National Institute of Dental and Craniofacial Research
Division of Intramural Research

Annual Report Summary

2001

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Fiscal Year 2001 Annual Report

National Institute of Dental and Craniofacial Research

Overview

Henning Birkedal-Hansen, Scientific Director

National Institute of Dental and Craniofacial Research
Division of Intramural Research
Overview

The past year witnessed a high level of activity that resulted in many exciting and innovative findings by our scientists. The creativity of the individual scientists and the scientific leadership at the Branch, Section and Unit levels resulted in important new insights and advances at both the conceptual and technological levels. This Annual Report highlights the scientific advances made by the seven Branches and four single-standing units which represent the organizational infrastructure for our 35 independent investigators. DIR scientists published nearly 300 articles in FY2001. A significant proportion of these were published in high-profile biomedical research journals *(Cell, PNAS, Nature Medicine, Nature Genetics, J. Cell Biol, J. Biol Chem. etc)* suggesting that our findings reach a broad range of scientists in many fields both inside and outside the dental, oral and craniofacial research community.

Dr. Lawrence Tabak, the NIDCR Director, took office shortly before the beginning of the fiscal year. He has continually provided strong support and direction to the Division's research program. Dr. Tabak has emphasized the importance of staying close to the Institute's core mission as we plan for future programmatic changes. The Director has met one on one with all independent scientists and attended Branch Chiefs meetings on a regular basis in order to fully understand the depth and breadth of the Division's activities.

Several programmatic changes deserve mention. In August of 2001, the Division of Extramural Research (DER), NIDCR was reorganized. As part of the reorganization, the oral health promotion arm of the former Oral Health Promotion, Risk Factors and Molecular Epidemiology Branch (OHPRFMEB) was moved organizationally from the Division of Intramural Research to the DER's newly created Division of Population and Health Promotion Sciences, under the leadership of Dr. Dushanka Kleinman, Deputy Director, NIDCR. Also as part of the reorganization, Dr. Albert Kingman, NIDCR Chief Statistician and the head of the NIDCR Biostatistics Core, was relocated from the DIR to the DER. Dr. Kingman's biostatistical services remain available to everyone in the Institute including DIR scientists. Another OHPRFMEB investigator, Dr. Deborah Winn, left the DIR to accept a position at the NCI. Her programs are being phased out or assumed in the newly reorganized DER. Two tenure-track investigators, Dr. Ashok Kulkarni and Dr. Nicholas Ryba, were granted tenure following review by the NIDCR Promotion Committee and the NIH Central Tenure Committee. In each case the NIH Central Tenure Committee recognized their outstanding scientific contributions.

The building renovations have accelerated and over a 12-18 month period a new state-of-the-art, expanded animal facility will be created in the basement of Building 30. At the same time, we are pursuing two opportunities for further programmatic expansion, one in the Twinbrook area of Rockville, MD and the other at the National Naval Medical Center. These initiatives may lead to acquisition of additional space in one or both locations within the next two to three years.

During the past year the Craniofacial and Skeletal Developmental Biology Branch was successfully reviewed by the Board of Scientific Counselors (BSC). The BSC noted many strengths in the Branch and were highly complimentary of their scientific programs, including

the clinical initiative. The Board was also highly laudatory of Dr. Robey's leadership. As a consequence, the Branch will continue to receive strong support for its programs. Dr. Francis Macrina rotated off the BSC and the NIDCR is indebted to Dr. Macrina for his energetic and thoughtful leadership as a member and most recently as its Chair. Dr. Macrina will continue to provide guidance and leadership to the Institute as a member of the National Advisory Dental and Craniofacial Research Council (NADCRC). We are indeed fortunate that Dr. Lynda F. Bonewald, University of Missouri, Kansas City, has accepted Dr. Tabak's invitation to serve as Chair of the BSC. Dr. Bonewald brings seasoned scientific leadership qualities in addition to impressive research credentials in skeletal biology.

DIR scientists also were highly active in organizing scientific meetings and workshops, being invited to present at international scientific meetings and giving invited presentations at universities across the country. In addition, our scientists continue to develop an extensive network of national and international collaborative projects with colleagues in the United States and abroad. These activities are detailed annually in a separate document "Interactions with the Scientific Community."

Henning Birkedal-Hansen
Scientific Director

Craniofacial Developmental Biology and Regeneration Branch

Kenneth Yamada
Hynda Kleinman
Yoshihiko Yamada

CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH 2001

The Craniofacial Developmental Biology and Regeneration Branch (CDBRB) focuses on generating new research breakthroughs to (a) understand the mechanisms of normal and abnormal craniofacial development and function at genetic, molecular, and cell biological levels, (b) discover new genes, biologicals, and biomimetics relevant to diagnosis, repair, and therapy, and (c) develop creative, biologically based methods to replace tissues that are defective or damaged. Particular emphasis is placed on the interface between cells and extracellular molecules. Our mission spans the range from basic research to clinical, and from normal development to anomalies, wound healing, cancer, and AIDS. CDBRB researchers are exploring important fundamental questions in developmental biology and related fields, such as the molecular and cell biological mechanisms of morphogenesis, formation and functions of extracellular matrix and its receptors, signaling from the cell surface to the nucleus for novel gene induction, cellular differentiation, wound repair, and cancer cell growth and metastasis. These ongoing basic research innovations will provide the basis for novel translational and patient-oriented applications. This past year, our researchers continued to produce a variety of exciting research advances and to receive international recognition. We also continued to place high priority on training younger scientists to become independent leaders in academia and industry. In addition, we provided extensive service and citizenship activities on behalf of NIDCR, NIH, and our research fields.

Researchers in the CDBRB have made substantial progress and exciting scientific breakthroughs during the past year as reflected in the 54 publications in our annual report bibliography. Several selected research advances are highlighted below. Project reports from each Section provide more comprehensive summaries of the major new findings in our Branch.

CDBRB initiated the Oral and Craniofacial Genome Anatomy Project (OC-GAP) to catalogue genes expressed in oral and craniofacial tissues and to discover novel genes important for tooth, oral, and craniofacial development. Previously, CDBRB researchers discovered ameloblastin, a tooth-specific, developmentally regulated gene associated with enamel formation and linked to the congenital disorder amelogenesis imperfecta. Expression patterns of cDNA clones from mouse tooth germ and craniofacial cDNA libraries have been analyzed using cDNA microarrays. Several new genes have been identified as expressed predominantly in specific developing tissues such as tooth and hair follicles. These genes have been further characterized by expressing them in tissue culture and in mice. In addition, gene expression profiling of multiple different stages of salivary development using microarray and SAGE approaches has identified a variety of additional genes that may be of potential importance in gland development. CDBRB members also serve as Project Officers of a major contract with Washington University, St. Louis, in association with research teams at Johns Hopkins University and the Necker-Enfant Malades Hospital in Paris, to discover and catalogue expression patterns of human craniofacial genes that are active in specific tissues during early development. All clones, cDNA libraries, and antibodies will continue to be made freely available to dental, craniofacial, and other investigators to promote research in the area.

Our knowledge about gene regulation and the extracellular matrix is being used to examine pathology in animal models and in human diseases. For example, the Molecular Biology Section has created gene knockout mice to study the biological roles of perlecan and ameloblastin.

Perlecan-deficient mice showed severe chondrodysplasia, with radiographic and clinical features remarkably similar to a lethal autosomal recessive disorder in humans termed dyssegmental dysplasia, Silverman-Handmaker type (DDSH). In fact, mutations were found in the perlecan gene (*HSPG2*) of patients with DDSH. The mutations cause a frameshift, resulting in a truncated protein core that is not secreted by the patient's chondrocytes but is degraded to smaller fragments within cells. Thus, DDSH is caused by a functional null mutation of perlecan. In contrast, ameloblastin knockout mice showed defective enamel formation, suggesting an important role for ameloblastin in amelogenesis. In addition, transcription factors that regulate cartilage collagen genes have been identified and characterized.

Laminin and laminin peptides have been implicated by CDBRB members in differentiation, tumor growth, and metastasis. Active sites of laminin chains have been identified in a variety of biological processes using synthetic peptide and recombinant protein approaches by the Molecular and Cell Biology Sections. Many of these peptides and molecules have potent cell type-specific effects on cell adhesion, angiogenesis, salivary gland differentiation, and tumor metastasis. For example, one peptide can cause tumor cells to metastasize to the liver and has no effect on angiogenesis, while another peptide increases lung colonization five-fold and stimulates angiogenesis. The receptors for both have been identified: the former is a cell surface heparan sulfate-containing molecule, and the latter is an integrin. These studies may lead to the development of new therapeutic reagents.

The Cell Biology Section has identified potent extracellular regulators of cell migration and tumor metastasis. Ongoing studies on thymosin beta-4 have established roles in endothelial, keratinocyte, and corneal epithelial cell migration. It decreases inflammation and accelerates wound repair in a rat skin model and in the cornea, suggesting its potential for promoting human wound healing. A novel function for osteonectin was identified as supporting breast and prostate cancer cell metastasis to bone and induction of proteases. The cellular receptor has been identified as an integrin, and the amount of this integrin may serve as a diagnostic marker for metastatic breast and prostate cancer.

Molecular responses to extracellular matrix are being characterized in human salivary gland (HSG) cells and fibroblasts by the Cell Biology and Developmental Mechanisms Sections. When cells are placed on extracellular matrix proteins in cell culture, they show dramatic changes in gene expression and protein biosynthesis. More than thirty genes were identified as induced by adhesion of salivary cells to collagen, fibronectin, or basement membrane extract, and many of them are novel. Two classes have been studied in detail. Expression of the metallothionein gene was found to be induced at least six-fold by laminin. When metallothionein expression in salivary gland tumor cells is increased by transfection, the acinar structures formed in vitro are larger, and the tumors formed in vivo are smaller and more differentiated. This gene is likely to have a role in salivary differentiation. FGF receptors are also important in early gland development and may be important in gland regeneration. Regulatory molecules involved in the salivary cell transcriptional response to extracellular matrix were found to include the protein kinase C isoforms gamma and delta. These studies are building the knowledge base necessary to develop creative therapeutic approaches for the repair or replacement of salivary glands and other tissues. For example, CDBRB is collaborating with the Gene Therapy and Therapeutics Branch to develop a first-generation artificial salivary gland.

