB-hosted international symposium on HIV-1 Infection, Mucosal Immunity and Pathogenesis in September 1997. Many scientific achievements as highlighted below underscore the ongoing research efforts of the Branch.

Infectious and parasitic diseases are the leading cause of death worldwide. Understanding infectious pathogens, their unique attributes and their virulence factors provides insight into mechanisms of disarming them. One of these infectious pathogens is HIV, and OIIB scientists have made several important discoveries in the understanding of HIV infection and in the therapy of HIV disease. For example, in an experimental model of HIV disease, OIIB scientists showed that human chorionic gonadotropin (hCG) could prevent the HIV-dependent syndrome characterized by skin lesions, wasting and death in homozygous HIV transgenic mice. Discontinuation of hCG results in the reappearance of HIV mRNA and protein with wasting and death. These studies have been extended to other hormones, such as luteotropic hormone, which is closely related to hCG and which by inhibiting HIV mRNA and protein, prevents wasting and death in the transgenic mice.

In other novel therapeutic approaches, investigators have succeeded in expressing anti-HIV antibody genes intracellularly in $CD4^+$ T cells. The intracellular antibodies bind to HIV antigens and block maturation which in turn dramatically inhibits viral replication. Using these expression systems, investigators have extended this project to a CD8 derived cytokine, interleukin 16. $CD4^+$ T cells, transfected with IL-16, which inhibits HIV mRNA expression, are resistant to HIV infection. The application of these approaches is not only useful for studying

basic intracellular biological processes, but may also be useful for treating HIV and other diseases.

New insights into toxin-fusion proteins also suggest new opportunities for therapeutic intevention in HIV disease. Anthrax toxin protective antigen protein (PA) binds to receptors on the cell surface, is cleaved by a cell surface protease to capture either lethal factor (LF) or edema factor (EF) and enter an acidic compartment from which LF and EF escape to the cytosol and kill the cell. Having solved the structure of PA, fusion proteins were generated containing gp120, the envelope protein of HIV-1. These fusion proteins enabled presentation of specific gp120 peptide epitopes on the cell surface which stimulated cellular immunity to HIV. This system may provide a unique and effective way to induce immunity to HIV.

In additional studies focusing on the regulation of HIV production, co-infection with opportunistic pathogens was shown to influence viral replication. By *in situ* hybridization, unprecedented levels of HIV were detected in co-infected tissues. Striking concentrations of HIV-1 RNA signal were evident within *P. carinii* or *M. avium* infected lymph nodes, and the majority of HIV-positive cells expressed macrophage-specific markers. These novel observations on the dynamic relationship between opportunistic pathogens, macrophages and high levels of viral replication suggest that co-infection is an impetus for HIV production, and that macrophages contribute to plasma viremia, particularly in late-stage HIV disease. Importantly, these observations suggest that opportunistic infection therapy may not only diminish the opportunistic pathogens, but also decrease viral burden.

Beyond the study of HIV, other microorganisms are being investigated in OIIB as causative or contributory agents in the etiology of a variety of oral diseases including dental caries, gingivitis, and periodontitis. The pathogenicity of Gram-positive streptococci and of Gram-negative anaerobes (*Fusobacterium, Leptotrichia ssp.*) can be attributed in part, to the generation of organic acids and toxic sulfur derivatives during fermentation of dietary sugars and amino acids. In defining the genetic and molecular mechanisms for regulation of fermentation in oral bacteria, two novel enzymes (phospho-α- and phospho-β-glucosidase) from *F. mortiferum* were cloned, sequenced, and expressed. By encoding the genes (*malH* and *pbgA*) of these unique enzymes from fusobacteria into suitable vectors, plasmid or transposon constructs can be used for the manipulation and transfer of genetic information in pathogenic oral bacteria. In translational studies, chemical synthesis of unique chromogenic and fluorogenic phosphorylated substrates enabled development of assays for detection of α- and β-phosphoglycosylhydrolase activities.

The etiologies of dental caries and periodontal diseases are closely associated with dental plaque, the biofilm that develops from microbial colonization of the tooth surface. Colonization is initiated by a limited number of gram positive species, primarily different viridans streptococci and actinomyces. The formation of this community most likely depends on an array of specific adhesive interactions including those mediated by GaINAc or Gal sensitive adhesins. Coaggregation receptors comprise a family of structurally related cell wall polysaccharides recognized by lectins located on the surface of the coaggregation partner cells. The antigenic cross reactivity of different receptor polysaccharides correlates with the non host-like features of these molecules prompting studies to identify, characterize and compare gene clusters. Through the characterization of transposon insertion mutants lacking receptor polysaccharide production

and genetic complementation, it becomes possible to characterize genes for polysaccharide biosynthesis in viridans streptococci, and then to develop unique molecular approaches to assess and ultimately, interrupt the recognition role of these molecules in microbial colonization and biofilm formation.

Research in this Branch continues to focus on the cellular and molecular mechanisms by which the host mobilizes and modulates cellular inflammatory reactions in defense against these infectious agents and other foreign antigens. Defining these pathways identifies targets for agonists, antagonists and/or therapeutic intervention in pathogenic conditions. In a multi-disciplinary approach, mechanisms of integrin adhesion, chemotaxis, signalling, mediator synthesis and apoptosis are explored *in vitro* and extended into experimental animal systems including gene targeting models (gene knockouts and transgenics) and models of genetic susceptibility to chronic inflammatory diseases. For example, administration of bacterial cell walls induces arthritis and liver fibrosis in genetically susceptible rodents and provides a model to explore all phases of a host immune response and the consequences of its dysregulation. One objective of this research is to identify known and novel molecules expressed during the development of inflammation including members of the chemokine superfamily. During the past year, mutated forms of selected chemokines with sequence modifications predicted to produce receptor antagonism (ra), have been engineered which block chemotaxis and control leukocyte accumulation at sites of inflammation.

Another promising avenue for manipulation of host defense is through requisite signal transduction pathways. The activation of mast cells and basophils by immunoglobulin cell surface receptors results in the release of an array of mediators and serves as a model for exploring signal transduction by receptors on other immune cells. Emphasis on understanding the protein tyrosine phosphorylations that occur during cell activation have revealed the critical nature of the protein kinases, Syk and FAK and their substrates. Moreover, the level of protein phosphorylation of a molecule is due to the balance between protein tyrosine kinase and phosphatase activities and new insights have been uncovered in the role of the phosphatases in initiating, maintaining and regulating signal transduction. New studies reveal that the SH2 domain containing protein tyrosine phosphatases SHP-1 and SHP-2 associates with FcεRI, and that SHP-2 also associates with the adhesion molecule PECAM-1 that is tyrosine phosphorylated by activation. Identification of these signaling molecules suggests target molecules for the control of immune cell activation.

