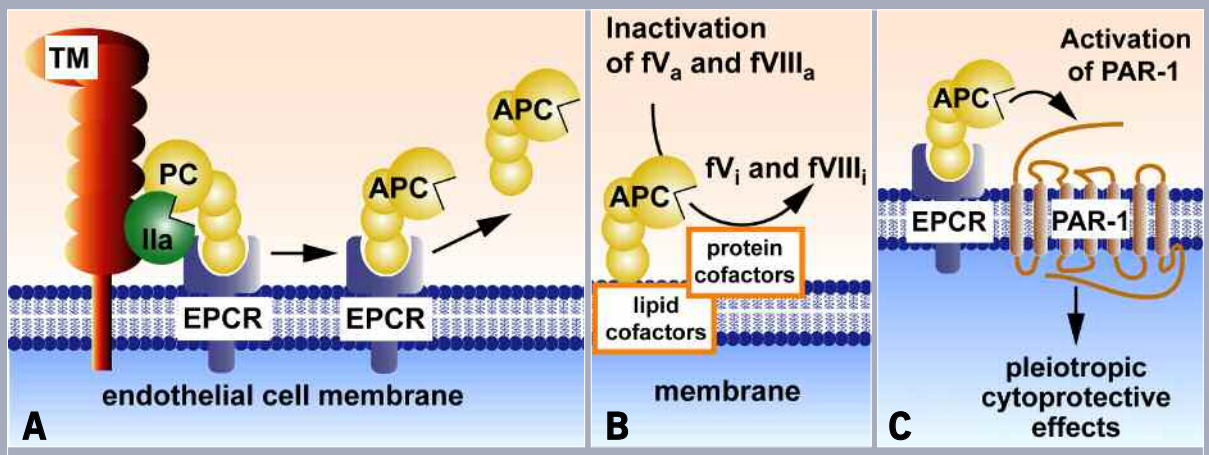
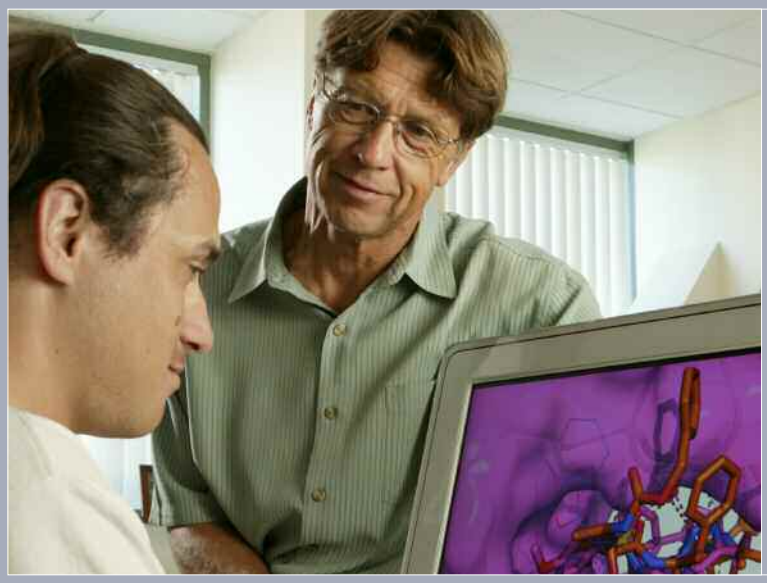
Molecular and Experimental Medicine



Protein C (PC), a serine protease zymogen, and its enzymatically active derivative, activated protein C (APC), are normal components of human blood. Discovery of the pathologic effects of hereditary PC deficiency in humans at Scripps Research helped lead to clinical trials that resulted in approvals of both PC and APC as drugs by the Food and Drug Administration. The decrease in mortality in patients with severe sepsis treated with recombinant APC gave impetus to new directions for basic and preclinical research on APC. A, APC is generated by the enzymatic action of thrombin (IIa) on endothelial cell membranes, where the endothelial PC receptor (EPCR) and thrombomodulin (TM) reversibly bind the reaction components. B, Dissociation of APC from EPCR (A) allows expression of APC anticoagulant activity involving proteolytic inactivation of 2 blood clotting cofactor proteins, Va (fV_a) and VIIIa ($fVIII_a$), by APC to yield the inactivated cofactors fV_i and $fVIII_i$. C, In contrast, binding of APC to EPCR on cells mediates expression of the multiple direct cellular activities of APC that require the 2 cellular receptors EPCR and protease-activated receptor 1 (PAR-1). Because of its pleiotropic activities, APC not only is useful in treatement of sepsis but also is promising as a potential treatment of other complex conditions such as thrombosis, ischemic stroke, and chronic neurodegenerative disorders. This figure was orignally published in Blood. Mosnier, L.O., Zlokovic, B.V., Griffin, J.H. The cytoprotective protein C pathway. Blood 109: 3161, 2007. Work done by Laurent Mosnier, Ph.D., research associate, in the laboratory of John H. Griffin, Ph.D., professor.



Michael Giffin, Ph.D., Research Associate, and Bruce E. Torbett, Ph.D., Associate Professor

DEPARTMENT OF MOLECULAR AND EXPERIMENTAL MEDICINE

STAFF

Ernest Beutler, M.D.*
Chairman and Professor
Head, Division of Hematology and Genetics

Masahiro Aoki, M.D., Ph.D.
Adjunct Assistant Professor

Hiroshi Asahara, M.D., Ph.D.
Assistant Professor of Molecular Medicine

Bonno N. Bouma, Ph.D.
Adjunct Professor

William D. Bugbee, M.D.
Adjunct Associate Professor

Joel N. Buxbaum, M.D.
Professor
Head, Division of Research Rheumatology

Dennis A. Carson, M.D.
Adjunct Professor

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Assistant Professor

Francis V. Chisari, M.D.
Professor
Head, Division of Experimental Pathology

Clifford W. Colwell, Jr., M.D.
Adjunct Professor

Laura M. Crisa, M.D.
Assistant Professor of Molecular Medicine

Arthur D. Dawson, M.D.
Adjunct Professor

Albert B. Deisseroth, M.D., Ph.D.
Adjunct Professor

Gregory J. del Zoppo, M.D.**
Associate Professor of Experimental Medicine

Thomas F. Deuel, M.D.
Professor
Head, Division of Molecular Oncology

Darryl D'Lima, M.D.
Assistant Professor

Darlene J. Elias, M.D.
Adjunct Associate Professor

Brunehilde Felding-Habermann, Ph.D.
Associate Professor

Kelly A. Frazer, Ph.D.
Associate Professor of Translational Genomics

Mitchell H. Friedlaender, M.D.
Adjunct Professor

Jeffrey S. Friedman, M.D., Ph.D.
Assistant Professor

Theodore Friedmann, M.D.
Adjunct Professor

Andrew J. Gale, Ph.D.
Assistant Professor

Roberta A. Gottlieb, M.D.***
Associate Professor
San Diego State University
San Diego, California

John H. Griffin, Ph.D.**
Professor
Head, Division of Translational Vascular Medicine Research

Andras Gruber, M.D.
Adjunct Assistant Professor

Luca G. Guidotti, D.V.M., Ph.D.
Associate Professor

Asa B. Gustafsson, Ph.D.
Adjunct Assistant Professor

Anne M. Hanneken, M.D.
Associate Professor

Mary J. Heeb, Ph.D.**
Associate Professor

James A. Hoch, Ph.D.
Professor
Head, Division of Cellular Biology

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Professor Emeritus

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Adjunct Professor

Shaun Phillip Jackson, Ph.D.
Adjunct Associate Professor

Eric F. Johnson, Ph.D.
Professor
Acting Head, Division of Biochemistry

Thomas J. Kipps, M.D., Ph.D.
Adjunct Professor

Lawrence E. Kline, D.O.
Adjunct Associate Professor

James A. Koziol, Ph.D.
Professor
Head, Division of Biomathematics

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Adjunct Professor

Thomas J. Kunicki, Ph.D.**
Associate Professor

Pauline L. Lee, Ph.D.
Associate Professor of Molecular Medicine

Stuart A. Lipton, M.D., Ph.D.
Adjunct Professor

Martin Lotz, M.D.
Professor
Head, Division of Arthritis Research

Christopher Lee Marsh, M.D.
Adjunct Associate Professor

David A. Matthews, Ph.D.
Adjunct Professor

Robert McMillan, M.D.
Professor Emeritus

William E. Miller, M.D.
Adjunct Assistant Professor

Richard Milner, M.D., Ph.D.
Assistant Professor

Kevin V. Morris, Ph.D.
Assistant Professor

Sarah Shaw Murray, Ph.D.
Assistant Professor of Translational Genomics

Jorge J. Nieva, M.D.***
Assistant Professor
Billings Clinic
Billings, Montana

Marta Perego, Ph.D.
Associate Professor

Paul J. Pockros, M.D.
Adjunct Assistant Professor

K. Michael Pollard, Ph.D.
Associate Professor of Molecular Medicine

Giuseppe Remuzzi, M.D.
Adjunct Professor

Zaverio M. Ruggeri, M.D.**
Professor
Head, Division of Blood Cell and Vascular Biology