The Developmental Mechanisms Section has also used fibroblastic cells to characterize the molecular mechanisms of cellular responses to extracellular matrix molecules. Fibroblast proliferation was found to be regulated not only by the MAP kinases, but also by the small GTPase RhoA. A novel regulatory pathway was identified originating with integrins and growth factors on the cell surface leading to RhoA, then to stimulation of cyclin D1 and suppression of p21 in the nucleus. Cooperation between this pathway and the well-known Ras-MAP kinase signaling pathway can explain the potent synergism between fibronectin and growth factors in stimulating cell cycle progression. Interaction of cells with a three-dimensional extracellular matrix provides an even stronger stimulus to cell proliferation, which is stimulated two-fold compared to standard tissue culture substrates. In fact, distinctive effects of three-dimensional matrices on cell adhesion structures, cell attachment, shape, and migration were also identified. The phosphorylation state of cell adhesions could be modified experimentally using a new enzyme delivery system. A novel molecular chimera linking a targeting molecule with the protein kinase Src via leucine zippers induced site-specific modification of focal adhesions. These findings provide insights into mechanisms for regulating and modifying cell adhesion and proliferation that should eventually benefit cell-based tissue engineering.

Besides publishing extensively, our Branch distributes its research materials widely by licensing, donating to repositories, and providing numerous gifts to research colleagues. Products generated by CDBRB members that were licensed by companies included Matrigel, invasion substrates, and monoclonal antibodies against integrins. CDBRB has donated hundreds of cDNA clones to the ATCC and completed about 75 new Material Transfer Agreements with extramural researchers over the past year to provide our reagents. Members of the Branch have also received support from outside organizations. NASA provided funds to study salivary gland cell differentiation in microgravity, and non-NIH salary support for postdoctoral members of CDBRB has come from various sources.

CDBRB members continue to be invited as featured speakers at a variety of international meetings and symposia. Examples from this past fiscal year included two American Association for Cancer Research symposia (H. Kleinman and K. Yamada), 3rd Workshop on Heritable Disorders of Connective Tissues (Y. Yamada), Keystone Symposium on Cell Migration and Invasion (K. Yamada), International Symposium on Orthopedic Surgery in Nagoya, Japan (Y. Yamada), and Gordon Research Conferences. Our members continue to serve on the editorial boards of a number of leading journals. Examples include J. Cell Biology (K. Yamada, Editor, and H. Kleinman, board member); Cancer Research (H. Kleinman, Associate Editor); J. Cellular Physiology (K. Yamada, Editor); Matrix Biology (Y. Yamada and K. Yamada, Associate Editors); J. National Cancer Institute (H. Kleinman, Associate Editor); J. Biological Chemistry (Y. Yamada, board member), J. Cell Science (K. Yamada, board member), and a variety of other editorial boards. Members also served on various review panels, on the Board of the Metastasis Research Society (H. Kleinman), and on the Council of the International Society for Matrix Biology (H. Kleinman and K. Yamada). CDBRB members provide extensive service on more than two dozen NIH and NIDCR committees, including the NIH Senior Biomedical Research Service Policy Board, the NIH Committee on Scientific Conduct and Ethics Committee, and the NIH Diversity Council.

CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH
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Craniofacial Epidemiology and Genetics Branch

Scott Diehl

CRANIOFACIAL EPIDEMIOLOGY AND GENETICS BRANCH
2001

The mission of the Craniofacial Epidemiology and Genetics Branch (CEGB) is focused on using epidemiological strategies and approaches to enhance understanding of the hereditary and environmental causes of dental, oral and craniofacial diseases and disorders. Such knowledge can be used to improve diagnosis, prevention and therapy. Most of the research conducted in the Branch involves clinical research conducted on human subjects. Limited studies are conducted using animal models. Basic research involves the development and evaluation of new laboratory (genomic) assays and statistical methods designed to improve the power and efficiency of these research approaches, followed by application to ongoing studies being conducted in the Branch and elsewhere. Current studies of CEGB staff include large surveys of oral and systemic health based on interviews and examinations, case-control and cohort designs, and recruitment of nuclear and extended families. The focus of these investigations range from assessing health effects of amalgam, characterizing the oral manifestations of HIV and the oral physiology of aging, and improving understanding of the etiology of periodontitis and other causes of tooth loss, oral cancer, nasopharyngeal carcinoma, cleft lip and palate and other craniofacial disorders. Most studies are designed to evaluate both genetic variation and the behavioral and environmental risk factors associated with these diseases. Three Senior Investigators have conducted research in the CEGB of the Division of Intramural Research (DIR), NIDCR during the past year: Scott R. Diehl, Ph.D., Deborah M. Winn, Ph.D., and Albert Kingman, Ph.D. Most of the CEGB staff work at offices and computer facilities located in the Natcher Building on the main NIH campus. In addition, Dr. Diehl's gene mapping laboratory is located a short distance away at the National Naval Dental Center.

Molecular Genetic Epidemiology

The mission of Dr. Diehl's research program is to increase our understanding of the etiology of dental, oral and craniofacial disorders by using the gene mapping strategies of association (disequilibrium) and linkage. Nearly all of his studies involve complex diseases, where multiple susceptibility genes are involved, and where gene-environment interactions are common. Although the majority of his studies utilize molecular assays such as marker polymorphisms or mutation analyses of candidate genes, several projects investigate familial aggregation of diseases and disease-related phenotypes without incorporation of molecular data. Analyses include key behavioral risk factors such as diet, smoking and alcohol consumption. These are treated both as covariates to disease risk and as genetically heritable phenotypes of interest themselves. The gene mapping strategy requires the following components, all of which have been successfully implemented by Dr. Diehl's research team: design and implementation of large scale field studies to obtain biospecimens and risk factor assessments for cases and appropriately matched controls; establishment of a laboratory capable of high-throughput genomic assays for both highly polymorphic DNA markers and candidate gene single nucleotide polymorphisms (SNPs); bioinformatics systems for management of biospecimens, molecular assay data, and clinical and risk factor information; and construction of computer hardware and software systems capable of conducting the thousands of statistical analyses required for genome wide studies by integrating a diverse array of computer programs in a user-friendly, semi-automated environment.

Dr. Diehl has collaborations with clinicians and epidemiologists throughout the world that have led to the successful establishment of several large patient collections for his research. Oral cancer cases and controls have been recruited in Europe, North American and Asia, using both case-control and family-based sampling designs. A gene mapping and risk factor study of nasopharyngeal carcinoma has been implemented in Taiwan, based primarily on multiplex families (families with 2 or more affected members). A large study of syndromic and non-syndromic cleft lip and palate using both simplex and multiplex families has been completed at three clinical sites in the U.S. Studies of oral clefts in humans have been complemented by Quantitative Trait Locus (QTL) analyses of a mouse model of teratogen-induced clefting. New studies of pain using human subjects suffering from phantom limb pain and the autotomy animal model have been initiated in collaboration with scientists at the NIDCR and from Israel. A study of Kartagener syndrome has led to the mapping of a disease gene using families obtained through collaboration in Poland. Kartagener syndrome is a form of primarily ciliary dyskinesia that has chronic sinusitis as a craniofacial manifestation. A whole genome scan and analyses of several candidate genes is being conducted for a study of early onset periodontitis using families collected in the U.S., Chile, and Israel.

Completion of these major, long-term studies, several of which are currently in subject recruitment phases, will be the focus of much of Dr. Diehl's research team's efforts during the next couple of years. Improvements in genomic technologies, especially the exciting developments in the area of single nucleotide polymorphisms (SNPs) can be expected to vastly increase the speed and scope of analyses that will be feasible for gene mapping laboratories. Dr. Diehl is committed to keeping his laboratory at the cutting edge of these new methods. Considering these likely technological advances in laboratory capabilities, ensuring future access to large numbers of biospecimens with high quality risk factor and demographic data will become an even more essential priority if this research program is to remain highly productive. Long term planning is especially important in this field, since it can take many years to establish collaborations, design and carry out large epidemiological studies.

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Craniofacial and Skeletal Diseases Branch

Pamela Gehron Robey
Larry W. Fisher
Marian F. Young

CRANIOFACIAL AND SKELETAL DISEASES BRANCH
2001

We have continued to concentrate our efforts on gaining an in-depth understanding of the skeleton, which we define as bone, cartilage, teeth, and their associated soft tissues. The rationale for this emphasis is based on the critical role that skeletal tissues play, not only in oral health, but also for general health. Genetic and acquired disorders of the skeleton clearly impact on the public at large. For the most part, genetic disorders of the skeleton are currently untreatable. Furthermore, the incidence of acquired disorders is increasing at a rapid rate due to the fact that we are outliving the durability of our skeletons. Our goal is to apply cutting edge basic, translational and clinical research in order to impact current medical practice.

To accomplish this goal, our studies focus on the cells, genes and macromolecules of skeletal tissues in order to elucidate the fundamental mechanisms that govern development, growth and maintenance of the normal skeleton, and to determine the pathogenic mechanisms in genetic and acquired skeletal disorders. These studies are performed by three Principal Investigators who are highly collaborative, not only within the Branch, NIDCR and NIH, but also worldwide. As can be seen from the research summaries, each group is focused on specific aspects of skeletal metabolism that has positioned the Branch to address major physiological questions, to devise new hypotheses, and to test them through in vitro and in vivo analyses, and through clinical observation.

The Skeletal Biology Section, directed by Dr. Pamela Gehron Robey, has focused on stem cells that form hard tissues, in particular, bone marrow stromal stem cells (BMSSCs), which have the ability to form bone, cartilage, hematopoiesis-supportive stroma, associated fat cells, and perhaps other connective tissues as well. As such, they are important mediators of skeletal metabolism in the post-natal organism. The Section has continued to define their biochemical nature by studying the types of integrins that they possess and the matrix molecules that they associate with, and the role that these cell-matrix interactions play in osteogenic differentiation. These studies revealed that at least five different integrins could be detected, and that the $beta_1$-containing integrins are the predominant class on BMSSCs. Furthermore, the cells prefer binding to fibronectin, vitronectin, laminin and type IV collagen as opposed to other matrix molecules that are found in the bone marrow microenvironment. By using specific blocking antibodies, cell-matrix interactions via the $beta_1$-containing integrins were found to be essential for differentiation of BMSSCs into mature bone-forming cells. In other studies, the Section has applied the techniques that they have developed to isolate and characterize stem cells in other tissues. For the first time, it was shown that adult dental pulp contains stem cells that can form a dentin/pulp-like organ upon in vivo transplantation. In addition, adherent cells with the ability to form bone and fat were also identified for the first time in the peripheral blood of different animal species, including humans. These studies indicate that hard tissue adult stem cells are more widely distributed than had been previously imagined. Comparison of these different stem cell populations by immunophenotyping and microarray analysis indicates that they all are amazingly similar, yet able to form such different tissues. Genes that are differentially expressed are currently being further characterized to determine their function within a given stem cell population.