Molecular mechanisms of signal reception and transduction in immune cells often parallel pathways evident in signaling for the sensations of taste and smell. Little is known about the molecular mechanisms involved in taste sensation, although both sweet and bitter taste detection involves G-protein mediated processes. Taste-tissue cDNA libraries have been analyzed to characterize gene expression in the circumvallate papilla. Studies of the molecular components of olfactory signal transduction in the main olfactory epithelium and vomeronasal organ have identified a new family of as many as 300 putative pheromone receptor genes expressed at high levels in small sub-populations of these neurons. The expression patterns and properties of the putative odorant and pheromone receptors suggest that small groups of receptor cells with distinct ligand binding properties exist and are responsible for odor discrimination. Moreover,

these receptors provide molecular markers for dissection of mechanisms involved in olfactory signaling.

Signal transduction molecules are also involved in the pathophysiology of autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM). In this regard, 5 novel diabetes-related genes have been isolated from a pancreatic beta cell subtraction library, including IA-2β which is a member of the protein tyrosine phosphatase family and structurally related to IA-2. Autoantibodies to IA-2 and/or IA-2β appear years before the development of clinically apparent IDDM and are, therefore, highly predictive markers for identifying individuals likely to develop clinical disease. In these clinically relevant studies, it has been shown that the presence of autoantibodies to more than one islet cell autoantigen is even more highly predictive than autoantibodies to a single autoantigen.

In this inaugural year, the Oral Infection and Immunity Branch has made significant progress in the understanding of oral and mucosal infections and diseases. As the Branch begins its second year with the initiation of a clinical research program, new opportunities and insights will become part of our research portfolio.

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Oral and Pharyngeal Cancer Branch

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ORAL AND PHARYNGEAL CANCER BRANCH

1997

Cancer of the head and neck area is the sixth most common neoplastic disease in the developed world, representing a very serious health problem based on annual morbidity and mortality rates. This form of cancer share many common traits with those from other tissues. However, a number of issues pertaining to diagnosis, etiology, tumor promotion, genetic and environmental risk factors are unique and distinct, thus meriting a high-impact research effort in this area. The molecular and etiological factors involved in the development of head and neck tumors, including oral cancers, are still largely unknown.

OPCB scientists work on several complementary aspects of cancer cell biology, in an effort to understand the molecular basis for malignant transformation as well as to use this knowledge to develop molecular markers of disease progression and novel therapeutic approaches for oral malignancies. Our goal is scientific excellence in addressing the ravaging problem of oral cancer. During the current reporting period, progress has been made in a number of research efforts at the OPCB.

- **Identification of molecules that uniquely describe stages of progression from normal to malignant**

In spite of the high frequency of oral cancer, little is known about the molecular events that contribute to the initiation and progression of this disease. Deletion or mutation of p53 characterizes benign and malignant neoplasms from a broad spectrum of tissue types, including oral mucosa. Although several studies have demonstrated p53 aberrations in squamous cell carcinomas of the head and neck (HNSCC), the functional contribution of such mutations to tumor development in this system remains unproven. Studies focussed on determining the likely role of HNSCC-derived p53 mutants on tumor establishment and progression revealed that the vast majority of these molecules were not able to induce p53-dependent gene expression. On the contrary, they blocked the trancriptional activity of wild-type p53 alleles. Furthermore, these HNSCC-p53 mutants were tumorigenic when expressed in a variety of *in vivo* systems. These data suggest that p53 mutants make a functional contribution to tumor development, via dominant-negative and/or gain-of-function mechanisms.

- **Novel signaling pathways involved and normal and aberrant cell growth**

In the past two years, work from a number of laboratories has uncovered the existence of a novel family of enzymes closely related to MAP kinases, known as Jun kinases (JNKs), which phosphorylate the N-terminal transactivating domain of the *c-jun* proto-oncogene product thereby increasing its transcriptional activity. Recent efforts at the OPCB have led to the identification of key components of the pathway connecting cell surface receptors to JNK, and to the discovery that JNK is potently activated by a number of human cancer-causing genes (oncogenes), including *dbl*, *ost*, and *vav*. How cell surface receptors activate the small GTP-binding Rac1 was not known, but it was noticed that in certain cells, but not in others, receptors of the tyrosine kinase class induce JNK activation in a Rac1-dependent manner. Recent work established that the normal counterpart of the *vav* oncogene, the Vav proto-oncogene product,

can behave as a tyrosine-phosphorylation-dependent guanine nucleotide exchange factor for Rac1. This finding represents the first example of an exchange factor for small GTP-binding proteins regulated directly by tyrosine phosphorylation, thus raising the possibility of the existence of a novel signal transduction pathway linking the membrane to the nucleus. This biochemical route was found to play a role in signaling from T and B cell receptors in T and B lymphocytes, respectively, and also during antigen-induced activation of mast cells and basophils. Thus these findings are likely to have important implications in cancer as well as in the study of those mechanisms controlling the immune response, allergic reactions and inflammatory processes.

- **Signaling from cell surface receptors to the c-*jun* promoter involves the MEF2 transcription factor: evidence for a novel JNK-independent pathway**

The *jun* gene family of nuclear proto-oncogenes appears to be critical for cell cycle progression, and to play a central role in normal and cancerous cell growth. As such, expression of c-*jun* is rapidly induced by most mitogenic stimuli. Focussing on the pathway regulating the activity of the c-*jun* promoter by cell surface receptors, it was found that the transcription factor MEF2 controls c-*jun* promoter expression through a yet to be identified pathway, which is distinct from that of JNK.