Enrique Saldivar, M.D., Ph.D.
Adjunct Assistant Professor

Daniel R. Salomon, M.D.
Associate Professor

Nicholas J. Schork, Ph.D.
Professor

Alessandro Sette, Ph.D.
Adjunct Professor

Farhad F. Shadan, M.D., Ph.D.
Adjunct Assistant Professor

Sanford J. Shattil, M.D.
Adjunct Professor

Alexander R. Shikhman, M.D., Ph.D.
Adjunct Assistant Professor

Inmaculada Silos-Santiago, M.D., Ph.D.
Adjunct Associate Professor

Gregg J. Silverman, M.D.
Adjunct Professor

Ronald A. Simon, M.D.
Adjunct Professor

Jack C. Sipe, M.D.
Associate Professor

Donald D. Stevenson, M.D.
Adjunct Professor

Eng M. Tan, M.D.
Professor Emeritus

Eric J. Topol, M.D.
Professor of Translational Genomics

Bruce E. Torbett, Ph.D.
Associate Professor

Susan L. Uprichard, Ph.D.***
Assistant Professor
University of Illinois
Chicago, Illinois

Kottayil I. Varughese, Ph.D.***
Associate Professor
University of Arkansas
Little Rock, Arkansas

Peter K. Vogt, Ph.D.
Professor
Head, Division of Oncovirology

Matthias G. von Herrath, M.D.
Adjunct Associate Professor

Jill M. Waalen, M.D.
Assistant Professor

Xiaohua Wu, Ph.D.
Assistant Professor

Takao Yagi, Ph.D.
Associate Professor

Dong-Er Zhang, Ph.D.
Professor

STAFF SCIENTISTS

Andreas G. Bader, Ph.D.***
Asuragen, Inc.
Austin, Texas

Joseph R. Biggs, Ph.D.

Stephanie Cherqui, Ph.D.

Mei-Hui Hsu, Ph.D.

Chengqun Huang, M.D., Ph.D.***
San Diego State University
San Diego, California

Jennifer L. Johnson, Ph.D.

Sunil M. Kurian, Ph.D.

Patrizia Marchese, Ph.D.

Tsaiwei Olee, Ph.D.

Brian Savage, Ph.D.

Uzen Savas, Ph.D.

Rachel D. Schrier, Ph.D.

Stefan Wieland, Ph.D.

Akemi Yagi, Ph.D.

Ming Yan, Ph.D.

SENIOR RESEARCH ASSOCIATES

Hiroshi Deguchi, M.D., Ph.D.

Yuichi Kamikubo, Ph.D.

Joseph S. Krueger, Ph.D.

Laurent O. Mosnier, Ph.D.

Deirdre M. O'Sullivan, Ph.D.

Shuhei Otsuki, Ph.D.

Natalie M. Pecheniuk, Ph.D.

Keith Stephenson, Ph.D.

Subramanian Yegneswaran, Ph.D.

RESEARCH ASSOCIATES

Eun-Young Ahn, Ph.D.

Shinichi Asabe, Ph.D.

Marcy R. Auerbach, Ph.D.

Agnieszka A. Augustyniak, Ph.D.

Dong Bai, Ph.D.

Yajnavalka Banerjee, Ph.D.

Jennifer L. Barber-Singh, Ph.D.

Cristina Bongiorni, Ph.D.

Kristen E. Bower, Ph.D.***
CovX Research L.L.C.
San Diego, California

Nathan R. Brady, Ph.D.***
German Cancer Research Center
Heidelberg, Germany

Christoph Burkart, Ph.D.

Anna E. Cartier, Ph.D.***
University of California
San Diego, California

Giulio Cattarossi, Ph.D.

David M. Cauvi, Ph.D.

Yunchao Chang, Ph.D.

Yue Chen, Ph.D.

Guofeng Cheng, Ph.D.

Xiu Li Cong, M.D., Ph.D.

Chinh T. Dao, Ph.D.

Adam Denley, Ph.D.***
Freehills Patent Attorneys
Melbourne, Australia

Alejandra R. Diaz, Ph.D.***
Universidad Nacional del Sur
Buenos Aires, Argentina

Jonathon M. Flanagan, Ph.D.***
St. Jude Children's Research Hospital
Memphis, Tennessee

Tatsuya Fukushima, Ph.D.

Michael J. Giffin, Ph.D.

Shawn Patrick Grogan, Ph.D.

Marco Gymnopoulos, Ph.D.

David Habart, M.D., Ph.D.

Jonathan Hart, Ph.D.

Wolf-Achim Hassenpflug, M.D.***
Universitätsklinikum Hamburg-Eppendorf
Hamburg, Germany

Dominik R. Haudenschild, Ph.D.

Matteo Iannacone, M.D.***
Harvard Medical School
Boston, Massachusetts

Masanori Isogawa, M.D.

Tatsuo Ito, M.D.***
Memorial Sloan-Kettering Cancer Center
New York, New York

Hao Jiang, Ph.D.

Daisy Vanitha John, Ph.D.

Sohye Kang, Ph.D.***
Amgen
Thousand Oaks, California

Sharookh B. Kapadia, Ph.D.***
Gilead Sciences, Inc.
Foster City, California

Pablo G. Landart, Ph.D.

Alan Yueh-Luen Lee, Ph.D.

Shi-Sheng Li, Ph.D.

Miao-Chia Lo, Ph.D.

Jiann-Kae Luo, Ph.D.***
Regeneron Pharmaceuticals, Inc.
Rensselaer, New York

Mihaela Lorger, Ph.D.

Holly N. Maier, Ph.D.***
Amylin Pharmaceuticals, Inc.
San Diego, California

Nathalie Malo, Ph.D.

Mathieu Marella, Ph.D.

Florent M. Martin, Ph.D.

Shinobu Matsuura, Ph.D.

Sebastien Mauen, Ph.D.

Yasunori Mishima, M.D.***
Kurume University School of Medicine
Kurume, Japan

Eiko Nakamaru-Ogiso, Ph.D.***
University of Pennsylvania
Philadelphia, Pennsylvania

Christian J. Nievera, Ph.D.

Maria S. Ninniri, Ph.D.

Akiko Okumura, Ph.D.

Fumihiko Okumura, Ph.D.

Petra Pavlickova, Ph.D.

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Christina H. Swan, Ph.D.

Hendrik Szurmant, Ph.D.

Ken Takahashi, M.D.

Noboru Taniguchi, M.D., Ph.D.

Jesus Torres-Bacete, Ph.D.

Jaroslav Truksa, Ph.D.

Masanao Tsuda, Ph.D.***
Burnham Institute for Medical Research
La Jolla, California

Ji Wang, Ph.D.***
M.D. Anderson Cancer Center
Houston, Texas

Robert A. White, Ph.D.

Adam C. Wilson, Ph.D.

Tetsuo Yamashita, Ph.D.***
Kagawa University Faculty of Medicine
Kagawa, Japan

Xia Yang, Ph.D.

Zhengyi Ye, Ph.D.

Xiaoyan Yin, Ph.D.

Antonella Zampolli, Ph.D.

Li Zhao, Ph.D.

Jin Zhong, Ph.D.

Weiguo Zou, Ph.D.***
Harvard School of Public Health
Boston, Massachusetts

Masahiko Zuka, M.D., Ph.D.***
Kanazawa University
Ishikawa, Japan

SCIENTIFIC ASSOCIATES

Fanny E. Almus, Ph.D.

Jose A. Fernandez, Ph.D.

Terri P. Gelbart, B.S., M.T.

Oxana A. Malakhova, M.S.

Byoung Boo Seo, Ph.D.

** Joint appointment in The Skaggs Institute for Chemical Biology*

***Joint appointment in Department of Cell Biology*

**** Appointment completed, new location shown*

Ernest Beutler, M.D.

Chairman's Overview

It has been a dynamic year for the Department of Molecular and Experimental Medicine. Two of our senior faculty members, both of whom had been in the department continually since their postdoctoral training, collectively 38 years, assumed senior responsibilities in other institutions. Roberta Gottlieb became director of the Bioscience Center at San Diego State University and Gregory Del Zoppo accepted an appointment as professor of medicine at the University of Washington in Seattle. This seeding of other institutions with outstanding investigators is part of our mission. At the same time, it has given us an opportunity to provide much-needed additional space for our departmental core facilities and for investigators whose programs have expanded.