The Skeletal Clinical Studies program, currently under the direction of Dr. Pamela Gehron Robey, has continued to focus on two aspects: the role of BMSSCs in skeletal disease, and the use of BMSSCs for tissue regeneration. Patient accrual is continuing in four clinical protocols (97-DK-0055, 98-D-0145, 98-D-0146, 99-D-003) for the study and treatment of fibrous dysplasia of bone (FD) and the McCune-Albright Syndrome (MAS). FD/MAS is known to arise from a post-zygotic mutation in the GNAS1 gene (R201C and R201H) which regulates adenylyl cyclase and ultimately results in excess cAMP production, which profoundly affects the ability of BMSSCs to differentiate and form bone. FD is found in a broad range of severity, ranging from monostotic (single bone) to polyostotic (many bones) and often in association with the MAS, which in addition to FD has multiple endocrinopathies and skin hyperpigmentation. To date, 87 patients have been enrolled and studied extensively through clinical chemistries and various imaging modalities, and the clinical data is currently being closely analyzed. Of note, it was found that nearly half of the patients are hypophosphatemic, which could contribute significantly to the osteomalacia that we previously reported to be a common feature in FD bone. In addition, virtually all of the patients also exhibited a mild renal tubulopathy that consisted of abnormal vitamin D metabolism, proteinuria and aminoaciduria. Due to the lack of increase in urinary cAMP, which would be expected if renal cells are also mutated, we hypothesize that FD bone secretes a factor(s) that impinges upon renal function in a similar fashion to what is observed in oncogenic osteomalacia, and current studies are testing this hypothesis. Given the fact that mutated BMSSCs are the cause of genetic skeletal dysplasias, they can also be considered the potential cure. The program has continued to optimize the ex vivo expansion conditions of BMSSCs and their transplantation for tissue regeneration. It was determined that if fetal bovine serum is removed from the cells prior to transplantation, as would be required for use in human patients, bone formation was actually increased. Furthermore, it was determined that the size of hydroxyapatite/tricalcium phosphate particles used as a carrier also influences the amount of bone formation. In addition to use in direct orthotopic bone regeneration, it was also determined that the ex vivo expanded cells could be used to generate vascularized bone grafts, a technique that has major utilization in reconstructive surgery. These studies served as the basis for development of pre-clinical trials in a canine calvarial defect model, and for several trial runs on generating cells in the Division of Transfusion Medicine's core facility in the NIH CC. The long-term canine study required by the FDA has recently been completed, and the data is being inserted into an Investigational New Drug (IND) application that will be resubmitted in the coming fiscal year.

The matrix proteins of bones and teeth play key roles in the structure and functions of these tissues. The objective of the Molecular Biology of Bones and Teeth Unit, lead by Dr. Marian F. Young, was to study their function and regulation using a combination of in vitro and in vivo analyses. To determine the function of matrix proteins in vivo, transgenic animals that are deficient in one or more Small Leucine Rich Proteoglycans (SLRPs) were generated. SLRPs are an expanding family of proteoglycans found in the extracellular matrix that contains tandem repeats of a motif rich in leucine. Previously a biglycan deficient mouse (*bgn* KO) was generated and was found to fail to develop peak bone mass and developed osteoporosis. A dramatic age-dependent decrease in osteogenic precursors was observed in the *bgn* KO animals compared to normal littermates, and once these precursors matured into osteoblasts, a decrease in expression of bone matrix proteins was also noted, providing a plausible cellular mechanism leading to osteoporosis. Because there may be functional redundancy between different SLRPs

that are co-expressed in bone and cartilage, another transgenic animal was also developed that is deficient in both fibromodulin (another SLRP) as well as biglycan (*bgn/fbn* DKO). Abnormal collagen fibrils have been observed in both *bgn* KO mice and *fbn* KO mice. *Bgn/fbn* DKO mice are fertile and viable. But compared to age-matched *wt* and single knockout animals, one-month old *bgn/fbn* DKO mice exhibited a decreased flexibility of their knee and ankle joints, thereby impairing their gait. Radiographs and histological analysis demonstrated that sesamoid bones form within tendons, and also revealed osteoarthritis in the medial compartment of the knee by three months of age. Preliminary ultrastructural analysis of the patella tendon confirmed changes in collagen fibril size and morphology. It is theorized that abnormal collagen fibrils result in biomechanical alterations of the tendons in the *bgn/fbn* DKO mice, and are the common origin of the gait impairment, ectopic ossification and premature osteoarthritis noted in these mice. Future analysis will aim at providing a molecular mechanism to explain the gait problems, tendon alterations and osteoarthritis observed in the absence of *bgn* and *fbn* by biomechanical analysis, in vitro cell-based studies, and laser capture microdissection along with mouse specific custom microarrays to assess patterns of gene expression.

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 Matrix Biochemistry Unit, headed by Dr. Larry W. Fisher, has been performing a variety of experiments and collaborations to help determine the structure-function relationship of several of the more interesting non-collagenous proteins. During the last year, the Unit has focused predominantly on the structure/function relationships of the family of human chromosome 4-linked proteins that Dr. Fisher has named SIBLINGs (Small Integrin-Binding LIgand, N-linked Glycoproteins). Using a replication-deficient adenovirus system, milligram quantities of normal and mutant bone sialoprotein (BSP), dentin matrix protein 1 (DMP1) and osteopontin (OPN) have been generated in BMSSCs. The fluid phase NMR structures of the recombinant BSP and OPN were shown to be flexible throughout their entire length by our NIDCR colleague, Dr. Dennis Torchia. Furthermore, two biological functions for at least three SIBLING members were uncovered. First, BSP, OPN and DMP1 all rapidly form strong (nM), 1:1 complexes with complement Factor H. When the SIBLING first binds to either the vitronectin receptor (all three) or CD44 (DMP1 and OPN) on cell surfaces and then binds Factor H, the cell is protected from lysis by complement. Blocking the action of Factor I with an antibody destroys this protective property. It was also found that the SIBLING-Factor H complex in serum must be disrupted before the SIBLING can be properly quantified. The NIH has patented the assay using this approach for the detection of BSP, OPN and other SIBLINGs made by tumors in cancer patients. In addition, the Unit has developed several novel approaches to identifying and quantifying the somatic mutations in biopsies, cultured cells and paraffin sections in support of the clinical program studying the FD/MAS disorder.

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Gene Therapy and Therapeutics Branch

Bruce Baum
Indu S. Ambudkar
John Chiorini
R. James Turner

GENE THERAPY AND THERAPEUTICS BRANCH
2001

The Gene Therapy and Therapeutics Branch (GTTB) is a model of translational research, providing a bench to clinic continuum. The GTTB has both a primary tissue-specific focus, asking questions related to salivary gland biology, pathology and management, as well as a primary applications focus, gene transfer technology. The GTTB remains committed to the notion that significant advances in clinical care will come from our understanding of biological mechanisms and our inter-disciplinary approach. This reporting period has seen substantial scientific progress.

The production of salivary fluid is due to neurotransmitter stimulation of transepithelial secretion of Cl^- by acinar cells. A Na^+-K^+-$2Cl^-$ cotransporter, NKCC1, located in the acinar basolateral membrane drives much of this Cl^- flux. This transporter is the rate-limiting step in salivary secretion. The Membrane Biology Section (MBS) continues to concentrate on understanding the structure, function and regulation of this important transport protein. The NKCC1 molecule consists of large cytosolic N and C termini (35 kDa and 50 kDa, respectively) on either side of central hydrophobic domain (50 kDa) containing 12 membrane-spanning segments. The latter contains the regions primarily responsible for ion transport and substrate affinity, while the cytosolic N and C termini are primarily involved in transport regulation. The MBS hypothesized that regulatory changes in the NKCC1 termini (e.g., phosphorylation) must be transmitted to the membrane spanning domain through some sort of inter-molecular interaction. This year, using the yeast two-hybrid-system, the MBS generated evidence for interactions of the N terminus with itself, and with the C terminus, as well as several possible interactions between the termini and intracellular loops.

To identify and characterize the functional regions of NKCC1 the MBS has used multiple experimental approaches. Recently, using cysteine scanning mutagenesis, a NKCC1 mutant (A483C) has been generated that is particularly interesting since it renders NKCC1 sensitive to inhibition by several sulfhydryl reagents that have no effect on the wild type transporter. This residue (A483) is located in membrane spanning region 6, a highly conserved portion of the protein that previous MBS studies showed was critical for function. To determine the precise location, as well as functional properties, of membrane spanning segments in NKCC1 the MBS has used a chimeric vector to test for signal anchor or stop transfer activity. This vector contains multiple glycosylation sites appended onto the C terminal end of putative NKCC1 membrane spanning sequences being tested. Since the glycosidation of this peptide could be readily detected by a significant shift in the apparent molecular weight of the resulting recombinant protein, the integration and orientation of the putative membrane spanning segments can be easily determined. This method can be used in an intact cell system and is presently being applied to further explore the membrane integration of NKCC1 as well as that of the water channel AQP1, whose integration into the membrane during protein synthesis is controversial.

Neurotransmitter stimulation of sustained salivary fluid secretion depends on the activation of store-operated Ca^{2+} entry (SOCE). The molecular mechanism involved in and mediating this process is unknown for all non-excitable cell types. GTTB's Secretory Physiology Section (SPS) is focused on understanding such Ca^{2+} influx using salivary gland cells as models. SPS scientists

continue to make exceptional progress on their studies of the transient receptor potential *(trp)* gene family as possibly encoding the store-operated Ca^{2+} influx channel (SOCC). For example, Ca^{2+} influx through Trp3 channels is supposedly regulated by a secretion-like coupling mechanism involving a reversible interaction between the inositol trisphosphate receptor (IP_3R) and Trp3 that is enabled by trafficking of the endoplasmic reticulum to or away from the plasma membrane. The SPS used a number of agents to probe the effect of cytoskeletal reorganization on the localization and function of Trp3 and key Ca^{2+} signaling proteins. For example, calyculin A, which purportedly disrupts Trp3-IP_3R interactions, induces formation of a condensed actin layer at the plasma membrane and the internalization of Trp3, the $G\alpha_{q/11}$subunit, phospholipase C (PLC) β, and caveolin-1. Jasplakinolide, an actin-stabilizing agent, also induced internalization of Trp3 and caveolin-1. From such studies it appears that Trp3 is assembled in a caveolar Ca^{2+} signaling complex with IP_3R, the Ca^{2+} pump from the endoplasmic reticulum (ER), $G\alpha_{q/11}$, PLCβ, caveolin-1, and ezrin. Conditions that result in stabilization of actin diminish Trp3 activation due to internalization of the Trp3-signaling complex, and do not disrupt IP_3R-Trp3 interactions. These data suggest that localization of the Trp3-associated signaling complex depends on the status of the actin cytoskeleton.