- **Signaling from G protein-coupled receptors to MAP kinase involves the novel PI 3-kinase γ**

Whereas the tyrosine-kinase class of growth factor receptors regulates MAP kinases in a multistep process that involves the small GTP-binding protein, $p21^{Ras}$, the mechanism(s) involved in MAP kinase activation by cell surface receptors acting through heterotrimeric G proteins has been poorly understood. In this regard, members of OPCB have shown that the βγ subunits, not Gα, act in a Ras-dependent manner to stimulate MAP kinases, and these findings prompted the search for molecules linking βγ dimers to Ras. Recently, it was found that a novel form of phosphatidylinositol 3-kinase (PI3K), PI3K-γ, plays a critical role linking G protein-coupled receptors and βγ to Ras and MAP kinase. This research is expected to have important implications in the search for novel targets for therapeutic intervention in neoplastic diseases.

- **Novel molecules mediate mitogenic signaling through tyrosine kinase growth factor receptors**

It was previously established that eps8 is a direct substrate of the epidermal growth factor receptor (EGFR). Unlike other SH2 domain-containing substrates of EGFR such as Grb2, the interaction between eps8 and EGFR was found not to involve phosphotyrosine residues on the receptor. Recent studies suggest that eps8 plays a role in the proliferation pathway of EGFR, and have identified constitutive phosphorylation of eps8 in human tumor cell lines. A parallel effort to elucidate the cellular function of eps8 has focused on the SH3 domain of eps8. SH3 domain, a protein domain for protein-protein interactions, has been shown to play diverse roles including regulation of enzyme activity, intracellular targeting, recruitment of substrates, and assembly of multisubunit enzymes. The SH3 domain of eps8 was crystallized, and shown to be a unique member of that SH3 domain family, as it can exist as an interwinded dimer. To identify eps8 SH3 domain binding protein, a human expression library was screened by a novel approach, and

three unique human cDNA clones were isolated. One of these clones, designated as e3B1 for eps8 SH3 domain binding protein 1, was shown to associate with eps8 *in vivo*. Data were obtained to suggest that one of the functions of the EGFR-eps8 mitogenic signaling pathway might involve the inactivation of the growth-inhibitory activity of $p65^{e3B1}$.

- **Deoxyhypusine synthase: Its crystal structure, mechanism of catalysis activity, and essential requirement for yeast viability**

Deoxyhypusine synthase catalyzes the first step of hypusine biosynthesis, the NAD-dependent transfer of the aminobutyl moiety of spermidine to the ε-amino group of a specific residue of the eIF-5A precursor protein to form deoxyhypusine. Inactivation of the deoxyhypusine synthase gene in yeast causes loss of cell viability, indicating that the hypusine modification is vital for *in vivo* eIF-5A activity and cell proliferation. The physical and catalytic properties and the reaction mechanism of the enzyme were characterized, leading to the identification of an active site residue, (Lys^{329}), of the human enzyme that is involved in enzyme-intermediate formation, and thereby is critical for catalysis. X-ray crystallography of human deoxyhypusine synthase in a complex with NAD has revealed its tetrameric structure and the NAD binding site. Molecular modeling of the active site with known inhibitors related to spermidine, currently underway, is expected to provide new leads in the design of more effective, structure-based inhibitors for this enzyme that may represent novel anti-proliferative agents. One such recently prepared compound, 1-guanidino-7-aminooctane, is a better inhibitor than 1-amino-7-guanidinoheptane, the best inhibitor used in OPCB's claims for a U.S. patent on the use of deoxyhypusine synthase blockers in cell growth inhibition.

- **Cancer-derived immunosuppression factors**

It was recently found that the C4 domain of gp120 from HIV is in an alpha helical conformation in the parent protein and that CD4, the receptor for HIV, binds to the hydrophobic face of this helix. In addition, synthetic peptide-based alpha helical constructs can bind to CD4 on CD4+ cell surfaces and, in doing so, activate intracellular signaling pathways via $p56^{lck}$, a well-known cell activation tyrosine kinase. Other groups have found that, in response to CD4-mediated signaling, IL-2 transcription factors are phosphorylated and the result is that the cell no longer is capable of making IL-2. More recently, it was described the ability of the intracellular protein, p53, to bind an inhibitor, MDM2, via an interaction that involved an amphipathic alpha helix from p53. The composition of the p53 hydrophobic surface was investigated and compared to the C4 from the gp120 hydrophobic surface. A Trp-Leu motif was found, that was similar the Trp-Val motif described in C4, thus raising the possibility of p53 binding to CD4, a phenomenon that has not described in the literature. Although p53 was not expected to bind an extracellular receptor such as CD4, it was shown that a small percentage of patients suffering from various types of cancer have antibodies to p53 in their sera, thus indicating that p53 was probably being secreted from the cancer cells *in vivo* where, outside of the cell, it was immunogenic. That reinforced the notion that p53 could have access to and bind to CD4 *in vivo*. Using commercially available p53 constructs it was found that CD4 does bind to intact p53 in an ELISA-type of format. During the course of those studies, an assay to detect the presence of CD4-binding proteins in serum was developed. A simple test is now being further developed for clinical uses to assay for the presence of possible immunosuppression(CD4-binding) factors in the serum from cancer patients.

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Pain and Neurosensory Mechanisms Branch

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PAIN AND NEUROSENSORY MECHANISMS BRANCH
1997

The Pain and Neurosensory Mechanisms Branch (PNMB) conducts a multidisciplinary research program aimed at improved understanding and treatment of pain. Studies range from evaluating molecular responses to tissue injury and elucidating the mechanisms of peripheral tissue inflammation, including subsequent changes within the nervous system, to evaluating novel drugs and clinical hypotheses about pain and its control in human models of acute and chronic pain. The hallmark of the Branch's research program is the integration of basic and clinical research which permits not only a rapid transfer of new findings from the laboratory to the clinic, but also foster basic research based on clinical problems. This integrative approach provides an optimal environment for training clinicians and basic researchers in the principles and methods of pain research across a spectrum spanning basic molecular mechanisms to the clinical management of pain. In addition, the Branch's senior investigators participate widely in speaking, writing, and collaboration with professional organizations and academic institutions to transfer emerging scientific information to the training of clinicians and the treatment of patients.