This year we have also been able to accommodate an important new initiative spearheaded by our clinical colleagues at Scripps Health, namely, the application of advanced genomic techniques to the understanding and, ultimately, the treatment of human disease. The internationally known cardiologist Eric Topol has been appointed chief of genomic medicine and translational science for Scripps Health and will perform some of the basic studies in this department. The implementation of this program has made possible the recruitment of a number of other leading investigators in genomics. Nicholas Schork and Kelly Frazer, along with the junior staff that they have recruited, bring expertise in biomedical informatics and in the rapidly evolving technology of high-throughput DNA analysis. These programs fit well with the widespread activities in genetics already extant in the Department of Molecular and Experimental Medicine and provide us with much needed expertise in these important new areas.

Details of the accomplishments of our faculty can be found in the pages following this overview, and as has been my usual practice, I do not try to summarize the diverse work of my colleagues here. Instead I focus on one important approach to the study of human health, an approach that is widely used in our department. Alexander Pope (1688–1744) in his *Epistle II: Of the Nature and State of Man With Respect to Himself, as an Individual* wisely observed, "The proper study of mankind is man." This statement is as true today as it was then. But the study of complex diseases of mankind in humans is difficult, both because of ethical constraints and because of the heterogeneity of mankind. Thus, even though our ultimate aim is to understand the diseases of humans, animal models have been a valuable resource in our investigations of human disease.

Historically, animal models of infectious disease have been a cornerstone of understanding the pathogenesis of these diseases. Indeed, 1 of Koch's 4 postulates, the classical criteria for the demonstration that a microorganism plays a causative role in a disease, is the reproduction of the disease in a "healthy organism." Although this "organism" has occasionally been a human, as in the fatal self-inoculation of the infectious organism by Chagas to reproduce Arroya fever, experimental animals are almost universally used for this purpose. But in recent years, the use of animal models has been expanded to include the growth of tumors in mice and other hosts and the study of genetic diseases that either occur spontaneously or are induced by targeted disruption of genes or the development of transgenic animals.

The faculty members of the Department of Molecular and Experimental Medicine strive to better understand human disease and consequently make extensive use of animal models, always, of course, in strict conformity with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Francis Chisari and his colleagues focus on the pathogenesis of the hepatitis viruses. In some of these studies, the classical approach is used, and experimental animals are infected with the virus. In other studies, an innovative method pioneered by Dr. Chisari is used; transgenic

animals in which a part of the virus replicates have been created, so that the pathogenesis of the disease can be studied under carefully controlled conditions.

Brunehilde Felding-Habermann is using a mouse model to study treatment of breast cancer. In her studies, human tumors are allowed to grow in a genetically immunocompromised mouse. This method makes it possible to study treatment strategies; Dr. Felding-Habermann is investigating an antibody that she and her colleagues have developed. Ultimately, the antibody may prove to be useful in the treatment of human tumors.

Thomas Deuel and his colleagues are studying the growth of mammary tumors in mice. Their aim is to define the factors that may stimulate tumor growth, particularly the role of pleiotrophin, a cytokine that Dr. Deuel discovered.

A number of research groups in the department are using mouse models to study genetically determined human disease. Joel Buxbaum and his group are studying a common polymorphism of the gene encoding transthyretin that causes heart disease in African Americans. He has created a transgenic mouse model in which this human mutant transthyretin is overproduced. These studies could lead to better understanding of this disease. Extending these studies, the Buxbaum group has created mice that carry both a human Alzheimer's disease gene and the overexpressed transthyretin gene. Interestingly, overexpression of the transthyretin gene appears to suppress the effect of the Alzheimer's gene, a surprising effect that is well worth exploring.

A number of genes are known to be involved in iron homeostasis in humans. In work in my laboratory, we make extensive use of mice in which some of these genes have been disrupted or mutagenized. Included are the genes encoding HFE, transferrin receptor 2, β_2-microglobulin, and hemojuvelin. These studies have given us valuable information on the roles of each of these components in the iron signaling pathway. In collaboration with Bruce Beutler's group in the newly formed Department of Genetics, we have been investigating a mutation induced by *N*-ethyl-*N*-nitrosourea in a gene encoding transmembrane serine protease 6 (*Tmprss6*). The lack of this gene, previously of unknown function, causes iron deficiency. We are trying to determine whether mutations of this gene also exist in humans and whether such mutations contribute to the high incidence of iron deficiency.

Tom Kunicki and his colleagues observed that mice that lack an important collagen receptor have a bimodal bleeding response. A positional cloning effort in collaboration with scientists at the Genomics Institute of the Novartis Research Foundation led to the discovery of a gene, *klf4*, that modifies the hemostatic response of these mice. The role of this newly discovered polymorphism in human disease remains to be elucidated.

Animal models have played a critical role in our understanding of human disease. An animal model, the pancreatectomized dog, led directly to appreciation of the fact that the pancreas was involved in diabetes, and this finding was soon translated into the discovery of insulin and the saving of hundred of thousands of lives. Animal models made possible the whole field of tissue transplantation. They have been critical in the development of vaccines to protect us against infectious diseases. The illusion that a computer could substitute for an animal is just that—an illusion. Biology is simply too complex to make using a computer rather than an animal a reality. The study of human diseases and the use of animal models continue to be essential efforts to improve human health, and such models help scientists at Scripps Research better understand disease.

Division of Arthritis Research

Martin Lotz, M.D., Division Head

Adult Stem Cells in Articular Cartilage

S. Grogan, M. Lotz

Cartilage injury, limited repair capacity, and aging-associated changes in cartilage cells and extracellular matrix are major risk factors for osteoarthritis, the most prevalent joint disease. Mesenchymal stem cells (MSCs) have been studied extensively for potential therapeutic use in tissue engineering to repair cartilage injury. Recent findings suggest that MSCs are present in mature human articular cartilage. The role of resident MSCs in cartilage repair is unknown. Cells expressing MSC markers are prominent in the clusters of proliferating cells characteristic of osteoarthritic cartilage. These cells express high levels of inflammatory mediators and markers of aberrant chondrocyte differentiation, suggesting that their activation contributes to the pathogenesis of arthritis.

Our understanding of chondrogenesis and the molecular mechanisms that regulate MSC differentiation is incomplete. Identification of factors that repress and activate chondrogenesis may lead to the development of techniques and technologies that will allow use of MSCs for tissue repair and for the treatment of osteoarthritis.

Published data and our preliminary findings strongly suggest that the Notch signaling pathway is a critical mediator of differentiation toward the chondrocytic lineage. We have shown that Notch and its downstream targets Hes-1 and Hey-1 regulate chondrogenesis through novel interactions with Sox9, the principal prochondrogeneic transcription factor.

Mechanotransduction in Chondrocytes

D. Haudenschild, D. D'Lima, M. Lotz

Mechanical forces regulate chondrocyte proliferation, survival, differentiation, gene expression, and biosynthetic responses. The type and duration of mechanical stimulation determine the outcome of the cellular responses, which in their extreme manifestations can range from cell proliferation to cell death and from matrix formation to matrix destruction. We measured the actin reorganization that occurs in response to dynamic compression of agarose-embedded chondrocytes, tested whether Rho kinase is required for the actin cytoskeletal reorganization induced by dynamic compression, and investigated whether dynamic compression alters the intracellular localization of Rho kinase and actin-remodeling proteins in chondrocytes.

Dynamic compression of agarose-embedded chondrocytes induced actin cytoskeletal remodeling and caused a significant increase in punctate actin structures. Rho kinase activity was required for these cytoskeletal changes; dynamic compression in the presence of Rho kinase inhibitor did not induce punctate actin structures. Dynamic compression increased the amount of phosphorylated Rho kinase, as shown by immunofluorescence confocal microscopy. The genes for the chemokine CCL20 and inducible nitric oxide synthase were the ones most highly upregulated by dynamic compression, and this response was reduced by the Rho kinase inhibitor hydroxyfasudil.

In conclusion, we found that dynamic compression induces changes in the actin cytoskeleton of agarose-embedded chondrocytes, and we developed a method to measure these changes. Furthermore, we showed that Rho kinase activity is required for compression-induced actin reorganization and gene expression.

GLUT1 and Chondrocyte Homeostasis

A. Shikhman, D. Brinson, M. Lotz

Articular cartilage is an avascular tissue that receives its nutrients and oxygen by diffusion from blood vessels in the subchondral bone and from synovial fluid. Energy generation in cartilage strongly depends on glucose supply. Transmembrane transport of glucose is facilitated by a group of highly specialized glucose transporter proteins termed GLUTs. Human articular chondrocytes express at least 6 different GLUTs, including GLUT1, GLUT3, GLUT6, GLUT8, GLUT10, and GLUT11.