The SPS previously showed that expression of a truncated form of hTrp1Δ (with a deletion of the C terminus, encompassing amino acids 664-793) increased thapsigargin- and carbachol-activated Ca^{2+} influx via the SOCE. The Trp1 C-terminus mediates a Ca^{2+}-dependent feedback inhibition of SOCE, and this truncation attenuates the inhibition. Additionally, full-length Trp1, but not the truncated hTrp1, is able to bind calmodulin in a Ca^{2+}-dependent manner. This finding led the SPS to propose that calmodulin binding to the Trp1 C-terminus acts as a Ca^{2+} sensor for the regulation of SOCE. The SPS has also shown that SOCC activity is tightly regulated by $[Ca^{2+}]$ in the cytosol and in the ER, i.e by IP_3R and ER Ca^{2+} pump activity. Very recent studies examining single channel currents induced by the expression of Trp1 in HSG cells demonstrate that Trp1-induced channels have Ca^{2+} and Na^{+} conductances quite similar to the endogenous SOCC, supporting previous SPS evidence that Trp1 is a component of the SOCC in these cells.

The newly established AAV Biology Unit (AAVBU) has made remarkable progress. The focus of this group's activity is on understanding the interactions of adeno-associated virus (AAV) with its host cell. As a helper-dependent parvovirus, AAV requires a considerable degree of host cell support for transduction and life cycle events, much of which is poorly understood. The AAVBU staff has cloned two novel AAV serotypes (AAV4 and AAV5) and shown in vitro and in vivo that these vectors may be useful to mediate gene transfer to cells and tissues poorly transduced by the commonly utilized serotype 2 AAV vectors. For example, a recombinant serotype 5 vector is ~50 fold more efficient in mediating gene transfer to cultured human airway epithelia than an AAV2 vector. These new viral isolates are being studied both as natural mutations of other serotypes for understanding the biology of this genus of virus and because of their unique cell tropism, as novel vectors for gene transfer. While heparin sulfate proteoglycans are important in AAV2 binding and transduction, AAV4 and AAV5 interact with the cell surface by a distinct mechanism. AAVBU studies indicate these latter serotypes bind to the cell surface via another charged carbohydrate, sialic acid. Treatment of cells with neuraminidase, competition with soluble sialic acid conjugates or resialyation experiments with specific sialyltransferases all indicate that serotypes 4 and 5 both require 2-3 sialic acid linkages for

efficient binding and transduction. However, and in keeping with their different cell tropism, AAV4 requires an O-linked 2-3 sialic acid while AAV5 requires a 2-3 N-linked sialic acid.

The Gene Transfer Section (GTS) studies clinically relevant applications of gene transfer to salivary glands. Historically, much of this work has employed replication-deficient recombinant adenoviral vectors. These vectors are useful for establishing proofs of clinical or biological principle. However, adenoviral-mediated transgene expression is transient and vectors elicit both direct cytotoxicity and a potent immune response. Therefore, we have continued to expand our efforts to develop alternative means of transferring genes to salivary glands.

For example, during this reporting period we considerably extended our work with recombinant AAV serotype 2 (rAAV2) vectors. Last year we reported the construction of a rAAV2 encoding human (h) interleukin (IL)-10, rAAVhIL10. During this reporting period we examined the biological efficacy of the vector-directed hIL-10 *in vivo* by employing a stringent test, using IL-10 knockout (KO) mice, which are exquisitely sensitive to lipopolysaccharide, developing endotoxic shock. Administration of relatively modest levels of rAAVhIL10 (10^{10} genomes) to IL-10 KO mice resulted in stable hIL-10 secretion into the bloodstream, which at 8 weeks gave median serum levels of 0.5-1.0 pg/ml. Acute endotoxic shock led to a 33% mortality rate, and severe morbidity, in untreated IL-10 KO mice, while no mortality and little morbidity were seen in IL-10 KO mice administered rAAVhIL10 seven weeks earlier. This vector, thus, maybe useful for *in vivo* applications requiring sustained IL-10 expression, such as we suggest for the management of Sjögren's syndrome (SS). Additionally, we continued our efforts towards the practical application of the hybrid adeno-retroviral vector that we first described two years ago. In particular, we focused on addressing a key question about this vector: is the long-lived transgene product expression observed with AdLTR-luc derived from the integrated transgene or from transgene present in surviving, episomally localized vector? We studied this question *in vitro* using two different experimental approaches. Both types of experiments strongly supported the conclusion that following infection of epithelial cells with AdLTR-luc, long-lived luciferase expression is derived from the integrated transgene.

Over the last three years we have also tried to address the particularly severe salivary hypofunction suffered by patients who lack any parenchymal cells, and thus are not candidates for gene therapy. For these patients we have worked to develop an "artificial salivary gland" that could be implanted intraorally. This device has been designed to have a biodegradable substratum, a luminal coating of an extracellular matrix protein, and a monolayer of epithelial cells. In the present reporting period we examined the tissue compatibility of two biodegradable substrata potentially useful in fabricating such a device, poly-L-lactic acid (PLLA) and poly-glycolic acid coated with PLLA, subjacent to oral mucosa. We also examined the optimal cell organizational behavior for this device in order to secrete fluid, i.e., the cells must be polarized, displaying tight junctions and being capable of generating osmotic gradients.

Our Sjögren's Syndrome Clinic conducts clinical studies relevant to this condition. We are testing the general hypothesis that immunomodulatory treatments may favorably alter the course of SS. This year in a placebo-controlled, randomized, clinical trial (RCT) of hydroxychloroquine we found improvement in stimulated salivary flow and several clinical laboratory measures. This suggests that salivary flow in SS indeed may be improved by immunomodulatory therapy.

A placebo-controlled RCT of thalidomide (as a TNF-α inhibitor), begun during the previous reporting period, was stopped this year because we observed excessive adverse effects, including sedation, postural hypotension, diffuse peripheral edema, peripheral neuropathy, and skin rashes, in the initial group of patients studied. Currently, a relatively new biological reagent, etanercept, also an inhibitor of TNF-α action, is being tested in a RCT in SS patients, while another RCT of dehydroepiandrosterone (DHEA), based on hypothesis that sex hormones exert immumomodulatory effects, is nearing completion.

GENE THERAPY AND THERAPEUTICS BRANCH
2001 BIBLIOGRAPHY

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Oral Infection and Immunity Branch

Sharon M. Wahl
John Cisar
Jerry Keith
Paul Kolenbrander
Steve Leppla
Abner Notkins
Nick Ryba
Reuben Siraganian
John Thompson

ORAL INFECTION AND IMMUNITY BRANCH
2001

The 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, immune and sensory responses provide the basis for dissecting the interactions between pathogens, noxious agents and the host defense system. These multifaceted approaches define fundamental mechanisms of host defense, how these pathways become dysregulated in disease, and how to intervene for the benefit of the host.

The Oral Infection and Immunity Branch (OIIB) continues to generate diverse and exciting advances in our research portfolio. The basic research programs within the Branch provide discoveries and innovative approaches for translational and clinical directions. During the past year, Senior Investigators in the Branch have received recognition for their innovative research and their commitment to sharing their discoveries, transgenic animals and novel reagents with the extramural community. The Branch initiated two new opportunities for interactions with our extramural colleagues: the OIIB Distinguished Speaker Series in which internationally recognized extramural investigators are invited to the NIDCR to lecture and meet with members of the Branch, and the Distinguished Alumni Award in recognition of successful Branch alumni whose careers serve as role models for our Fellows. Our inaugural awardee, hosted by the Fellows, was Dr. Edward Janoff, Professor of Medicine at the University of Minnesota School of Medicine, who presented a seminar on his acclaimed research and then met with the Fellows to provide insight and advice on career opportunities after NIDCR. In turn, recognition of the accomplishments of OIIB Senior Investigators was evident by their numerous invitations to organize, chair and speak at national and international meetings, opportunities to participate on editorial boards, invitations to edit books and write authoritative chapters, and to serve as officers in scientific societies. Within the Branch, OIIB Employee of the Quarter Awards also recognized special achievements. Mentoring young investigators continues to be a major emphasis of the Branch which includes our joint training programs with Children's National Medical for Infectious Disease Fellows and with the Department of Periodontology at the University of Maryland for Periodontal Disease Fellows.

Within the Branch, there have been stellar accomplishments and remarkable progress in many of the multiple unique and diverse research programs during the past year through both independent and collaborative endeavors. Important new findings and scientific breakthroughs, including those featured in prestigious journals such as *Cell*, *Immunity*, *Proceedings of the National Academy of Sciences*, *Nature Medicine*, *Journal of Experimental Medicine and Journal of Biological Chemistry* are highlighted in this Annual Report. In a stunning new breakthrough featured on the cover of *Cell*, members of the OIIB Taste and Smell Unit identified the mammalian sweet taste receptor, a G protein coupled receptor, T1R3 encoded at the *Sac* locus. In compelling studies, expression of the *Sac* gene in a nontaster transgenic mouse strain transformed the sweet-insensitive animals to tasters. Moreover, heterologous expression of T1R3 in combination with T1R2 resulted in a functional sweet taste receptor confirming that

T1R3 is an essential component of sweet taste transduction through a heteromultimer complex, unique amongst chemoreceptors.

In addition to mammals, microorganisms also use dietary sugars as a source of nutrients. Sucrose is the precursor for glycan synthesis that facilitates attachment of *S. mutans* to the tooth surface, and subsequent fermentation of the disaccharide to lactic acid initiates demineralization of tooth enamel. In this context, the five isomers of sucrose may serve as potential substitutes for dietary sucrose because they are less sweet, are not metabolized by *S. mutans* and thus, noncariogenic. Although *S. mutans* fails to utilize the sucrose isomers, recent discoveries in OIIB document that certain bacteria can indeed metabolize these isomers and through sophisticated techniques, the enzymes responsible for hydrolysis have now been identified. Moreover, the genes encoding sucrose-6-phosphate hydrolase and phospho-alpha-glucosidase have been cloned and sequenced from *K. pneumoniae*, but homologous genes have not been detected in *S. mutans*, consistent with the failure of *S. mutans* to utilize the five sucrose isomers. These novel observations may pave the way towards selective targeting of pathogenic microbes.