The PNMB completed the transition during the past year from the former Neurobiology and Anesthesiology Branch with three sections operating with a single budget under the Branch Chief to six units/sections acting under the scientific leadership of senior staff members with independent resources. The independence and challenge presented by this new organization and the opportunities for PI-initiated research has already yielded increased scientific vigor and productivity. The number of publications, an indirect measure of scientific productivity, during fiscal year 1997 (N=52) is comparable to the number of publications during fiscal year 1995 (N=58) despite a reduced Branch budget and two fewer senior investigators. The Branch continues to operate a large clinical research program, the Pain Research Clinic, in the Magnuson Ambulatory Clinical Research Facility under the scientific direction of Drs. Max, Dionne and Gracely. Research conducted in this clinic is based on observations made in the Branch's basic laboratories as well as uses novel and prototypic drugs to test emerging scientific hypotheses in man, representing a true 'bench to bedside' continuum. Numerous publications in respected peer-reviewed scientific journals attest to the Branch's continued scientific productivity. Dr. Max received the prestigious Wilbert Fordyce Clinical Research Award this year from the American Pain Society in recognition of his contributions to the understanding and treatment of neuropathic pain. The work of the Branch was also featured in a nationally broadcast segment of the NBC's Evening News. Highlights of research findings by Branch investigators during the past year are presented below.

Molecular basis for hyperalgesia: Hyperalgesia has continued as a theme common to the work of much of the Branch's efforts, both basic and clinical, during the past year. Continuing work on the molecular genetics of hyperalgesia has lead to description of a model for nociceptive specific regulation of the dynorphin gene based on observations of a dynorphin promoter (DYNCRE3) in the spinal cord. This work is being extended in collaboration with investigators from NCI to further characterize nociception induced changes in gene regulation. It has also been observed that nociception activates STAT (signal transducers and activators of

transcription) transcription factors in the primary afferent neuron, the first neuron in the pain response, at a timepoint when rats exhibit thermal hyperalgesia following experimentally induced tissue injury. Current efforts are focused on determining which STAT proteins are activated by persistent pain. These mechanisms may be important for producing abnormal pain sensations in chronic pain states such as allodynia and hyperalgesia.

Role of the NMDA receptor in hyperalgesia: A second continued focus of the Branch's activity during the past year is the role of the N-methyl-D-aspartate (NMDA) receptor in the development of hyperalgesia following the release of excitatory amino acids in the spinal cord following nociceptive barrage. Antisera to a subunit of the NMDA receptor (NR1) has been used to map the NMDA system in the spinal cord and compare it to the substance P (neurokinin 1) receptor. Preliminary immunocytochemical data suggest that a phosphorylated receptor protein of this subunit is upregulated in spinal cord neurons by peripheral inflammation. Several clinical trials conducted in the past year examined the role of the NMDA receptor in neuropathic pain. Based on previous observations that the low affinity NMDA receptor antagonist dextromethorphan is analgesic in patients with painful diabetic neuropathy, a new larger study was initiated comparing dextromethorphan to memantine, another low affinity receptor blocker. Preliminary results of this study have confirmed the initial observation of dextromethorphan efficacy but have failed to demonstrate any evidence of analgesic activity for memantine. Animal studies have suggested that another class of excitatory gluatamate receptors, AMPA/kainate receptors, play a role in mediating pain but the receptor subtypes have remained unclear. PNMB investigators carried out phase 1 toxicity and dose-finding studies demonstrating that AMPA/kainate receptor blockade reduces pain and hyperalgesia evoked by intradermal capsaicin by about 50% at doses which did not produce side effects, suggesting promise for similar compounds as analgesics. By combining evidence from this study and animal studies with other selective AMPA/kainate antagonists, it is hypothesized that a kainate receptor subtype (GluR5) mediates analgesic effects, while another receptor subtype (GluR1-4) mediates sedation and other side effects.

Spinal processing and control of nociception: The dorsal horn of the spinal cord remains an important focus of the laboratory's basic research efforts as this is the first site of synaptic processing for nociceptive information with many basic molecular and physiologic processes occuring which alter nociceptive transmission from the periphery to higher centers in the nervous system. Descending control by higher centers on spinal nocieptive neural circuits represents a mechanism whereby the brain can control nociceptive messages from the periphery that are processed in the spinal cord. Using an animal model of spinal transection with hind paw inflammation, it was observed that descending afferents on spinal neural circuits inhibit the response of opioid neurons to noxious stimulation in the periphery. Parallel studies examining the role of brainstem neurons which use either serotonin or norepinephrine as neurotransmitters, suggest that removal of all descending axons has a dynamically different effect than selectively removing aminergic input.

Animal studies in rats with chronic constriction injury evaluated the role of nerve growth factor in pain following nerve injury. Application of nerve growth factor at the site of the nerve injury delayed the development of hyperalgesia, suggesting that nerve growth factor is important in the expression of hyperalgesia following nerve injury leading to neuropathic pain. Parallel studies

compared the consequences of nerve inflammation with minor injury to gross neural injury produced by the chronic constriction injury model in rats. The results suggest that inflammation produces a centrally-mediated increase in A beta-sensitivity and the unusual situation of A beta-mediated pain while gross nerve injury results in a combination of centrally-mediated increased sensitivity and peripherally-mediated loss of A beta function. Clinical studies of the consequences of inflammation on orofacial nerve function demonstrated that both increased mechanical and electrical sensitivity at two days post injury (oral surgery) which returned to normal by 8 days. These results indicate that inflammation and nerve injury may result in a continuum of effects ranging from increased tactile sensitivity to mechanical allodynia. These studies also suggest that electrical stimulation provides useful diagnostic information for the evaluation of chronic orofacial pain syndromes, possibly distinguishing between inflammatory and nerve injury etiologies.

Hyperalgesia in the cortex: Cortical mechanisms of pain and hyperalgesia also continued to be an active area of investigation during the past year. Neurons in the primary somatosensory cortex of monkeys responding to intradermal injections of capsaicin were classified as either wide dynamic range or nociceptive specific neurons using previously characterized criteria. The results of this investigation suggest that nociceptive neurons are common in the somatosensory cortex, contrasting with the long held view that nociceptive neurons are rare in this area. The somatotopic organization of nociceptive neurons were also studied and indicate that the wide dynamic neurons are distributed over a wider area of the somatosensory cortex than the nociceptive specific neurons and sheds light on the role of these neurons in providing information about localization and the cortical activation that accompany noxious stimulation.