GLUT1 is the most abundant glucose transporter in human articular chondrocytes. Expression of GLUT1

is increased in cartilage affected by osteoarthritis and in chondrocytes activated by cytokines and growth factors in vitro. Inhibition of GLUT1 mRNA and protein expression with specific small interfering RNA inhibits IL-1–induced production of nitric oxide, suppresses growth factor–stimulated transmembrane thymidine transport and chondrocyte proliferation, and enhances growth factor–induced production of hyaluronan and expression of hyaluronan synthase type 2.

The effects of GLUT1 on chondrocyte proliferation and thymidine transport are mediated via AMP-activated protein kinase. However, GLUT1-regulated hyaluronan synthesis does not depend on this kinase.

To determine the role of GLUT1 in the initiation of intracellular signaling, we studied specific plasma membrane proteins with binding affinity for GLUT1. Mass spectrometry of chondrocyte lysates immunoprecipitated with antibodies to GLUT1 revealed that annexin II was the main plasma membrane protein that reproducibly coprecipitated with GLUT1. In addition, using Western immunoblotting with antibodies to annexin II, we found that annexin II was present in GLUT1 coprecipitates. Immunoprecipitation of annexin II resulted in coprecipitation of GLUT1. Interaction between GLUT1 and annexin II was also shown by confocal microscopy. The interactions between GLUT1 and annexin II depend on interactions between their oligosaccharide side chains.

Transcriptional Regulation of Cartilage Development

T. Ito, N. Taniguchi, M. Tsuda, H. Asahara

Chondrogenesis and cartilage development are tightly regulated processes in which multipotential mesenchymal stem cells differentiate into chondrocytes to form cartilage. This process is initiated by commitment to the chondrogenic lineage and condensation of the stem cells, followed by differentiation of the cells into chondrocytes, a change associated with cartilage-specific gene expression. Such activity is regulated transcriptionally both spatially and temporally, such that transcription factors have dynamic expression patterns during chondrogenic differentiation. Subsequently, chondrocytes proliferate and secrete a cartilage-specific matrix to form the cartilage anlagen. We examined 2 molecules, coactivator-associated arginine methyltransferase 1 (CARM1) and high mobility group box 1 protein (HMGB1), as critical regulators of endochondral ossification.

Cartilage development is regulated by the transcription factor Sox9, but the molecular mechanisms that underlie this activity remain unclear. We found that CARM1 regulates chondrocyte proliferation via arginine methylation of Sox9. Mice lacking the gene for CARM1 had delayed endochondral ossification. Conversely, cartilage development in CARM1 transgenic mice was accelerated. CARM1 specifically methylates Sox9 at its HMG domain in vivo and in vitro. These results establish a role for CARM1 as an important regulator of cell proliferation during development.

HMGB1 has dual roles. First, as a nuclear factor, it alters chromatin formation and regulates gene expression. Second, extracellular HMGB1 released from damaged cells acts as a cytokine and chemoattractant. However, the role of extracellular HMGB1 in physiologic conditions has not been fully understood. We discovered that mice lacking the gene for HMGB1 have severely impaired endochondral ossification during embryogenesis. HMGB1 is secreted from cultures of cartilage, and the protein is specifically located in the cytosol of hypertrophic chondrocytes. Recombinant HMGB1 promotes osteoclast migration as well as endothelial cell migration, suggesting that extracellular HMGB1 regulates endochondral ossification as a chemoattractant, at least in part. Taken together, these data provide evidence for a critical role of HMGB1 in skeletal development.

PUBLICATIONS

Davis, D.K., Goltz, D.H., Fithian, D.C., D'Lima, D. Anatomical posterior cruciate ligament transplantation: a biomechanical analysis. Am. J. Sports Med. 34:1126, 2006.

D'Lima, D.D., Patil, S., Steklov, N., Chien, S., Colwell, C.W., Jr. In vivo moments and shear after total knee arthroplast. J. Biomech., *in press.*

D'Lima, D.D., Patil, S., Steklov, N., Slamin, J.E., Colwell, C.W., Jr. Tibial forces measured in vivo after total knee arthroplasty. J. Arthroplasty 21:255, 2006.

Gordon, A.C., D'Lima, D.D., Colwell, C.W., Jr. Highly cross-linked polyethylene in total hip arthroplasty. J. Am. Acad. Orthop. Surg. 14:511, 2006.

Hall, J., Copp, S.N., Adelson, W.S., D'Lima, D.D., Colwell, C.W., Jr. Extensor mechanism function in single radius vs multiradius femoral components for total knee arthroplasty. J. Arthroplasty, *in press.*

Hardwick, M.E., Pulido, P.A., D'Lima, D.D., Colwell, C.W., Jr. e-Knee: the electronic prosthesis. Orthop. Nurs. 25:326, 2006.

Hiraoka, K., Grogan, S., Olee, T., Lotz, M. Mesenchymal progenitor cells in adult human articular cartilage. Biorheology 43:447, 2006.

Patil, S., D'Lima, D.D., Fait, J.M., Colwell, C.W., Jr. Improving tibial component coronal alignment during total knee arthroplasty with use of a tibial planing device. J. Bone Joint Surg. Am. 89:381, 2007.

Tam, H.K., Srivastava, A., Colwell, C.W., Jr., D'Lima, D.D. In vitro model of full-thickness cartilage defect healing. J. Orthop. Res., *in press.*

Taniguchi, N., Yoshida, K., Ito, T., Tsuda, M., Mishima, Y., Furumatsu, T., Ronfani, L., Abeyama, K., Kawahara, K., Komiya, S., Maruyama, I., Lotz, M., Bianchi, M.E., Asahara, H. The stage-specific secretion of HMGB1 in cartilage regulates endochondral ossification. Mol. Cell. Biol., *in press.*

Temple, M.M., Bae, W.C., Chen, M.Q., Lotz, M., Amiel, D., Coutts, R.D., Sah, R.L. Age- and site-associated biomechanical weakening of human articular cartilage of the femoral condyle. Osteoarthritis Cartilage, *in press.*

Zhao, D., Banks, S.A., D'Lima, D.D., Colwell, C.W., Jr., Fregly, B.J. In vivo medial and lateral tibial loads during dynamic and high flexion activities. J. Orthop. Res. 25:593, 2007.

Zhao, D., Banks, S.A., Mitchell, K.H., D'Lima, D.D., Colwell, C.W., Jr., Fregly, B.J. Correlation between the knee adduction torque and medial contact force for a variety of gait patterns. J. Orthop. Res. 25:789, 2007.

DIVISION OF BIOCHEMISTRY

Eric F. Johnson, Ph.D., Division Head

Cytochrome P450: Regulation, Structure, and Function

E.F. Johnson, K.J. Griffin, M.-H. Hsu, R.L. Reynald, S. Sansen, Ü. Savas

Enzymes in the cytochrome P450 superfamily primarily serve 2 purposes in human physiology. Some P450s catalyze specific biotransformations in autocrine, paracrine, and endocrine signal transduction pathways. A second, relatively large group of P450 monooxygenases play defensive roles by converting toxic compounds to less toxic forms that are more soluble and more easily excreted than are the parent compounds. Each xenobiotic-metabolizing P450 generally oxidizes structurally diverse substrates, leading to a wide-ranging protective capacity for elimination of toxic chemicals. Often the expression levels of these enzymes are increased in response to exposure to xenobiotics or altered physiologic states. We wish to understand how the structural diversity and genetic regulation of P450s that metabolize xenobiotics contribute to a person's ability to avoid the adverse effects of environmental chemicals or alter the clearance and bioavailability of therapeutic drugs.

Although extensive information on the conditional expression of P450 genes in experimental animal species is available, in humans the transcriptional responses of P450 genes to environmental stimuli and to physiologic changes are poorly understood. To address this problem, we use human cell lines, primary cultures of human cells, and transgenic mice to study mechanisms that regulate human family 4 P450 genes. These genes encode enzymes that are involved in both signal transduction and the metabolism of endogenous lipids and xenobiotics. Studies with cell lines are providing new information about endocrine and autocrine signal transduction pathways that govern the conditional expression of these genes in response to nutritional, hormonal, and xenobiotic signals.

Research is in progress to test whether more complex physiologic conditions such as pregnancy or caloric restriction alter the expression of the human enzymes in transgenic mice. We produced 2 independent transgenic mouse strains containing the human gene for CYP4A11 and extensive flanking intergenic regions. We found that the gene is expressed predominantly in kidney and liver at concentrations observed in human tissue samples. The expression of the transgene is elevated in response to fasting or exposure to agonists of the peroxisome proliferator-activated receptor α. Interestingly, the basal level of CYP4A11 expression is lowered in mice that do not express this receptor.