Excess sucrose not only represents a pathway to caries, but its inappropriate metabolism in humans plays a role in diabetic pathogenesis. In new studies to define the underlying autoimmune mechanisms of diabetes, transgenic mice were utilized to define epitopes on IA-2, a transmembrane protein tyrosine phosphatase-like molecule which is a major autoantigen in type 1 diabetes, and a widely used diagnostic and predictive marker of disease. In these studies, humanized HLA transgenic mice were generated in which mouse class II was genetically inactivated (knockout) and then replaced with human HLA DQ8 which is frequently present in diabetic patients. Immunization of the mice with IA-2 peptides resulted in identification of five DQ8 specific IA-2 peptides, which could stimulate T cell-mediated immunity and may be vaccine candidates to induce immunological tolerance.

Innate and adaptive immune responses to antigens and infectious microorganisms are essential to host defense, and clearance of the infectious agent is typically associated with apoptosis of activated T cells, resolution of the response, and return to homeostasis. Deletion of antigen-specific T cells is also fundamental to immune tolerance and/or unresponsiveness. In striking new studies, TGF-beta, a potent immunoregulatory molecule, was found to control the life and death decisions of T lymphocytes. Both thymic and peripheral T cell apoptosis was increased in mice null for TGF-beta compared to wildtype littermates. In an unexpected finding, TGF-beta was localized to mitochondria within wildtype T cells and the absence of TGF-beta resulted in disruption of mitochondrial membrane potential, which marks the point of no return in a cell condemned to die. Even more surprisingly, this TGF-beta dependent viability appeared dissociable from the TGF-beta receptor-Smad3 signaling pathway. Not only do these novel findings have importance in understanding T cell selection and tolerance, but also for defining mechanisms of programmed cell death in general.

Immune and other mammalian cells are also killed via bacterial toxins. *B. anthracis* produces a tripartite anthrax toxin, the components of which function coordinately to bind cell receptors, enter cytoplasmic vesicles, and escape to the cytosol to alter cAMP and cleave MAP kinase kinases. The unique ability of the protective antigen (PA) to bind to cell surface receptors where it is cleaved by the surface protease furin can be exploited as a targeting device for cell

specificity. In this regard, replacing the furin site with sequences cleaved by tumor associated plasminogen activator protease generates a mutant toxin which becomes specific for tumor cells, is ineffective in plasminogen activator deficient transgenic mice, and may provide a powerful anti-tumor modality. Additional studies have focused on further identification of virulence genes and on development of PA as a vaccine candidate. To this end, PA has been successfully expressed in transgenic tomato plants towards the goal of developing vaccine antigens for anthrax and as a model for other human infectious diseases.

Key to multiple inflammatory and immunological reactions are mast cells, which release an array of initiating and amplifying mediators. In order to dissect the cellular signaling events leading to mediator release, OIIB scientists have isolated mast cell variants that are defective in signaling as a crucial approach to unraveling signal transduction pathways. Crosslinking of the IgE receptor on one of these variants stimulated tyrosine phosphorylation without a sustained calcium influx implicating a defect upstream of phospholipase C gamma. Correction of the defect by Rho family members Cdc42 or Rac1 GTPase rescued the degranulation response demonstrating not only their essentiality but also their potential as interventional targets.

Microbe-microbe and microbe-host interactions and their consequences continue to be a research focus for members of OIIB. During the past year, investigators have cloned and characterized the *S. gordonii* gene (*hsa*) encoding the streptococcal surface component, Hs antigen, which binds to sialic acid termini of host glycoconjugate receptors. This is the first identification of a sialic acid binding adhesin from a viridans group streptococcus and is structurally predicted to consist of a receptor-binding domain attached to the cell surface by a molecular stalk. Insertional inactivation or deletion abolished Hs immunoreactivity. Both immunoreactivity and function could be restored by *hsa* plasmid expression in the mutant. Beyond its interaction with host cells, *S. gordonii* readily binds to enamel and grows independently and/or co-aggregates utilizing SspA and SspB binding proteins with other early colonizers. These other early colonizers, *S. oralis* and *A. naeslundii*, however, are dependent upon mutualistic interactions with one another to establish adherence and colonization, and as the underlying molecular adhesion mechanisms are defined, disruption of biofilm formation will be achievable.

ORAL INFECTION AND IMMUNITY BRANCH
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Oral and Pharyngeal Cancer Branch

J. Silvio Gutkind
Thomas Bugge
Myung Hee Park
Frank A. Robey
Adrian Senderowicz

ORAL AND PHARYNGEAL CANCER BRANCH

2001

Cancer of the head and neck area is the sixth most common neoplastic disease in the developed world, representing a very serious public health problem based on annual morbidity and mortality rates. The molecular and etiological factors involved in the development of head and neck tumors, including oral cancers, are still largely unknown. Members of our Branch work on complementary basic, translational, and clinical aspects of cancer research, in an effort to understand the molecular basis for malignant transformation as well as tumor invasion and metastasis, and to use this knowledge for the development of molecular markers of disease progression and novel therapeutic approaches for oral malignancies.

During the current reporting period, we have expanded the breadth and depth of our program by making a concerted effort in areas of direct relevance to head and neck cancer research. This includes the expansion of Dr. Adrian Senderowicz' program, whose recently established unit focuses on the development of novel "mechanism-based" therapeutic approaches for head and neck cancer patients, and the continuing efforts of Dr. Myung Hee Park, Dr. Frank Robey, Dr. Thomas Bugge, and Dr. J. Silvio Gutkind's teams, whose research programs address basic mechanisms involved in squamous carcinogenesis and tumor metastasis. These directions are expected to provide a better understanding of the still unknown molecular alterations that lead to oral malignancies, thus broadening the horizon on developing potential oral tumor markers and treatments modalities.

Our Branch has been highly productive during this year, and has made substantial contributions to the field, providing new concepts and shedding new light on questions of fundamental importance for cancer biology. We have also developed a large number of novel reagents, such as new genes, expression vectors, cell lines, and bioactive peptides of value to biomedical research, and provided them to hundreds of investigators in the U.S. and abroad. Another tradition of our Branch has been our high priority on the training of postdoctoral investigators to become independent leaders in the field; we have tried to continue and strengthen this commitment.

During the current reporting period, significant progress has been made in a number of research efforts at the OPCB. A variety of arbitrarily selected research advances are highlighted below. The progress report for each project provides a more comprehensive description of the major findings in our Branch.

We continued to make a concerted effort to apply state-of-the-art genomic approaches to investigate the molecular basis of oral cancer. Based on our successful use of laser capture microscopy (LCM) to procure specific cell populations from clinical samples from patients with head and neck squamous cell carcinomas (HNSCC), we have constructed a set of HNSCC-specific cDNA libraries, which have now been entered into the public collections of the Cancer Genome Anatomy Project (CGAP). These libraries were examined using recently developed bioinformatic tools. A total of 1080 genes were found in the tissues, of which 168 were previously unknown human genes. In order to examine which of these genes are frequently expressed in oral cancer, their DNAs were used to construct an oral-cancer-specific microarray,

to which was hybridized cDNA from 5 sets of oral cancers. In a study in process of submission, we found that a number of these newly discovered genes were highly expressed in these cancer lesions. Their putative role in the pathogenesis of HNSCC and/or their use as markers for early detection or as targets for pharmacological intervention in this disease can now begin to be evaluated. We have also made an effort to investigate protein expression profiles during HNSCC tumor development. In collaboration with the NCI, we have demonstrated the feasibility of utilizing antibody arrays for the study of protein expression in LCM-procured material from HNSCC. This work is expected to help identify gene products involved in the neoplastic process, as well as novel molecules representing clinically useful markers of oral carcinogenesis.

In an effort to understand the molecular and genetic mechanisms involved in oral carcinogenesis, we have characterized gene expression profiles in NHGK (normal human gingival keratinocytes), NHEK (normal human epidermal keratinocytes), IHGK (immortalized NHGK), HSG (human salivary gland line), SGT (human salivary gland carcinoma line) and several HNSCC's, using cDNA microarrays (NCI-OncoChip) containing 6720 sequence-verified cDNA elements representing 4900 independent genes. NHGK vs. NHEK microarray analysis indicates a high similarity between the gene expression patterns of the oral and the skin keratinocytes. Only 2% of the genes on the chip showed greater than two-fold difference in expression. Among 13 cytokeratins, only cytokeratin 19 showed a 2.5-fold overexpression in NHGK. IHGK vs NHGK microarray analysis showed an up-regulation of a number of growth factor/protein kinase/cell cycle regulators, in IHGK. SGT vs. HSG microarray analysis indicated up-regulation of several growth factors, cell cycle regulators, oncogenes, and cell surface adhesion molecules including FGFR2, CDC42, cyclin G2, Lyn, Jun, Met, syndecan-1, integrin A9, integrin alpha3, and laminin B1 in a salivary carcinoma cell line. Gene expression pattern of 11 HNSCC cell lines were analyzed with respect to a reference human cancer RNA pool. A number of genes were found to be overexpressed in most HNSCCs. The hierarchical clustering analysis of gene expression profile suggests two subgroups, group one consisting of HN4, HN12, HN31, HN30, HN6, HN17 and HN26 and group two consisting of HN22, HN13, HN8 and HN19. Group one overexpressed cysteine proteinase cathepsins L, cystatin A, urokinase-plasminogen activator, TIMP-3 and MMP14 suggesting that this group might be more metastatic than group two. Further analysis is needed to correlate the gene expression data with their phenotypes of cell lines, metastasic potential, clinical tumor stage, and the survival of the patients from whom these cancer cell lines were originated.

We have expanded our drug evaluation effort at the NIDCR, whose goal is to develop novel therapies aimed at improving the quality of life and life expectancy of oral cancer patients. One drug candidate, flavopiridol, which is a novel cdk inhibitor, has displayed potent antiproliferative activity in *in vitro* and *in vivo* models of HNSCC. Initial studies from our lab demonstrated that flavopiridol could induce cell cycle arrest, apoptosis, and differentiation. The basic mechanisms by which flavopiridol exerts these remarkable biological effects were investigated. Furthermore, the first Phase I trial of bolus flavopiridol in cancer patients was recently completed in collaboration with the NCI. Based on the encouraging results of this trial, we opened a protocol entitled "Phase II trial of daily bolus flavopiridol for five consecutive days in patients with recurrent/metastatic squamous cell carcinoma of the Head and Neck (SCCHN)" in collaboration with NCI and NIDCD. Thus far, we have treated one patient. Parallel basic, translational and clinical efforts have been also made using UCN-01, a novel protein kinase C (PKC) inhibitor, and perifosine, a novel oral alkylphospholipid that modulates signal transduction pathways.