Supraspinal processing of pain in humans: Positron emission tomography (PET) provides a powerful tool for defining neural networks that subserve pain, the interaction between these networks, and the functional abnormalities introduced by chronic pain in select pain populations. One study completed in the past year documents that acute pain induces a decrease in global cerebral blood flow, a novel finding which points to a previously unidentified sympathetic regulation of the cerebral vasulature in acute pain/stress states. The global blood flow decrease may have important implications for surgical operations, anesthesia, pain-related syncope, and technical aspects of imaging studies of pain. A second completed study explored the relationships between painful heat stimuli and regional brain activation using methods developed to make correlations between regional brain activation with subjects' ratings of pain intensity. The results of this study suggest that distributed neural sites in both cerebral hemispheres contribute to some sensory-discriminative pain processes, which contrasts with traditional theories of unilateral pain processing, and may provide a better understanding of attentional and cognitive aspects of pain processing in humans. New studies initiated this year are attempting to make a transition from experimental pain to clinical pain in studies with experimental ischemic pain and clinical pain from third molar extractions. Preliminary results suggest that post-ischemic dysesthesia is associated with activation of the contralateral somatosensory cortex and anterior cingulate cortex. This observation emphasizes the central consequence of clinical pain and dysesthetic conditions and may provide a method for the experimental analysis of supraspinal processing of these conditions.

Blocking the development of hyperalgesia in humans: The clinical significance of the development of hyperalgesia in clinical pain was evaluated in two studies conducted in the oral surgery model of acute pain and inflammation. A study was completed this year in which local anesthesia was administered prior to or following oral surgery conducted under general anesthesia to result in four groups of patients which differed in the time of nociceptive barrage thought to contribute to the development of central hyperalgesia. In distinction to previously published clincal studies, it appears that postoperative pain occurring after surgery is more important in the developement of hyperalgesia at 24 and 48 hours following surgery than the brief, intense nociceptive barrage that occurs during surgery conducted without any local anesthesia. This observation suggests that use of preventive analgesic manipulations in the immediate postoperative period will not only minimize perioperative pain but may also reduce pain at later time points. A parallel study, however, did not detect any clinical advantage to combining the effects of the NMDA receptor blocker dextromethorphan with an orally administered opiate due to a significant side effect liability

Development of novel therapeutics for pain using basic science findings: Three areas have been targeted in the past year to create new treatments for chronic pain. Adenovirus-mediated gene transfer to the nervous system has been evaluated by direct injections into the trigeminal ganglion and appears feasible. Several therapeutic viruses have been constructed which should confer an analgesic phenotype on the spinal cord or delete spinal cord receptors that transduce nociceptive stimuli. Preliminary results have been favorable examining recombinant cytotoxic toxin-ligand fusion proteins as a means to selectively lesion spinal cord neurons involved in pain transmission. Based on results in behavioral pharmacology studies, attempts have been initiated to develop pharmacologic agonist that selectively target the kappa2 opioid receptor. These studies are initiating a new era in pain research which bridges the continuum from 'molecules to man' and holds promise for the development of novel therapies which overcome the limitations of current pharmacologic and surgical interventions for chronic pain.

PAIN AND NEUROSENSORY MECHANISMS BRANCH
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Gene Targeting Facility

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GENE TARGETING FACILITY

The main focus of the GTF is to delineate precise *in vivo* functions of the specific genes involved in neurodegenerative diseases, metabolic defects and immune disorders. The focus involves isolation and characterization of murine genes, their disruption in embryonic stem cells by homologous recombination and generation and analysis of the gene knockout mice.

Phosphorylation of Neuronal Cytoskeleton in Neurodegenerative Diseases

Phosphorylation process plays an important role in the structural organization of neuronal cytoskeleton. Synthesis, transport and assembly of neurofilament (NF) proteins are regulated by specific kinases that extensively phosphorylate different motifs. The phosphorylation of NF proteins is developmentally and spatially regulated. Most of this phosphorylation takes place in the Lys-Ser-Pro (KSP) repeats in the carboxyl-terminal tail domain of NF-M and NF-H. This phosphorylation is believed to stabilize the NF network in the axon, and to affect the axonal transport and conduction velocity in the neurons. Some KSP sites of this tail domain in NF-H have been suggested to be phosphorylated by cyclin dependent kinase-5 (Cdk5). Cdk5 also phosphorylates tau protein and this phosphorylation occurs exclusively at the same sites found in the tau protein from Alzheimer's disease brain. Abnormal NF phosphorylation has also been associated with neurodegenerative diseases.

In order to delineate precise roles of specific kinases in neurodegenerative process in vivo, we have initiated generation of the gene knockout mouse models for specific kinases and their activator subunits. Cdk5 null mouse exhibited unique brain lesions including a lackcortical laminar structure and cerebellar foliation. Additionally, the large neurons in the brain stem and in the spinal cord showed chromatolytic changes with the accumulation of NF immunoreactivity. Subsequent analysis of Cdk5 null phenotype revealed abnormal migration of cortical neurons. Most importantly, the abnormal migration of the neurons in these mice was not found to be associated with abnormal reelin expression indicating an inedpendent pathway through which Cdk5 exerts its effects on the neuronal migration and corticogenesis.

Mouse Models of Inherited Metabolic Disorders

Fabry disease is an inherited X-linked recessive metabolic disorder resulting from the deficient activity of the lysosomal enzyme, a-galactosidase A (AGA). In affected hemizygous males, the progressive deposition of substrate in lysosomes of vascular endothelial and smooth muscle cells causes occlusive vascular disease. To date there is no specific treatment for this condition. Both enzyme replacement and gene therapy are under consideration, but carrying out these trials in human will be difficult and time-consuming. An animal model for Fabry disease will be valuable to develop such therapeutic regimes.

In order to generate an animal model we disrupted AGA genomic locus in mouse embryonic stem cells by homologous recombination and generated AGA null mice. Although these mice showed a complete lack of AGA activity, they appeared clinically normal at 10 weeks of age. Ultrastructural analysis revealed concentric lamellar inclusions in the kidneys, and confocal microscopy using a fluorescent-labeled lectin specific for a-D-galactosyl residues showed accumulation of substrate of in the kidneys as well as in the cultured fibroblasts. Lipid analysis

revealed a marked accumulation of the substrate in the liver and the kidneys. These findings indicate the similarity of the pathophysiological process in the AGA null mice and in patients with Fabry disease. Moreover, using biscistronic MDR vectors containing human AGA cDNA we have corrected AGA deficiency and accumulation of a-D-galactosyl residues in the cultured fibroblasts from the AGA null mice.