We also discovered that the human long-chain fatty acid ω-hydroxylase, CYP4F2, is induced in primary cultures of human hepatocytes and in cell lines by several statins, drugs used to lower serum levels of cholesterol. The induction of CYP4F2 could contribute to the reported reduction by statins of long-chain fatty acids that accumulate in X-linked adrenoleukodystrophy. The induction of CYP4F2 by statins could also aid in the treatment of patients with Refsum's disease, a congenital deficiency in the oxidation of branched-chain fatty acids that is exacerbated by dietary phytanic acid. The ω-hydroxylation of phytanic acid by CYP4F2 enables further metabolism by β-oxidation.

In collaboration with C.D. Stout, Department of Molecular Biology, we are defining the atomic structures of individual human P450s to understand the structural basis for the broad yet unique catalytic selectivity of each enzyme. This information can be used to better understand the adverse effects of oxidation of drugs and toxins and the potential for metabolic drug-drug interactions. These consequences of multidrug therapies can be life threatening and contribute extensively to the attrition of promising new candidate drugs. Toxicity and poor metabolic properties are significant barriers to the development of new drugs.

Mammalian P450s are tethered to the endoplasmic reticulum by a transmembrane segment at the

amino terminus and by additional interactions of the catalytic domain with the cytoplasmic side of the membrane. Although membrane proteins are difficult to crystallize, we developed methods to express, purify, and crystallize genetically modified mammalian P450s that retain a native catalytic domain. Using this approach, we have determined the atomic structures of several of the most important human drug-metabolizing P450s: 1A2, 2A6, 2C8, 2C9, and 3A4. Through these studies, we determined how the flexibility of the P450s and the diversity of their amino acid sequence shape catalytic specificity. Our recent publication of the structure of P450 1A2 provides the first structure of a family 1 P450. This structure indicates that family 1 P450s are highly adapted for the oxidation of large aromatic hydrocarbons, which are often produced by combustion and are generally carcinogenic (Fig. 1). The structure of the enzyme's active site complements the active sites of family 2 and 3 P450s.

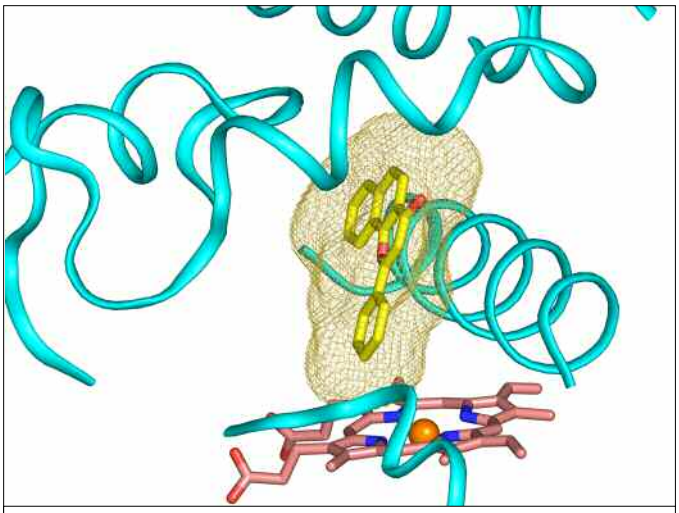

Fig. 1. The substrate-binding cavity (mesh surface) of human cytochrome P450 1A2 is narrow and well suited for large planar molecules like 7,8-benzoflavone (stick figure with yellow carbons), which was cocrystallized with the protein. Molecular oxygen is reduced by the heme prosthetic group (stick figure with pink carbons) to form a reactive intermediate that oxygenates the substrate. Parts of the protein backbone are shown as a cyan ribbon.

The P450 2A6 is the principal nicotine-detoxication enzyme and can also activate the tobacco smoke–specific carcinogen nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone to its carcinogenic form. Several reports indicate that because of the increased side effects of nicotine, persons who are genetically deficient in 2A6 activity are less likely to smoke than are persons not genetically deficient in this activity. In collaboration with J. Cashman, Human Biomolecular Research Institute, La Jolla, California, we are developing inhibitors of P450 2A6 that could reduce smoking behavior and diminish the likelihood of tobacco-related lung cancers.

PUBLICATIONS

Hsu, M.-H., Savas, Ü., Griffin, K.J., Johnson, E.F. Human cytochrome P450 family 4 enzymes: function and regulation. Drug Metab. Rev., *in press*.

Hsu, M.-H., Savas, Ü., Griffin, K.J., Johnson, E.F. Regulation of human cytochrome P450 4F2 expression by sterol regulatory element-binding protein and lovastatin. J. Biol. Chem. 282:5225, 2007.

Sansen, S., Hsu, M.-H., Stout, C.D., Johnson, E.F. Structural insight into the altered substrate specificity of human cytochrome P450 2A6 mutants. Arch. Biochem. Biophys., *in press*.

Sansen, S., Yano, J.K., Reynald, R.L., Schoch, G.A., Griffin, K.J., Stout, C.D., Johnson, E.F. Adaptations for the oxidation of polycyclic aromatic hydrocarbons exhibited by the structure of human P450 1A2. J. Biol. Chem. 282:14348, 2007.

Yano, J.K., Denton, T.T., Cerny, M.A., Zhang, X., Johnson, E.F., Cashman, J.R. Synthetic inhibitors of cytochrome P-450 2A6: inhibitory activity, difference spectra, mechanism of inhibition, and protein cocrystallization. J. Med. Chem. 49:6987, 2006.

Neutrophil Exocytosis and Extracellular Killing of Bacteria

S.D. Catz, D.B. Munafo, A.A. Brzezinska, B.A. Ellis, J.L. Johnson

ROLE OF RAB27 FAMILY MEMBERS IN EXOCYTOSIS OF NEUTROPHIL GRANULES

Neutrophils kill microorganisms via microbicidal products released into the phagosome or into the extracellular space. Neutrophils contain 4 types of excytosable storage organelles with different tendencies to undergo exocytosis: azurophilic, specific, and gelatinase granules and secretory vesicles. We are characterizing the secretory machinery that regulates the exocytosis of these organelles.

We recently showed that the small GTPase Rab27a and its effector JFC1 localize in a minor subpopulation of myeloperoxidase-containing granules (azurophilic) and that interfering with the Rab27a-JFC1 secretory machinery inhibits myeloperoxidase secretion in granulocytes. We also showed that Rab27a-deficient mice have impaired secretion of myeloperoxidase in vivo after intraperitoneal injection of lipopolysaccharide. Contrarily, mobilization of CD11b from intracellular granules (gelatinase granules and secretory vesicles) in response to the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine or lipopolysaccharide was not affected in Rab27a-deficient neutrophils.

Rab27b, a GTPase that shares 72% identity with Rab27a, is expressed in granulocytes and is upregulated in the absence of Rab27a. Granulocytes from mice deficient in Rab27b and from mice in which the genes for both Rab27a and Rab27b are inoperable mobilized CD11b in response to stimuli as efficiently as did wild-type controls. However, Rab27b deficiency impaired the mobilization of a subpopulation of specific granules expressing the IL-10 receptor. This finding suggests that Rab27b is involved in the mobilization of specific granules and that CD11b-containing vesicles are secreted independently of the Rab27 family members.

LOCALIZATION AND ACTIVITY OF THE NADPH OXIDASE ON NEUTROPHIL EXTRACELLULAR TRAPS

Neutrophils engulf microorganisms in a process known as phagocytosis. The bactericidal ability of these leukocytes relies on the antimicrobial peptides released into the phagosome and on the capacity of the neutrophils to generate reactive oxygen species. Many microorganisms escape phagocytosis-dependent killing. To overcome these escape tactics, neutrophils have developed alternative tools to kill bacteria. It has recently become apparent that extracellular neutrophil microbicidal components can be regulated by a novel mechanism: the formation of organized extracellular DNA fibers containing histones and other proteins with bactericidal ability. The composition of these neutrophil extracellular traps (NETs) and the bactericidal mechanism used are incompletely understood.

We tested the hypothesis that the NADPH oxidase is present and active on NETs. We showed that the oxidase is assembled on punctate structures distributed on NETs. Detection of NET-associated oxidase subunits was abolished by treatment with deoxyribonuclease and depended on cell stimulation. Using a superoxide-specific DNA-binding fluorescent probe, we found that superoxide anion is produced on NETs. Interfering with extracellular oxidase subunits by means of specific antibodies impaired extracellular bacterial killing. Our data support a role for NET-associated NADPH oxidase in the microbicidal mechanism used to combat nonphagocytosed microorganisms.

PUBLICATIONS

Johnson, J.L., Ellis, B.A., Munafo, D.B., Brzezinska, A.A., Catz, S.D. Gene transfer and expression in human neutrophils: the phox homology domain of p47*phox* translocates to the plasma membrane but not to the membrane of mature phagosomes. BMC Immunol. 7:28, 2006.

Munafo, D.B., Johnson, J.L., Ellis, B.A., Rutschmann, S., Beutler, B., Catz, S.D. Rab27a is a key component of the secretory machinery of azurophilic granules in granulocytes. Biochem. J. 402:229, 2007.