Both pharmacological agents display antitumor in *in vitro* and *in vivo* models of HNSCC. A new clinical/translational program at the NIDCR for the diagnosis, prognosis and treatment of premalignant oral lesions (leukoplakia), is currently under implementation.

Proteolytic modification of the extracellular matrix is essential for physiologic tissue remodeling, as well as for the progression of a number of chronic, degenerative diseases including cancer invasion, and metastasis. We have addressed the biochemistry, biology, and pathology of selected matrix degrading serine proteases, with particular interest in the plasminogen activation system. The urokinase plasminogen activator receptor (uPAR) is a high affinity cell-surface receptor for the urokinase plasminogen activator (uPA). uPAR focuses uPA-mediated plasminogen activation to the cell surface, and may also have additional non-proteolytic functions including cell adhesion, migration, uPA internalization, and signal transduction. We have focused our work on an uPAR-associated protein (uPARAP), which is a novel transmembrane glycoprotein that associates with ligand-bound uPAR, collagen-V, and possibly, matrix metalloprotease (MMP)-13. Of interest, like uPAR and MMP-13, uPARAP was overexpressed by transformed keratinocytes in squamous carcinoma of the skin. To directly explore the function of uPARAP in development, wound repair, and cancer, we have generated loss of function and gain of function mutations in mice, whose phenotype is under current investigation. In a parallel effort, we have taken advantage that uPAR and uPA are highly overexpressed in most human tumors, including HNSCC, to generate tumor cell-selective cytotoxins in collaboration with the Oral Infection and Immunity Branch. In particular, anthrax toxin-specificity was redirected by replacing the furin activation sequence of the toxin by a peptide sequence susceptible to specific cleavage by uPA. The potential use of this re-engineered anthrax toxin for *in vivo* imaging of cell surface protease activity, dissection of complex proteolytic activation pathways, monitoring the efficacy of protease inhibitors, and the selective ablation of tumor cells and activated inflammatory cells is under evaluation.

Receptors exhibiting an intrinsic tyrosine kinase (RTKs) activity or that are coupled to heterotrimeric G proteins (GPCRs) can effectively stimulate growth promoting pathways in a large variety of cell types and, if persistently activated, these receptors can also behave as dominant-acting oncoproteins. We have investigated the nature of the mitogenic and transforming pathways elicited by RTKs and GPCRs as an experimental system for uncovering novel biochemical routes participating in the transduction of proliferative signals. In this regard, expression of c-*myc* nuclear proto-oncogene is believed to play a central role controlling the expression and activity of cell cycle regulating molecules, and deregulation of c-*myc* function is often associated with neoplastic conversion. However, how growth factor receptors control c-*myc* expression has remained elusive. In a recent study, we have provided evidence of the existence of a novel signaling pathway by which RTKs stimulate c-*myc*. This biochemical route involves the phosphorylation of the Vav2 Rac-guanine nucleotide exchange factor (GEF) through Src, and the consequent activation of a Rac-dependent pathway that culminates with the nuclear expression of the c-*myc* proto-oncogene. In a parallel effort, we have also investigated how Rho GTPases exert their growth promoting effects. We found that Rho can potently stimulate the expression of the c-*jun* proto-oncogene, a finding that led to the discovery of a novel biochemical route linking Rho to the nucleus through the activation of a p38 isoform and the consequent stimulation of transcription from the c-*jun* promoter through the activation of transcription factors acting on distinct responsive elements.

ORAL AND PHARYNGEAL CANCER BRANCH

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Pain and Neurosensory Mechanisms Branch

Raymond Dionne
Richard Gracely
Michael Iadarola
Mitchell Max
M.A. Ruda

PAIN AND NEUROSENSORY MECHANISMS BRANCH
2001

The Pain and Neurosensory Mechanisms Branch (PNMB) conducts a multidisciplinary research program aimed at improved understanding and treatment of pain. Studies range from basic molecular and physiologic processes of nociceptive transmission, responses to tissue injury and peripheral inflammation (including subsequent plastic 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, translational and clinical research which permits not only a rapid transfer of new findings from the laboratory to the clinic, but also fosters basic research 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 contribute nationally and internationally to the transfer of emerging scientific information to the training of clinicians and the treatment of patients by speaking, writing, and collaborations with professional organizations, academic institutions, and patient advocacy groups.

The independence and challenges presented by the NIH-wide emphasis on PI-initiated research has increased scientific vigor and productivity in the Branch. 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 and Dionne. Research conducted in this clinic is often based on observations made in the Branch's basic laboratories as well as using novel and prototypic drugs to test emerging scientific hypotheses in man, representing a true 'molecules to man' continuum. Dr. Iadarola was awarded a Bench to Bedside award again this year to support preclinical safety studies of a cell deletion approach for treatment of intractable pain in humans to be conducted in collaboration with the Pain and Palliative Care Service. Drew Mannes joined the staff of the Department of Anesthesia and the PNMB and will be serving as principal investigator on this study as well as a gene transfer study that should be started during the next year. Dr. Gracely conducts collaborative research with The Chronic Pain and Fatigue and Research Center at Georgetown University using functional MRI to evaluate basic pain mechanisms in patients with fibromyalgia. Dr. Dionne received a Distinguished Scientist Award at the annual meeting of the International Association of Dental Research in June 2001. Publications over the past year in respected peer-reviewed scientific journals attest to the Branch's continued scientific impact. Highlights of research findings by Branch investigators during the past year are presented below.

Neuronal plasticity and altered gene expression for persistent pain states: Scientists in the Neuronal Gene Expression Unit (NGEU) have established a gene discovery program to better understand the molecular mechanisms underlying the transition to pain chronicity. A library of dorsal (sensory) versus ventral (motor) genes in the spinal cord after peripheral inflammation has been generated by subtraction cloning, sequencing and differential hybridization. This has yielded a set of genes whose expression is enriched for second order neurons involved in sensory and pain processing. A new gene that is up-regulated under these conditions codes for a secreted cysteine proteinase called cystatin C which was demonstrated to be elevated in the CSF of

females undergoing an emergency caesarian section after hours of labor. This finding illustrates the ability to discover new molecules or patterns of molecules secreted in humans in response to persistent pain based on molecular-genetic research in the laboratory, possibly leading to novel therapeutic targets for pain.

Blocking pain transduction at the vanilloid receptor: One of the most important molecules in pain transduction, the first step in pain sensation, is the vanilloid receptor 1 (VR1) which transduces the physical (heat) and chemical activation of receptors/ion channels in nociceptive primary afferent nerve endings located on skin and in deep tissues. In response to activation, nociceptors often release inflammatory substances that further enhance the pain signal, leading to sensitization of the peripheral nerve terminal. Investigators in the NGEU have demonstrated that the ability to activate the VR1 is conditional, depending in part on the pH of the extracellular fluid. This observation suggests how tissue damage and inflammation, which causes local acidic conditons, sensitizes VR1 to endogenous compounds, thereby maintaining pain sensation following tissue injury. In collaboration with Drs. Neubert and Dionne, the ability to block these changes by VR1 inactivation with capsaicin in humans has provided preliminary evidence of analgesia in the oral surgery model. Subsequent studies will use the oral surgery model to evaluate the analgesic potential for resiniferatoxin (RTX), an ultrapotent, long-lasting vanilloid. Parallel animal studies have demonstrated that epidural application of vanilloids deletes primary afferent cells to produce a long-lasting analgesia. Administration of RTX into or in the vicinity of spinal ganglia also deletes cells to produce analgesia, a possible strategy for the treatment of trigeminal neuralgia and other types of pain from nerve injury.

Genetic studies of acute and chronic pain: A collaborative effort between PNMB and the laboratory of Dr. David Goldman of NIAAA over the past year is evaluating the genetic contribution to acute and chronic pain. Subjects are phenotyped with experimental thermal pain and the cold pressor test followed by collection of blood samples for DNA extraction. A subset of subjects also have oral surgery performed under controlled conditions to permit assessment of their ratings of clinical pain and their responses to a fixed dose of an NSAID analgesic. For genotyping, allelic discrimination assays were developed for mu and delta opiate receptor gene variants. Screening of N=480 subjects did not reveal any differences in responsiveness to either thermal or cold experimental pain for mu opiate receptor genes but did demonstrate that delta opiate receptor gene alleles were associated with a significant increase in heat sensitivity in males, but not females. Screening for variants of the VR1 receptor alleles has revealed three single nucleotide polymorphisms, including one that is predictive of an amino acid change. These data provide preliminary evidence for a genetic contribution of the delta opiate receptor to pain perception in humans and for a potential genetic contribution for the VR1 receptor as well.

The Clinical Trials Unit also initiated a program to evaluate the genetic mechanisms of chronic neuropathic pain in collaboration with the Molecular Epidemiology Branch. Based on previous studies in a rodent model demonstrating recessive transmission of a trait for developing neuropathic pain after sciatic nerve injury, the intensity of pain behavior was studied in different inbred strains of mice. One strain was identified with high pain behavior and one with minimal pain behavior and 26 recombinant strains derived from a cross of these strains were evaluated for pain behavior in the sciatic nerve model. The recombinant strains varied widely in levels of pain behavior and resultant analysis localized the trait to a small part of one chromosome containing

several promising candidate genes. Future studies will more finely localize the responsible genes.

Evaluation of COX-2 selectivity at the site of tissue injury: The analgesic effect of selective cyclooxygenase-2 (COX-2) inhibitors is likely mediated through COX-2 expressed following tissue injury. Evaluation of the role of COX-2 in producing pain following tissue injury was evaluated by measuring local levels of prostaglandin E_2 – a product of both COX-1 and COX-2 – and thromboxane B_2 – a measure of COX-1 activity – using microdialysis probes placed under the surgical flap. The temporal profiles of these mediators following surgery and during pain onset were consistent with constitutive COX-1 activity and inducible COX-2 activity. Comparison of the selective COX-2 inhibitor celecoxib to the dual COX-1/COX-2 inhibitor ibuprofen demonstrated that celecoxib is selective for inhibition of COX-2 in humans with little detectable effects on products of COX-1. These studies permit assessment of mechanistic hypotheses of drug actions that are formulated in animal models and *in vitro* but not otherwise subject to confirmation in humans. The expression of COX-2 was evaluated in a parallel study with collection of tissue biopsies over time from pre-surgery to the onset of acute pain following oral surgery. Levels of COX-2 in comparison to a housekeeping gene first started to increase at 60 min post-surgery, reaching significant levels by 120 mins. This time course was similar to the measurement of PGE2 over time as a product of COX-2. These two studies support an involvement of COX-2 in the early phase of inflammation in the oral surgery model.