Cytokines and Growth Factors in Autoimmune Diseases

Cytokines and growth factors play critical roles in normal homeostasis of immune functions. TGF-ß1 is the dominant negative regulator of inflammatory responses. We have earlier generated TGF-ß1 null mice that exhibit multifocal inflammation associated with increased adhesion of leukocytes to endothelium, aberrant expression of MHC class-I and –II antigens, and auto-immune manifestations similar to Sjogren syndrome. To delineate role of TGF-ß1 in aberrant MHC expression, TGF-ß1 null mice were generated in the MHC-I and MHC-II deficient backgrounds. TGF-ß1 X MHC-I null mice exhibit increased longevity associated with reduced inflammation, diminished autoimmune manifestations and significantly elevated myelopoiesis. Although the TGF-ß1 X MHC-II mice exhibited lack of inflammation and autoimmune response, they showed neonatal mortality associated with increased myelopoiesis and metaplasia. These results indicate a dominant role of TGF-ß1 in leukocyte maturation and function.

Macrophage migration inhibitory factor (MIF) is a major protein constituent of the anterior pituitary gland released into the bloodstream during endotoxemia. For many years, MIF had been thought to be a T cell product associated with delayed-type hypersensitivity reactions. The identification of MIF as a pituitary "stress" hormone provides an important link in the regulation of systemic inflammatory responses by CNS. Additionally, there is compelling evidence that suggests a strong link between MIF and cell cycle suggesting MIF as an inhibitor in G0 phase. To dissect molecular roles of MIF, we have generated the mice heterozygous for the MIF locus. These mice are clinically normal and will be crossed to generate MIF null mice.

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Immunopathology Section

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IMMUNOPATHOLOGY SECTION

Role of Monocytes in Immunopathology

The focus of this project is on factors and signal transduction pathways involved in the modulation of the immune response, particularly in relationship to the human monocyte, that may contribute to the immunopathology associated with various disease states. Cytokines are well recognized as mediators which regulate the outcome or progression of disease states. Since multiple cytokines may be found at disease sites, it is important to determine the effect of a single cytokine as well as combinations of relevant cytokines on cell functions which contribute to the pathology of a particular disease. Thus we initiated studies to compare the effect of individual cytokines with that of multiple cytokines on the production of prostaglandins and matrix metalloproteinases by human monocytes which may impact on connective tissue and cancer related diseases. Cytokines, such as TNFα, GM-CSF, and IL-1β, were shown to differentially regulate matrix metalloproteinase (MMP) production by monocytes. When added alone they enhanced gelatinase B (MMP-9) and acted synergistically when added in combination. In contrast, these cytokines individually had little effect on interstitial collagenase (MMP-1), however when added in combination, particularly GM-CSF with either TNFα or IL-1β, they induced significant levels of MMP-1. The differential effect is related, in part, to the substantial stimulation of phospholipase A2 (PLA2) activity and subsequent production of PGE2 by the combination of cytokines. This is further substantiated by indomethacin inhibition of cytokine induced MMP-1 but not MMP-9. These findings indicate that the induction of MMP-1 by cytokines is tightly regulated by PGE2, unlike MMP-9.

Additional studies involving cytokines have examined the manner in which soluble HIV-Tat induces the production of MMP-9 by monocytes. Treatment of monocytes with HIV-Tat resulted in a significant increase in TNFα, IL-1β, IL-6 and IL-8. Inclusion of neutralizing antibodies to these cytokines revealed antibodies against TNFα and IL-1β, but not IL-6 and IL-8, significantly inhibited MMP-9 production. Moreover, the effects of HIV-Tat on MMP-9 production could be mimicked by TNFα and IL-1β, but not IL-6 or IL-8. Thus, the mechanism by which HIV-Tat induces monocyte MMP-9 involves the stimulation of TNFα and IL-1β.

The induction of monocyte MMPs by factors such as cytokines or LPS potentially involves multiple signaling pathways which may result in the differential regulation of MMPs depending on the stimulus. Our recent experiments have used LPS as the primary stimulus to examine the early signal transduction events that lead to monocyte MMP production. These studies have shown that LPS induces tyrosine kinase phosphorylation of MAP kinases which in turn phosphorylate cytosolic PLA2 (cPLA2) resulting in the release of arachidonic acid and the subsequent generation of PGE2 required for the induction of MMP-1. Utilization of specific inhibitors revealed that p38 is the major MAP kinase involved in phosphorylation of cPLA2 with p44/42 MAP kinase (ERK1/2) being involved to a lesser extent. Thus, at least part of the role in the regulation of monocyte MMPs by MAP kinases is due to their phosphorylation of cPLA2. Identification of cPLA2 as the specific phospholipase involved in the PGE2-cAMP dependent pathway for MMP production was demonstrated with a specific inhibitor (trimethylketone analog of arachidonic acid) of cPLA2.

In addition to the demonstration of tyrosine kinase regulation of monocyte MMPs, studies were also conducted to determine the role tyrosine kinases in monocyte apoptosis. The findings from this project demonstrated that interaction of CD40L, which is expressed on the surface of activated T cells, or antibodies against CD40 with CD40 on monocytes rescued the monocytes from undergoing apoptosis. The mechanism by which CD40L blocks apoptosis was shown to involve the induction of protein tyrosine kinase activity. Thus a tyrosine kinase signaling pathway is involved in the events controlling monocyte longevity.

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Matrix Metalloproteinase Unit

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MATRIX METALLOPROTEINASE UNIT

The Matrix Metalloproteinase Unit has made significant progress on a number of related projects during the past year:

Replacement/knock-out of the MT1-MMP gene
To improve the understanding of the biological role of MT1-MMP (MMP-14) in development and disease we have cloned the gene encoding MT1-MMP in the mouse and constructed a targeting vector that facilitates replacement of the endogenous mouse MT1-MMP gene in pluripotent embryonic stem cells (ES-cells) by homologous recombination. Using this approach the MT1-MMP gene was modified to introduce complete loss of MT1-MMP expression in transgenic mice generated from targeted ES cells. Our preliminary results indicate the homozygous (knock-out) mice possess a severe phenotype with more profound alterations than any previous MMP knock-out, including dramatically altered and retarded growth and early death. The phenotype is currently being explored in further detail.