Pacquelet, S., Johnson, J.L., Ellis, B.A., Brzezinska, A.A., Lane, W.S., Munafo, D.B., Catz, S.D. Cross-talk between IRAK-4 and the NADPH oxidase. Biochem. J. 403:451, 2007.

Preserving Vision in Glaucoma, Diabetes, and Macular Degeneration

A. Hanneken, J. Johnson

The ability to maintain the health of aging nerve cells and restore the health of injured nerve cells has great potential for preserving and improving visual function in patients with numerous eye diseases. We are identifying compounds that protect nerves from the type of injury that leads to visual loss in macular degeneration, diabetes, and glaucoma, 3 of the leading causes of blindness.

Macular degeneration leads to the death of both retinal pigment epithelial (RPE) cells and photoreceptor cells. Diabetes and glaucoma lead to the death of retinal ganglion cells. We are screening multiple different compounds for their ability to protect these cell types from the type of injury that is thought to cause visual loss in these diseases. This injury is known as oxidative stress and is caused by an overproduction of reactive oxygen species (ROS), which are byproducts of oxygen metabolism. Although many cells can protect themselves from toxic byproducts of oxygen, high concentrations of these compounds can overwhelm the body's natural defense mechanisms. During the past several years, we have identified a group of compounds that protect RPE cells and retinal ganglion cells from cell death induced by oxidative stress. Shown in Figure 1 is the healthy appearance of RPE cells that have survived oxidative injury because of the protective effect of luteolin, a natural compound found in certain plants. Luteolin also prevents cell death induced by oxidative stress in retinal ganglion cells.

Among the numerous risk factors associated with the development of macular degeneration are exposure to sunlight and high levels of lipofuscin (a byproduct of aging). To extend our initial studies into cellular models of macular degeneration, we have developed a light-based assay for identifying compounds that can protect RPE cells from the damage associated with light and aging pigments. In these experiments, RPE cells are grown in the presence of various components of lipo-

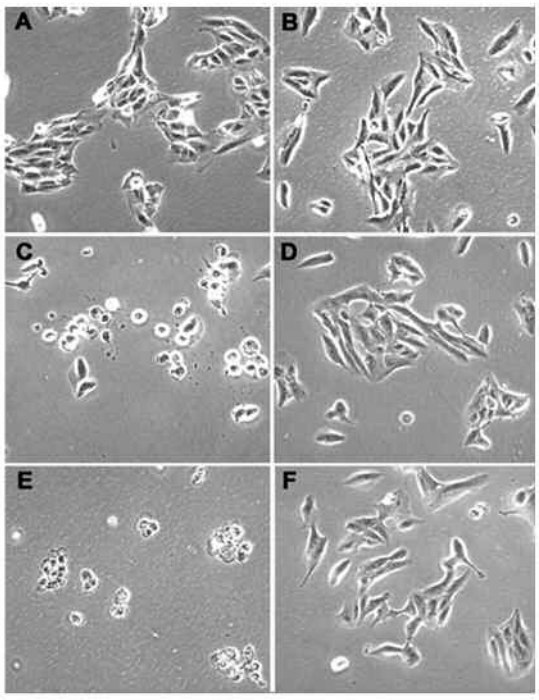

Fig. 1. Luteolin protects RPE cells from death induced by oxidative stress. A, Cells grown under normal conditions. B, Cells grown in the presence of luteolin alone. C, Cells dying after treatment with hydrogen peroxide. D, Cells surviving in the presence of luteolin and hydrogen peroxide. E, Cells dying after treatment with tert-butyl hydroperoxide. F, Cells surviving in the presence of luteolin and tert-butyl hydroperoxide.

fuscin and exposed to bright white or blue light. The levels of damaging ROS in the cells are measured after the light exposure. The cells containing various components of lipofuscin have high concentrations of ROS; the presence of protective compounds reduces these concentrations significantly. These effects are dose dependent. For example, Figure 2 shows the effect of

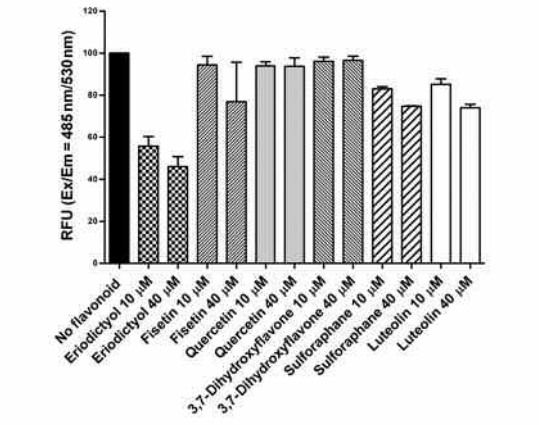

Fig. 2. Effect of various natural products on the accumulation of ROS in RPE cells exposed to light. RFU = relative fluorescence units.

various natural compounds on the reduction of ROS in RPE cells exposed to bright white light.

The mechanisms through which these beneficial effects occur are under investigation. Some compounds enhance the production of glutathione, the cell's primary defense against oxidative injury. Other compounds neutralize the production of ROS, which cause cellular injury and death. Additionally, some compounds activate cells' antioxidant response element, which induces the expression of genes that increase the cells' resistance to oxidative injury.

We are validating and expanding these results. Our goal is to identify additional compounds and combinations that have greater potency and efficacy than the previously tested compounds and combinations. We are planning to move into preclinical models of macular degeneration, diabetes, and glaucoma to translate these research findings into new potential therapies for clinical care.

This research is the result of a partnership formed between the Scripps Mericos Eye Institute and Scripps Research aimed at bringing together the promise of biomedical research and the practice of medicine.

PUBLICATIONS

Hanneken, A., Lin, F.-F., Johnson, J., Maher, P. Flavonoids protect human retinal pigment epithelial cells from oxidative stress-induced cell death. Invest. Ophthal. Vis. Sci. 47:3164, 2006.

NADH Dehydrogenases

T. Yagi, A. Matsuno-Yagi, B.B. Seo, E. Nakamaru-Ogiso, T. Yamashita, M. Marella, J. Barber-Singh, J. Torres-Bacete, P.K. Sinha

STRUCTURE AND FUNCTION OF NADH DEHYDROGENASES

The NADH-quinone (NADH-Q) oxidoreductases of the mitochondrial respiratory chain can be divided into 2 groups: the proton-translocating NADH-Q oxidoreductase (complex I) and the NADH-Q oxidoreductase lacking an energy coupling site (NDH-2). Mammalian complex I is composed of 45 unlike subunits, whereas NDH-2 is composed of a single polypeptide.

In one of our current projects, we are identifying the subunits that make up an inhibitor-binding pocket. We synthesized a photoreactive derivative of acetogenin, a complex I inhibitor, and found that the derivative specifically labeled subunit ND1, which is encoded by mitochondrial DNA.

In another project, to understand the biochemical basis for the function of yeast NDH-2 (Ndi1), we overexpressed Ndi1 in *Escherichia coli*. The Ndi1 purified from the membranes contained 1 FAD and had enzymatic activities comparable to those of original Ndi1. When extracted with detergent, isolated Ndi1 did not contain quinone. Reconstitution of the enzyme with quinone yielded a quinone-bound form. Quinone-bound Ndi1 had higher activity than did quinone-free Ndi1. Although both bound and free forms were inhibited by AC0-11, a quinone analog, the inhibitory mode for quinone-bound Ndi1 was distinct from that for quinone-free Ndi1.

The bound quinone was slowly released from Ndi1 by treatment with NADH or dithionite under anaerobic conditions. This release of quinone was prevented when Ndi1 was kept in the reduced state by NADH. When Ndi1 was incorporated into bovine submitochondrial particles, the quinone-bound form established the NADH-linked respiratory activity, which was insensitive to piericidin A but inhibited by potassium cyanate. Furthermore, Ndi1 produces hydrogen peroxide when isolated regardless of the presence of bound quinone, and this hydrogen peroxide was eliminated when the quinone-bound Ndi1 was incorporated into submitochondrial particles. The data suggest that Ndi1 bears at least 2 distinct quinone sites: one for bound quinone and the other for catalytic quinone (Fig. 1).

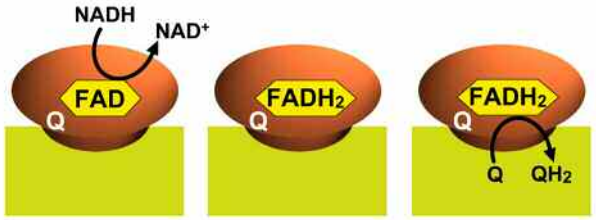

Fig. 1. A speculative mechanism of Ndi1. The reaction mechanism of Ndi1 is thought to follow ordered ping-pong. First, NADH binds to the enzyme, reduces FAD, and leaves as NAD^+ (left). When the enzyme is in the reduced state (center), quinone (Q) can bind to the catalytic site, accepts electrons from $FADH_2$, and is released as QH_2 (right).