Supraspinal activity from pain in fibromyalgia is associated with psychological traits: Patients with fibromyalgia completed the Beliefs in Pain Control Questionnnare and underwent fMRI in which blunt pressure was applied to the left thumb in alternating blocks of innocuous and painful intensities. The results of processed functional images for subjects during painful and non-painful pressures demonstrated that activity in the contralateral somatosensory cortex was highly correlated with the internal locus of control; belief in powerful doctors was associated with activation in the parietal cortex. These finding establish a link between psychological traits and brain activity, likely mediated by sensory mechanisms involved in selective attention and stimulus labeling in pathways involved in affective responses.

Evaluation of patients with chronic orofacial pain: A case series (N=32) evaluated patients with failed TMJ implants, many of whom continue to report severe pain, limitation of mandibular opening, and nonspecific systemic complaints. The failed TMJ implant patients appeared to have altered sensitivity to sensory stimuli, a higher number of tender points, a greater incidence of fibromyalgia diagnosis, increased self-reports of chemical sensitivity, and higher psychological distress. No evidence of systemic illness or autoimmune disease was observed in this series of failed TMJ implant patients. An association was observed between the present level of pain intensity and an increased incidence of fibropmyalgia that should be confirmed in a prospective cohort as a possible manifestation of central hyperalgesia secondary to nociceptive input.

These highlights of the studies summarized in the individual reports support the rationale and importance of conducting translational studies in humans to evaluate mechanisms of pain and analgesic strategies based on studies of nociceptive mechanisms in animal models and in vitro assays. Future studies by investigators in the Branch will extend these novel analgesic

mechanisms to block the molecular-genetic processes leading to the development of pain chronicity following tissue injury and to evaluate the clinical utility of cell deletion and gene transfer as chronic pain treatments.

PAIN AND NEUROSENSORY MECHANISMS BRANCH
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Functional Genomics Unit

Ashok Kulkarni

FUNCTIONAL GENOMICS UNIT
2001

Our Unit continues to produce exciting research advances in the quest of basic scientific knowledge in dissecting pathways pertinent to the disorders that affect craniofacial and dental systems as a result of genetic abnormalities and environmental factors. The Unit's primary research focus is centered on functional genomics. Since its inception in 1996, the key research work of the Unit has concentrated on the following studies:

In the first set of studies, we continue to characterize molecular roles of cyclin dependent kinase-5 (Cdk5) in neuronal phosphorylation and neuronal migration to gain insight into its role in a number of neurodegenerative disorders. We generated mice deficient in Cdk5 expression which exhibit perinatal mortality associated with gross lesions in the brains and spinal cords. These mice lacked normal stratification of the neurons along with cerebellar defoliation, accumulation of neurofilaments in the neuronal cell bodies and ballooned motor neurons. Further studies revealed a typical inverted cortex in these mice indicating a special "cell autonomous" role of Cdk5 in neuronal migration. We then reconstituted Cdk5 expression in the neurons to ascertain its role in non-neuronal cell types. We also generated Cdk5 "conditional" knockouts that exhibit abnormal gait, postures and reflexes mimicking ALS-like condition indicating a possible peripheral neuropathy. Our recent studies indicate a lack of facial nucleus in Cdk5 null mice associated with neuronal migration defects that seem to arise out of synergistic actions of Cdk5, reelin and mDAB. An exciting finding is that of involvement of Cdk5 in NMDA receptor biology.

In the second project, we continue to work on Fabry disorder because of its unique nature as a painful and fatal metabolic disorder and the challenges it presents in developing much needed therapeutic approaches. Following the cloning of the murine gene of alpha- galactosidase A, the gene involved in Fabry disease, we generated null mice, which exhibit lipid inclusions in the target organs typically seen in Fabry patients. Subsequent studies revealed that the aging of these mice accentuates and bone marrow transplantation ameliorates the phenotpye of these mice indicating a potential for BMT as a therapy for some of the Fabry patients. Additionally, we have now identified greater prevalence of dental and oral defects in Fabry patients and mutant mice.

In the third project, we have made rapid progress in characterizing role of amelogenin enamel formation by generating amelogenin null mice. These mice display unique phenotype which mimics amelogenesis imperfecta. We continue to delineate an in vivo role of dentin sialophospho protein gene (dspp) in dentinogenesis. We have cloned and characterized the structure, regulation and functions associated with the dspp gene products. We have developed a transgenic animal model with a reporter gene (ß-galactosidase) under the control of 5.7 kb 5' flanking dspp and the analysis of these mice validates this promoter for tooth specific expression of the candidate genes and Cre transgene. We have now generated dspp heterozygous mutant mice in order to obtain null mutants.

In order to understand precise role of transforming growth factor-ß (TGF-ß) in inflammation and tooth mineralization we have extended our studies to analyze the autocrine and endocrine roles

of TGF-ß isoforms. The initial findings from studies indicate ameliorating effects of circulating TGF-ß and also its dominant role in tooth mineralization.

FUNCTIONAL GENOMICS UNIT
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Immunopathology Section

Larry Wahl

IMMUNOPATHOLOGY SECTION
2001

Research in the Immunopathology Section focuses on the biological mediators and signal transduction pathways involved in the modulation of human monocyte functions that may contribute to the immunopathology associated with various disease states. Connective tissue destruction is associated with many diseases in which the monocyte/macrophage is a prominent cell. Since matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are believed to play a major role in the destruction and remodeling of connective tissue, a major emphasis has been placed on how these enzymes and inhibitors are regulated. Research is also conducted on role of MMPs and TIMPs in the regulation of cellular functions that are not directly related to connective tissue metabolism.

An understanding of how different biological stimulants invoke the signaling events leading to MMP production is important to the potential design of appropriate therapeutic interventions. Our previous and current work has compared how LPS and cytokines, such as TNF alpha and GM-CSF, signal to induce monocyte MMP production through their effect on mitogen activated protein kinases (MAPKs). These studies have demonstrated that LPS induces MMP-1 through the ERK 1/2 and the p38 MAPK pathways whereas MMP-9 synthesis occurs mainly through ERK 1/2. In contrast, only ERK 1/2 is involved in the induction of MMP-1 and –9 by the combination of TNF-alpha and GM-CSF. Moreover, the p38 pathway can serve as a negative regulator of ERK 1/2. Thus inhibition of p38 results in a substantial increase in MMP production, particularly MMP-1. Recent studies have demonstrated that stimulation of monocytes results in a sustained activation of ERK 1/2 (8 to 12 h). Thus, specific inhibitors of ERK 1/2 can be added 4 to 8 hr after stimulation of monocytes and still inhibit MMP production. These unique findings demonstrate that prolonged activation of ERK 1/2 is required for the induction of the synthesis of monocyte MMPs.

The association of monocytes/macrophages with many tumors has implicated these cells in the regulation of tumor progression. Activation of pro-MMP-2 is believed to be an important factor in tumor metastasis. Pro-MMP-2 activation is thought to occur through a trimeric complex between membrane type 1-MMP (MT1-MMP), TIMP-2, and pro-MMP-2 with the activation MMP-2 occurring through the interaction with an adjacent free MT1-MMP. We have shown that activated monocytes produce MT1-MMP but little or no MMP-2 or TIMP-2. When supernatants from tumor cells containing substantial amounts of TIMP-2 complexed with pro-MMP-2 are added to activated monocytes the MMP-2 is converted to the active form. It is hypothesized that the monocyte after binding the TIMP-2-MMP-2 complex has substantial free MT1-MMP remaining to activate MMP-2. This conclusion is supported by the inhibition of MMP-2 activation through the addition of TIMP-2 to monocytes. Moreover, blocking of monocyte MT1-MMP activity with antibodies prevents the activation of MMP-2, indicating that monocytes may be key regulators of the activation of tumor derived MMP-2. These findings indicate monocytes may have a significant role in the regulation of tumor progression. Additionally, our recent studies have provided further evidence for the requirement of a trimeric complex in the activation of MMP-2. The addition of equal molar concentrations of TIMP-2 and MMP-2 to MT1-MMP bearing monocytes resulted in optimal activation of MMP-2, whereas higher concentrations of TIMP-2 inhibited this activation.

The specific cytokines present at an inflammatory site are thought to determine the expression pattern and amount of MMPs produced. We have previously shown that TNF alpha or GM-CSF can induce MMP-9 by monocytes, however induction of MMP-1 requires both cytokines. Our recent cytokine studies have focused on how IFN-gamma affects the expression of monocyte MMP production in the presence of TNF-alpha and/or GM-CSF. While IFN-gamma alone has no effect on monocyte MMP-1 or MMP-9, when added with the combination of TNF and GM-CSF it causes a significant increase in MMP-1. We have shown that the ability of IFN-gamma to induce MMP-1 when added with GM-CSF occurs as a result of the induction of TNF-alpha as demonstrated by increased TNF-alpha production and inhibition of MMP-1 production by antibodies against TNF-alpha. In contrast to MMP-1, IFN-gamma regulation of monocyte MMP-9 is considerably different. Our recent data demonstrates that IFN-gamma inhibits TNF-alpha-induced MMP-9 synthesis through a mechanism involving caspases. This can be shown by the reversal of IFN-gamma mediated MMP-9 inhibition by caspase inhibitors, particularly inhibitors of caspase 8. Caspases are well know for their involvement in apoptosis, however this is not the mechanism by which IFN-gamma suppresses MMP-9 since monocyte cell death is not increased in the presence of IFN-gamma and TNF-alpha. These findings demonstrate a new and novel role for caspases in the regulation of MMPs. Future studies will focus on the specific mechanism(s) by which IFN-gamma mediates its effects through caspases.

IMMUNOPATHOLOGY SECTION
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Matrix Metalloproteinase Unit

Henning Birkedal-Hansen

MATRIX METALLOPROTEINASE UNIT
2001

The Matrix Metalloproteinase (MMP) Unit seeks to define the role of MMPs and their inhibitors in physiologic and pathologic processes. Recently, we have focused particular attention on membrane-bound metalloproteinases and their dual role in degrading the extracellular matrix and activating other members of the MMP family.

Targeting of the MT1-MMP Gene

Previous studies have causally linked the phenotypic defects including growth retardation, skeletal deformities, craniofacial defects, skin fibrosis, dwarfing and arthritis-like joint abnormalities of the MT1-MMP deficient mouse to inadequate degradation and remodeling of selected type I and type II collagen matrices. In spite of severe growth retardation and dwarfing, the growth plates go through a normal and essentially correctly timed developmental sequence including hypertrophy, resorption and bone formation for the first 40 days or so. Apparently the activation of MMP-13 which is essential for growth plate cartilage removal is not affected. This observation all but dispels a significant role by MT1-MMP in the activation of MMP-13, at least in the growth plate. These observations suggest that growth plate abnormalities are not a primary defect in these animals but come into play rather late in the already shortened lifespan of these animals. Studies conducted during the past year in collaboration with Dr. Wouter Beertsen in Amsterdam have shown that molars (but not incisors) display severe defects in root formation and tooth eruption.