Characterization of the Enamelysin gene
We have isolated, cloned, restriction digested, Southern blot analyzed, and sequenced the cDNA for murine *enamelysin* (MMP-20) by exploiting the information obtained by isolating the genomic clone using John Bartlett's cDNA clone of the pig homologue. Northern analysis reveals that expression of enamelysin is restricted to teeth and no transcripts have been found in any other organ including bone and cartilage. At this stage we are nearing completion of the murine enamelysin gene structure in preparation for targeting constructs that will permit ablation of the gene by homologous recombination.

Cellular Regulation and Activation of Collagen Degradation
The existence of 17 or more multiply expressed MMPs with broad and overlapping substrate specificity toward extracellular matrix substrates, and 4 or more broad-specificity inhibitors, render the dissection of individual proteolytic mechanisms inordinately complex. We have chosen to attempt to reconstitute collagen degrading activity in an inactive transfectable monkey cell line (COS7 cells). By recombinant linkage of MMPs to readily identifiable markers it is possible to monitor the success of transfection and to assess the functional outcome of expression of individual MMPs or combination of MMPs. It is commonly assumed the degradation of type I collagen fibrils is mediated by one of three (genuine) mammalian collagenases (MMP-1, MMP-8 and MMP-13) although recent findings may challenge this view in light of recent demonstrations of collagenolytic activity by Gelatinase A (MMP-2), Gelatinase B (MMP-9) and MT1-MMP (MMP-14). It is therefore a potentially rewarding strategy to examine the effect of introduction of these specific MMPs one at a time and in combination. So far this strategy appears to be successful. Transfection of the plasma-membrane-linked MT1-MMP cDNA (which is suspected of playing a significant regulatory role by initiating or mediating precursor activation of other MMPs) imparts collagen degrading ability to otherwise inactive COS7 cells.

An important priority of our program is to more fully understand the structure and function of the "cysteine-switch" which maintains catalytic latency in all MMPs (and ADAMS). Continuing collaborative studies with Dr. Jeffrey Engler and colleagues at the University of Alabama at Birmingham have shown, using stromelysin-1 (MMP-3) as a model, that the original "cysteine-switch" model requires some modification. X-ray crystallographic studies by others have confirmed the predicted structural arrangement, including the bonding of the unpaired Cys to the active-site Zn. Replacement by site-directed mutagenesis of this Cys in the propeptide blocks access to the active site

showing that activation with organomercurials thought to target the Cys residue, proceeds unaltered when this residue is replaced with Ser. Interestingly, however, the Cys-residue is held in place by a short Arg-Asp salt bridge. When this salt bridge is disrupted by site-directed mutagenesis, the ability to respond to organomercurial activation is lost, suggesting a mechanism somewhat different than that proposed in the original "cysteine-switch" model.

A series of studies have further expanded on the role of keratinocytes in the degradation of type I collagen fibrils. These cells degrade collagen fibrils effectively following induction of MMP expression either by certain growth factors and cytokines (TNF-a, EGF, TGF-a) or by phorbolester. Induced cells dissolve collagen fibrils rapidly. By use of specific synthetic and protein inhibitors we have shown that the process is MMP-dependent. Additional experiments using blocking and non-blocking, control monoclonal antibodies have shown that dissolution of the fibrils depend both on the uPA (through activation of plasminogen) pathway and on MMP-1 (interstitial collagenase) in that blocking antibodies to each can block the dissolution process.

Characterization and Biologic Function of TIMPs and TIMP genes.
A series of studies in collaboration with Jeffrey Engler's group at the University of Alabama at Birmingham, involving site-directed mutagenesis of the TIMP-I molecule were completed. These studies showed that the biologic and inhibitory activity of the molecule is highly resistant to single point mutations but also revealed that disruption of the important Cys1-Cys72 bond leads to ablation of activity.

Replacement of the TIMP-2 gene
Constructs have been made for the purpose of conducting gene replacement ("knock-out") experiments for TIMP-1, TIMP-2 and TIMP-3 in mouse embryonic stem (ES) cells. A less commonly used cell line HM1 (feeder-independent, HPRT-minus) is being used in these knockout experiments because of its phenotype. HM1 cells allow the use of positive selection for the HPRT gene when doing gene knockout experiments which can then later be selected against for the purpose of doing gene replacement procedures. These cells were tested in the NIDR Transgenic Facility for their ability both to form chimeric mice following microinjection into C-57 Black blastocysts as well as to contribute to the germline of chimeras produced. The HM1 cells produced high percentage HM1-phenotype chimeric mice which successfully produced offspring of the HM1-genotype.

TIMP-2 gene replacement clones in the outbred c57Bl6/129ReJ background and in the inbred 129ReJ background were produced which contain the exact genetic disruption initially targeted to the HM-1 cells. This leads to the production of a stable TIMP-2 mRNA species missing exons 2 and 3 which is translated into a mutant ~14 kd TIMP-2 polypeptide. The mutant TIMP-2 produced has minimal residual activity on a reverse zymogram (100-300 fold less than wild-type). The animals are outwardly unaffected by the lack of functional TIMP-2 and are reproduction competent. Fibroblast cell lines derived from gene replacement and wildtype mice both possess seemingly wild-type activity in the degradation of collagen fibrils, but the gene replacement cells differ somewhat in terms of response of Gelatinase-A to activating agents. Lack of TIMP-2 activity apparently is not an impediment to embryonic development, growth, reproduction or normal function in unstressed animals and the lack of this inhibitor creates only subtle phenotypic changes. The animals are therefore highly useful for experiments in wound healing, tumor growth and metastasis which challenge the function of MMP inhibitor activity.

Cloning and Characterization of the Murine TIMP-3 Gene

A murine gene replacement of TIMP-3 is being undertaken. Two different approaches are being pursued. One vector is being made which will knockout the first exon as well as the transcriptional start site of TIMP-3. This should result in no TIMP-3 being transcribed. The second approach involves a vector which will allow replacement with mutants of TIMP-3. TIMP-3 mutations have been implicated in a congenital disease in humans known as Sorsby's Fundus Dystrophy which can result in blindness. Making similar mutations in the mouse TIMP-3 gene might provide us with a mouse model of this disease.