MOLECULAR REMEDY OF COMPLEX I DEFECTS

Defects in complex I are involved in many human diseases. However, no remedies for the defects have been established. We have adopted a gene therapy approach that involves use of the gene *NDI1*, which encodes Ndi1, the yeast single polypeptide NADH dehydrogenase.

Recently, using rat dopaminergic cell lines, we investigated the protective effects of *NDI1* against the generation of reactive oxygen species (ROS) by inhibitors of complex I. Incubation of nontransduced control cells with rotenone elicited oxidative damage to mitochondrial DNA as well as lipid peroxidation. In contrast, oxidative stress was significantly decreased when the cells were transduced with *NDI1*. Furthermore, mitochondria from the *NDI1*-transduced cells had a suppressed rate of formation of ROS by the complex I inhibitors (Fig. 2). We conclude that Ndi1 can suppress overproduction of ROS from defective complex I.

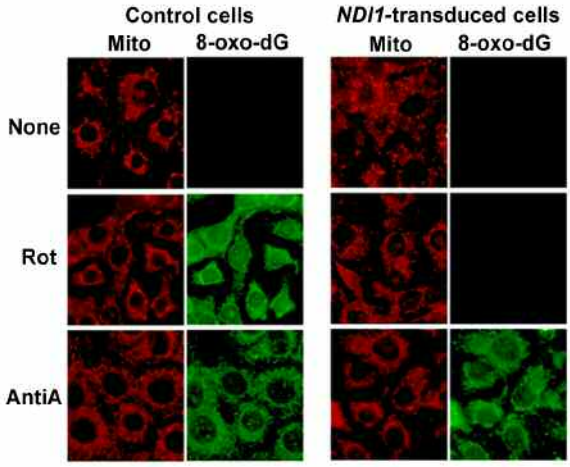

Fig. 2. Oxidative DNA damage by rotenone (Rot) and its prevention by Ndi1. In non–*NDI1*-transduced control cells, inhibition of complex I by rotenone or inhibition of complex III by antimycin (AntiA) triggers generation of ROS, resulting in oxidative modification of either mitochondrial or nuclear DNA or both as revealed by 8-oxo-deoxyguanine (8-oxo-dG) immunostaining. In *NDI1*-transduced cells, the DNA damage associated with inhibition of complex I, but not that associated with inhibition of complex III, was greatly reduced, indicating a protective effect of Ndi1. Mitochondria (Mito) were visualized by using MitoTracker.

Administration of 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) to mice and nonhuman primates causes a parkinsonian disorder. MPTP has been proposed to exert its neurotoxic effects through a variety of mechanisms, including inhibition of complex I, displacement of dopamine from vesicular stores, and formation of ROS from mitochondrial or cytosolic sources. However, the mechanism of MPTP-induced neurotoxic effects is still a matter of debate.

We used overexpression of Ndi1 in SK-N-MC cells and animals to determine the relative contribution of complex I inhibition in the toxic effects of MPTP. In cell culture, overexpression of Ndi1 abolished the toxic effects of 1-methyl-4-phenylpyridinium, the active metabolite of MPTP. Overexpression of Ndi1 through stereotactic administration of a viral vector harboring *NDI1* into the

substantia nigra protected mice against both neurochemical and behavioral deficits elicited by MPTP.

These data identify inhibition of complex I as a requirement for dopaminergic neurodegeneration and subsequent behavioral deficits induced by MPTP. Furthermore, combined with reports of a complex I deficit in patients with Parkinson's disease, our findings confirm the usefulness of MPTP in understanding the molecular mechanism that underlies neurodegeneration in Parkinson's disease.

PUBLICATIONS

Murai, M., Ishihara, A., Nishioka, T., Yagi, T., Miyoshi, H. The ND1 subunit constructs the inhibitor binding domain in bovine heart mitochondrial complex I. Biochemistry 46:6409, 2007.

Richardson, J.R., Caudle, W.M., Guillot, T.S., Watson, J.L., Nakamaru-Ogiso, E., Seo, B.B., Sherer, T.B., Greenamyre, J.T., Yagi, T., Matsuno-Yagi, A., Miller, G.W. Obligatory role for complex I inhibition in the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Toxicol. Sci. 95:196, 2007.

Schnermann, M.J., Romero, F.A., Hwang, I., Nakamaru-Ogiso, E., Yagi, T., Boger, D.L. Total synthesis of piericidin A1 and B1 and key analogues. J. Am. Chem. Soc. 128:11799, 2006.

Seo, B.B., Marella, M., Yagi, T., Matsuno-Yagi, A. The single subunit NADH dehydrogenase reduces generation of reactive oxygen species from complex I. FEBS Lett. 580:6105, 2006.

Shere, T.B., Richardson, J.R., Testa, C.M., Seo, B.B., Panov, A.V., Yagi, T., Matsuno-Yagi, A., Miller, G.W., Greenamyre, J.T. Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. J. Neurochem. 100:1469, 2007.

Yagi, T., Seo, B.B., Nakamaru-Ogiso, E., Marella, M., Barber-Singh, J., Yamashita, T., Matsuno-Yagi, A. Possibility of transkingdom gene therapy for complex I diseases. Biochim. Biophys. Acta 1757:708, 2006.

Yamashita, T., Nakamaru-Ogiso, E., Miyoshi, H., Matsuno-Yagi, A., Yagi, T. Roles of bound quinone in the single subunit NADH-quinone oxidoreductase (Ndi1) from *Saccharomyces cerevisiae.* J. Biol. Chem. 282:6012, 2007.

Division of Biomathematics

James A. Koziol, Ph.D., Division Head

Outcome Scales in Stroke

J.A. Koziol, G.J. del Zoppo

The development of stroke scales, such as the National Institutes of Health Stroke Scale (NIHSS), the Scandinavian Stroke Scale, or the Canadian Neurological Score, was motivated by the desire to describe the outcomes of stroke in terms of what clinicians understand most readily, the physical examination. Still, the issue arises as to how to handle the composite score represented by the NIHSS and similar scoring instruments. In particular, could some component of the NIHSS examination, or a neurologic examination in general, be a more sensitive indicator of outcome?

Operationally, the NIHSS score represents a reduction of all the data elements to a single score. Use of a summary score has benefits, including clinical validity (so long as each of the individual components that make up the score is a clinically important disease manifestation that has face validity), avoidance of multiplicity, and improved sensitivity because of expected reduction in measurement noise. Nevertheless, the fundamental question is whether or not the reduction of information from a large number of elements to an overall single quantity or score provides an adequate representation of the information available in the original clinical assessments. In particular, it is not at all obvious how to determine the optimal weights or, more generally, what constitutes the best method of combining the individual measures into a single index. One way to determine the appropriate set of weights is to use principal components analysis. An important initial step in this process is to examine the individual components or elements to ensure their reproducibility and validity; subsequent analyses should accommodate the ordinal and not numerical nature of the clinical scale.

The basic principles, methods, and terminology used in the evaluation of scales for clinical research are well established. And the use of composite scores for neurologic assessment is pervasive. For example, in one rating method for multiple sclerosis, the results of neurologic examination were converted into a weighted ordinal impairment scale. The various items that made up the impairment scale were not expected to be homogeneous, so it was not at all surprising that various components of the scale appeared more responsive than did the overall summary measure, and others, less responsive, to a treatment. Analogously, in clinical trials of treatment of stroke, certain components of the neurologic examination might be more sensitive to arterial recanalization than the summary NIHSS score and other components less sensitive. This possibility appears to be the case.

In a prospective study on dosages of recombinant tissue plasminogen activator (rt-PA), 93 of 104 patients with symptomatic documented cerebral arterial occlusions, infusion of intravenous rt-PA was completed within 5.4 ± 1.7 hours after the onset of signs or symptoms. Each patient was scored by the same neurologist at baseline and at 24, 48, and 72 hours and subsequently according to a neurologic examination based on the Harvard Stroke Registry. After rt-PA infusion, 4 patients

had complete recanalization, 31 had partial recanalization, and 58 had no recanalization as indicated by angiography at the end of the infusion.