Our continued studies on intramembranous bone formation have revealed a new paradigm for this form of ossification. It appears that all intramembraneous bone formation takes place on a scaffold of cartilage either in the chondrocranium, Meckel's cartilage (mandible, condyle, inner ear,) or clavicle. This cartilage primordium never calcifies but is dissolved by an only recently recognized process that is dependent on MT1-MMP.

Regulation and Activation of Collagen Degradation

Previous work has demonstrated the pivotal role of matrix metalloproteinase MT1-MMP in proMMP activation and fibrillar collagen turnover in fibroblasts. We have now identified an alternative cell surface-associated pathway for MMP activation and collagen dissolution that utilizes the plasminogen activation (PA) cascade and is independent of MT1-MMP. Primary keratinocytes have an absolute requirement for serum in order to dissolve a layer of collagen fibrils. Dissolution occurs in plasminogen-containing medium, but not in plasminogen-depleted medium. Keratinocytes can also degrade collagen fibrils under serum-free conditions when supplemented with plasminogen. Dissolution can be blocked by the exogenous additions of either plasminogen activator inhibitor-1 (PAI-1) or tissue inhibitor of metalloproteinases (TIMPs), demonstrating the requirement of both PA and MMPs for collagen dissolution. Aprotinin, but not α_2-antiplasmin, efficiently inhibited collagen dissolution demonstrating that cell-surface plasmin activity is critical for the degradation process. MT1-MMP deficiency did not affect collagen dissolution. Keratinocytes deficient in either uPA or tPA also retained the ability to breakdown collagen. Interestingly, however, collagen dissolution was completely eliminated in keratinocytes with combined deficiency in both uPA and tPA. Combined PA deficiency also completely blocked the activation of MMP-9 and MMP-13 by keratinocytes. In

wild type keratinocytes the activation of MMP-9 and MMP-13 could be prevented by exogenously added PAI-1 and aprotinin, but not by TIMPs, suggesting that plasmin activates the two MMPs directly. Since MMP-13, but not MMP-9, efficiently cleaves native fibrillar collagen we propose that the PA cascade promotes keratinocyte-mediated collagen breakdown via the direct activation of MMP-13 and possibly other fibrillar collagenases.

Targeting of Two other Membrane-Associated Matrix Metalloproteinases

MMP-19 and MT4-MMP (MMP-17) most likely are linked to the plasma membrane through GPI-anchors and thus distinct from all other members of the MMP family. To further explore the function of these enzymes, we targeted each of these genes for disruption in mice; MMP-19, in collaboration with Dr. Carlos Lopez-Otin, Oviedo, Spain. The mice, which are viable and do not display macroscopic pathological features, are being subjected to ongoing phenotypic analysis.

Targeting of a Matrix Metalloproteinase Specific for Tooth Development

Previous studies have shown that enamelysin (MMP-20) is expressed only by odontoblasts and ameloblasts, and only during certain stages of tooth development. These features make MMP-20 an attractive candidate for gene ablation strategies in pursuit of biologic function. In collaboration with Dr. John Bartlett at Forsyth Research Institute, Boston, we have uncovered a series of subtle but significant abnormalities in enamel formation and in the adhesion of enamel to dentin.

MATRIX METALLOPROTEINASE UNIT
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Molecular Structural Biology Unit

Dennis Torchia

MOLECULAR STRUCTURAL BIOLOGY UNIT
2001

The principal research goal of the Molecular Structurual Biology Unit (MSBU) is to elucidate the structure and dynamics of proteins and associated molecules at the molecular 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. Three projects are currently active (1) HIV-1 protease, as either the free enzyme or bound to a high affinity protease inhibitor (2) Ribosomal proteins S4 and L11, either free or bound to a target RNA molecule (3) VIAF, a protein that regulates apoptosis. Progress that has been made during the past year, relating the structure and dynamics of these molecules with their functions is discussed below.

HIV-1 protease

The mature, wild-type HIV-1 protease is a highly stable homodimer. Until recently, attempts to find conditions that shifted the equilibrium to favor formation of the monomer resulted in aggregation or in unfolding. However, we have shown that substituting a highly conserved Arg residue with a Lys residue yields a mutant, R87K, that is a monomer in solution. The chemical shifts of the amide NMR signals indicated that the fold of the R87K monomer was similar to that of the monomer in the wild-type protease homodimer. The dimer structure is thought to be stabilized primarily by a four-stranded beta-sheet made up of C-terminal residues 96-99 (the inner strands) and residues 1-4 (the outer strands) from each monomer. We investigated the folding, dimerization propensities and enzymatic activities of mutant proteases containing deletions of either one or both of the terminal strands. Our results show that both terminal strands stabilize the dimer, but that the inner C-terminal strands are essential for dimer formation. Further studies that identify regions of the monomer critical for dimer formation may assist in the design of novel protease inhibitors to overcome the problem of drug-resistance.

In order to better understand the process of ligand binding to the protease active site, we have characterized the fast dynamics of the flaps the cover the active site. We have found that in contrast with the inhibitor-bound protein, the tips of the free protease flaps are flexible on the subnanosecond time scale, facilitating access to the active site. The flap tips are rich in highly conserved Gly residues suggesting that this region could be a novel inhibitor target that could help overcome the problem of drug resistance.

In the course of our studies of protease sidechain flexibility, we developed a novel approach to check the internal consistency of 2H and 13C measurements of methyl flexibility. We showed that 2H- and 13C-derived order parameters agreed to better than 10%. In addition, we demonstrated a method of isotope enrichment, using a combination of 13C/2H labeled pyruvate and keto- butyrate that should prove useful in studying methyl dynamics of larger proteins.

Structure and dynamics of the ribosomal proteins S4 and L11

Binding of S4 to 16S rRNA is critical for the subsequent binding of other ribosomal proteins, and regulates its own translation and that of three other ribosomal proteins. Previous reports have described our solution structure of S4's mRNA binding domain (S4delta41). More recently we showed that the structure of the C-terminal domain of the intact protein is the same that of

S4delta41, while the highly flexible 45 N-terminal residues contain two conserved regions, $S_{12}RRL_{15}$ and $P_{30}YPP_{33}$, that adopt transiently ordered structures in solution. The recently determined crystal structure of the 30S ribosomal unit from *T. thermophilus* revealed that the two conserved arginines in the $S_{12}RRL_{15}$ segment adopt parallel conformations similar to that predicted by our NMR results. Moreover, the $P_{30}YPP_{33}$ segment adopts a polyproline II helical structure in the crystal, also as predicted by our NMR results. During the course of the structural work on the S4 N-terminus, severe overlap problems were encountered in the NMR spectra resulting from the flexibility of this S4 region. In addition, the high concentration of proline residues in the N-terminus further increased the assignment difficulties. We overcame these problems by developing two novel versions of standard experiments. ^{15}N relaxation data have been collected on both S4 and S4delta41,. These data were analyzed using both isotropic and anisotropic versions of the Model-free approach. These data indicate that S4 is somewhat less anisotropic than S4delta41, and further analysis is continuing in an effort to describe the overall hydrodynamic shape of the N-terminus in solution.

Ribosomal protein L11 is a strongly conserved component of the large ribosomal subunit, located in the L7/L12 stalk. L11 recognizes approximately 60 nucleotides from the 23S rRNA. We have previously determined the solution structure of the C-terminal RNA binding domain. Studies of full-length L11 are crucial to understanding in detail the mechanism of antibiotic binding and regulation at the GTPase center. We have therefore obtained nearly complete NMR signal assignments for the full length *Thermus thermophilus* L11. Using these assignments we have found two beta-strands and two helices in the N-terminal domain and three short beta-strands and three helices in the C-terminal domain, in good agreement with the consensus of the two L11 molecules in the crystal structure of the *T. maritima* protein bound to RNA. Our nearly complete set of NMR signal assignments opens the way to determine the three-dimensional structure and molecular dynamics of intact L11 in solution.

Structure/function study of VIAF, a protein that regulates apoptosis

VIAF is a member of a conserved protein family that associate with animal IAPs (inhibitor of apoptosis proteins). VIAF itself substantially protects cells from Fas- and Bax-induced apoptosis, while coexpression of VIAF, with suboptimal quantities of XIAP confers almost complete protection from these inducers. VIAF and XIAP activate JNK in a synergistic manner. Hence, VIAF is a novel cofactor which modulates the anti-apoptotic and signaling properties of the IAP family. In order obtain a basis for understanding the function of VIAF at the molecular level, we are solving the three-dimensional structure of VIAF using NMR spectroscopy. We have expressed VIAF in E..coli using the pET11 vector. Cultures were grown in 15N and 13C labeled minimal media, and the labeled protein was purified from inclusion bodies and refolded. NMR experiments have been recorded on VIAF dissolved in a 10 mM phosphate buffer, pH 7.4, at 35 °C. Nearly all backbone NMR signals have been assigned using 3D-heteronuclear experiments. These assignments have been used to predict the secondary structure of VIAF using the chemical shift index (CSI). Surprisingly the VIAF secondary structure is highly homologous to that of phosducin, in spite of the limited sequence homology shared by the two proteins. The predicted secondary structure of VIAF and the existing crystal structure of phosducin have been used to build an initial model for VIAF. This model has been tested by weakly orienting VIAF in a Pf1 phage liquid crystal medium and then measuring five different sets of residual heteronuclear dipolar couplings. Homology searches using these dipolar

couplings were conducted against seven-residue-fragments generated from a PDB structure file. The best fits for these dipolar couplings yielded a model of the protein structure that had the correct secondary structure, although additional data, in the form of NOE distance restraints are needed to obtain to determine the three dimensional structure of VIAF. In order to obtain the needed NOE restraints, proton resonance assignments have been extended to the amino acid sidechains. Currently we have assigned more than 90 % of the protons in the structured region of the protein, and are analyzing 3D and 4D NOESY data sets to obtain proton-proton distance restraints. We are encouraged by the fact that the VIAF model based upon the phosducin structure is consistent with the long range NOES that we have assigned so far. Upon solving the three dimensional solution structure of VIAF we plan to map out the interface on VIAF which interact with XIAP.

MOLECULAR STRUCTURAL BIOLOGY UNIT
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