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Molecular Structural Biology Unit

Dennis Torchia, Chief

MOLECULAR STRUCTURAL BIOLOGY UNIT

The principal research goal of the MSBU is to elucidate the structure and dynamics of proteins, RNAs and associated molecules at the atomic level in order to provide a basis for understanding function. The main research tool used in this work is high resolution multidimensional nuclear magnetic resonance (NMR) spectroscopy. Four projects are currently active (1) HIV-1 protease, free and bound to high affinity protease inhibitors (2) the cell attachment domain of fibronectin, consisting of the 9th and 10th type III fibronectin modules and (3) (a) the RNA recognition domain of ribosomal protein L11, L11-C, free and bound to the conserved 58 base region of the large subunit ribosomal RNA, and (b) the ribosomal binding protein, S4-delta-41. (4) MAP-30, a protein that specifically attacks HIV and tumor transformed cells. Below I summarize the progress that has been made in characterizing the molecular structure and dynamics of these systems as well as the prospective research for the coming year.

HIV-1 protease

Our work has focused upon studies of the protease complexed with two potent inhibitors, (a) DMP323, a member of a novel class of symmetric, specific and potent (K_i ~ 10-1000 pM) inhibitors in which a diol moiety is incorporated into a seven membered cyclic urea ring and (b) KNI272, an asymmetric mono-ol inhibitor having high affinity (K_i ca. 5 pM) and specificity for the protease. The asymmetry of the KNI272 inhibitor breaks the degeneracy of the protease monomer chemical shifts, and has enabled us to obtain monomer specific assignments, which have in turn allowed us to compare the internal dynamics and H/D exchange rates of backbone amides in separate monomers. Differences in monomer flexibility have been observed for three residues in the protease, and these differences in flexibility correlate with differences in structure. Molecular dynamics of a fully active, but stable protease mutant (Q7K, L33I, L63I) have also been studied and reveal that the flaps that cover the active site in the structures of the inhibited protein are highly flexible. In future work, we plan to compare the dynamics of active site side chains in the free and inhibited protein.

Fibronectin cell attachment fragment

Fibronectin is a large multidomain protein with numerous functions. The tenth module contains the RGD cell attachment sequence while the ninth module contains the synergy region. The domain consisting of the ninth and tenth type III modules has full fibronectin binding activity to the specific integrin receptor, $\alpha 5\beta 1$. We have recently attained our goal of determining the three dimensional structure of this two-module domain. Although the structures of the individual modules generally agree with the recently published crystal structure of the human fibronectin 7-10 fragment, there are clear and important differences between the solution and crystals structures. For example the RGD cell binding loop is well ordered in the crystal structure but is highly flexible in solution. Furthermore the 9th and 10th modules form a highly extended and well ordered structure in the crystal, whereas the available NMR data in solution agrees that the two modules form an extended structure, but one that allows significant flexibility in the relative orientation of the two modules. Hence, the RGD and SYNERGY regions are not rigidly positioned in the solution structures. We suggest that relative location of the RGD and SYNERGY regions are reasonably well defined in the cell attachment domain, and this is the

source of binding specificity. On the other hand, our work suggests that the cell attachment domain possesses sufficient flexibility to permit it to interact with integrins of varying structures.

Structures of Protein L11-C76/rRNA complex and the protein S4 delta41

The C-terminal of the RNA binding domain from ribosomal protein L11, L11-C76, recognizes a 58 nucleotide region of the large ribosomal RNA subunit that has been highly conserved during evolution. L11 and its interactions with rRNA are conserved in prokaryotes and eukaryotes and L11 is required for efficient protein synthesis. Following our determination of the three dimensional structure of the free L11 domain, we have now determined the structure of the domain bound to its rRNA target. We find that while most of the structure of the free protein is retained in the bound state, that the large flexible loop in the free protein becomes ordered in the protein-RNA complex. In addition NOESY data show that the loop makes direct contact with the rRNA. Other regions of the protein that interact with the RNA are a loop that bridges helices two and three, and helix three itself. These results provide the first experimental data about protein residues that are involved in rRNA interactions. In order to further characterize these interactions, we aim to solve the structure of the rRNA in the complex. To this end we have worked out methodology for obtaining 15N/13C labeled rRNA, and have prepared labeled samples of both a 12 residue model tetraloop RNA and the 58nt L11-C76 rRNA target. The tetraloop has been used to test multidimensional triple resonance pulse sequences which will be used to assign the signals of the RNA in the L11-C76/rRNA complex. Because of severe signal overlap in the RNA and the large size of the complex, 27kDa, determining the bound RNA structure will be a formidable task, and much effort has been devoted to determining solution conditions where the complex behaves as a stable, monomeric (non-aggregating) molecule. At this point or preliminary data encourage us to believe that it will be possible to determine the structure of the complex.

Binding of the protein S4 to 16S rRNA is critical for the subsequent binding of other proteins and mutations in S4 affect the accuracy of translation. S4 regulates its own translation and that of three other ribosomal proteins by binding to its own messenger RNA. Since deleting the first 41 residues of S4 (yielding S4 delta41) does not affect binding to RNA we have chosen to determine the structure of the truncated protein. We have obtained complete signal assignments and have delineated the secondary structure of the protein. So far, over 1000 interresidue distance restraints have been derived from NOESY data. These restraints together with X-PLOR show that S4 delta41 folds into a compact shape, containing a several long alpha helices and a four-stranded antiparallel beta-sheet. We expect that we will be able to solve its high resolution structure, using spectra separated into four dimensions and 3D spectra acquired at 750 MHz to improve resolution. Based on 15N T2 measurements, S4 delta41 appears to be relatively rigid on the picosecond-to-nanosecond time scale. This may reflect S4's role in ribosome assembly. A relatively rigid S4 may bind to a flexible 16S RNA and force it to adopt a conformation suitable for the subsequent binding of other ribosomal proteins. After the completion of a high resolution structure for the protein, we will probe its interactions with RNA. So far, a small RNA target, suitable for detailed structural studies, has not been identified. However, we may be able to identify residues affected by binding to large RNAs using chemical shift perturbations.

Structure/function study of Map30

Map30 is an anti-HIV and anti-tumor multi-functional protein extracted from the matured fruits and seeds of *Momordica charantia* (bitter melon). In order to understand its functionality at the molecular level, we have undertaken to determine its three dimensional solution structure. Although the size of the protein, 30kDa., makes this a formidable task, we have obtained more than 95% of $^{1}H/^{13}C/^{15}N$ chemical shift assignments, ca. 350 angle restraints and over 1500 NOEs which have enabled us to determine the protein's secondary structure and folding topology. The focus of the work has now shifted to determining additional NOEs in an iterative fashion in conjunction with X-PLOR structure calculations to determine the three dimensional structure of the protein.

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