In this trial, we found no significant difference between the 35 patients who had complete or partial recanalization and the 58 patients who had no recanalization for 3 of the categories of the neurologic examination: general characteristics, behavioral examination, and motor examination. The 2 groups differed significantly in the sensory examination. In order to investigate this difference further, the proportions of patients with abnormal findings for the hand and face sensation components of the sensory examination at each time point were observed (Fig. 1). Compared with patients

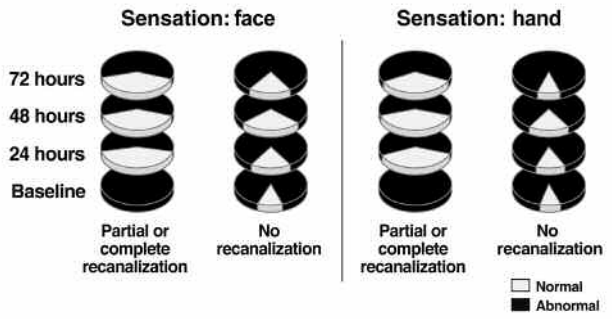

Fig. 1. Time changes in the hand and face sensation components of the individual sensorimotor evaluations for patients who had partial or complete recanalization (n = 35) vs no recanalization (n = 58) after treatment with rt-PA. Proportions of normal vs abnormal findings are depicted, where abnormal status comprises subjective decrease, partial loss of function, or severe loss of function.

who did not have recanalization, those who had complete or partial recanalization improved dramatically during the first 24 hours after rt-PA infusion. Thereafter, the sensory responses seemed stable within each group.

In this particular trial then, sensation seemed to be an early (24 hour) indicator of recanalization status, more so than the other components of the neurologic examination. Such fine distinctions may be obscured in a summary measure, particularly if little weight is given to the sensation domain in the summary.

In general, clinical changes associated with recanalization might be expected to be greatest in the first 24 hours after onset of stroke (especially if rt-PA is given within 3 hours of onset). Hence, a plausible scenario is that the recanalization group improves and the no-recanalization group does not in the first 24 hours, and the differences are then maintained over the next 2 days. Although the data tend to support this notion, we caution that only few patients achieve complete recanalization with intravenous infusion of rt-PA, and combining patients with complete and partial recanalization outcomes may dilute real effects of improved blood flow. More to the point, one might question the underlying premise that recanalization of a major artery should be associated with improved brain function. In the end, it would be useful to know which elements of stroke scales are most sensitive to recanalization.

PUBLICATIONS

Koziol, J.A., Feng, A.C. On the analysis and interpretation of outcome measures in stroke clinical trials: lessons from the SAINT I study of NXY-059 for acute ischemic stroke. Stroke 37:2644, 2006.

Division of Blood Cell and Vascular Biology

Zaverio M. Ruggeri, M.D., Division Head

Platelets in Hemostasis, Thrombosis, and Host Defense Mechanisms

Z.M. Ruggeri, F. Almus-Jacobs, R, Habermann, Y. Kamikubo, P. Marchese, R. McClintock, J. Orje, G.M. Podda, J. Roberts, B. Savage, A. Zampolli

Mammalian platelets, small anucleated cells with a diameter of 1–2 μm, are released from bone marrow megakaryocytes into circulating blood. Platelets play a key role in controlling bleeding from wounds but, in the context of degenerative vascular disease, may cause arterial thrombosis and life-threatening conditions such as myocardial infraction and stroke. In other vertebrates, the functions typical of mammalian platelets are carried out by thrombocytes, “white cells” similar in morphology to large lymphocytes, that have a diameter of 10–20 μm. The evolutionary reasons for the transition from thrombocytes to platelets are not known. Thrombocytes not only are involved in controlling bleeding but also have other activities typical of leukocytes, such as phagocytosis and production of inflammatory mediators and cytokines. Mammalian platelets have retained many activities relevant to host defense mechanisms, and their role in inflammation is the topic of continued research efforts.

During the past 20 years, my colleagues and I in the Division of Blood Cell and Vascular Biology have focused on understanding key mechanisms that explain the initiation and regulation of platelet aggregation and clotting in response to vascular injury. Our results have contributed to major advances in understanding the origin of bleeding disorders and the pathogenesis of arterial and venous thrombosis, which together are the leading cause of disease and death in developed countries.

Currently, in collaboration with W. Ruf, Department of Immunology, we are defining the mechanisms responsible for the regulated integration of platelet adhesion and aggregation that occur in activation of the coagulation system and fibrin deposition during thrombus formation. The clots that occlude coronary and cerebral arteries are the result of a fundamental defense process—arresting hemorrhage—that has gone astray for loss of regulation at many potential checkpoints; these clots essentially reflect excessive function of both platelets and coagulation. Our goal is to understand all the interactions that occur in flowing blood exposed to an altered vascular surface that lead to the unregulated deposition of platelet and fibrin clots.

In other studies, we are collaborating with L.G. Guidotti, Department of Molecular and Experimental Medicine, to develop his initial hypothesis that platelets play a key role in immune-mediated processes. Using original mouse models of acute viral hepatitis that he had developed with F.V. Chisari, Department of Molecular and Experimental Medicine, Dr. Guidotti found that platelet depletion reduces the accumulation of virus-specific cytotoxic T lymphocytes (CTLs) in the liver and, consequently, organ damage. This animal model mimics what happens in humans affected by viral hepatitis, when T lymphocytes that recognize viral antigens expressed on liver cells become responsible for the process that replaces functioning liver with scar tissue.

In a first study, we found that transfusion of normal but not activation-blocked platelets to platelet-depleted mice restored accumulation of T lymphocytes and severity of disease. Because it was apparent that platelets are not required for the normal antigen recognition and killing functions of CTLs, these findings suggested that platelets play an essential role in directing lymphocytes to the sites of viral accumulation and inflammation. We verified the validity of this hypothesis with additional studies that have confirmed a more general function of platelets in viral clearance.

We found that in mice infected with different isolates of lymphocytic choriomeningitis virus, a mild hemorrhagic anemia develops, which becomes severe and eventually lethal in animals depleted of platelets or lacking integrin β_3. Lethal hemorrhagic anemia is mediated by virus-induced IFN-α/β that causes platelet dysfunction, mucocutaneous blood loss, and suppression of erythropoiesis. In addition to the life-threatening hemorrhagic anemia, platelet-depleted mice do not mount an efficient CTL response and clear the virus. Transfusion of functional platelets into these animals reduces hemorrhage, prevents death, and restores CTL-induced viral clearance in a manner partially dependent on CD40 ligand. These results indicate that upon activation, platelets expressing integrin β_3 and CD40 ligand are required to protect the host against the induction of a lethal hemorrhagic diathesis dependent on IFN-α/β and to clear lymphocytic choriomeningitis virus infection through CTLs.

Our specific interest in this project stems from the observation that platelet activation is required to direct T lymphocytes to their target sites. This requirement implies that platelets must be able to recognize specifically the occurrence of virus-induced pathogenic processes, presumably through markers of inflammation directly or indirectly presented onto vascular surfaces, and in turn signal the location to T lymphocytes for arrest and extravasation. Once these mechanisms are defined in detail, it may be possible to use drugs that modulate platelet function to influence the course of viral infection and immunopathogenic processes mediated by T lymphocytes.

PUBLICATIONS

Bergmeier, W., Piffath, C.L., Goerge, T., Cifuni, S.M., Ruggeri, Z.M., Ware, J., Wagner, D.D. The role of platelet adhesion receptor GPIbα far exceeds that of its main ligand von Willebrand factor in arterial thrombosis. Proc. Natl. Acad. Sci. U. S. A. 103:16900, 2006.

Donadelli, R., Banterla, F., Galbusera, M., Capoferri, C., Bucchioni, S., Gastoldi, S., Nosari, S., Monteferrante, G., Ruggeri, Z.M., Bresin, E., Scheiflinger, F., Rossi, E., Martinez, C., Coppo, R., Remuzzi, G., Noris, M.; the International Registry of Recurrent and Familial HUS/TTP. In-vitro and in-vivo consequences of mutations in the von Willebrand factor cleaving protease ADAMTS13 in thrombotic thrombocytopenic purpura. Thromb Haemost. 96:454, 2006.

Kasirer-Friede, A., Moran, B., Nagrampa-Orje, J., Swanson, K., Ruggeri, Z.M., Schraven, B., Neel, B.G., Koretzky, G., Shattil, S.J. ADAP is required for normal $\alpha_{IIb}\beta_3$ activation by VWF/GP Ib-IX-V and other agonists. Blood 109:1018, 2007.

Kisucka J., Butterfield, C.E., Duda, D.G., Eichenberger, S.C., Saffaripour, S., Ware, J., Ruggeri, Z.M., Jain, R.K., Folkman, J., Wagner, D.D. Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. Proc. Natl. Acad. Sci. U. S. A. 103:855, 2006.

Konstantinides, S., Ware, J., Marchese, P., Almus-Jacobs, F., Loskutoff, D., Ruggeri, Z.M. Distinct antithrombotic consequences of platelet glycoprotein Ibα and VI deficiency in a mouse model of arterial thrombosis. J. Thromb. Haemost. 4:2014, 2006.

Reininger, A.J., Heijnen, H.F.G., Schumann, H., Specht, H.M., Schramm, W., Ruggeri, Z.M. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. Blood 107:3537, 2006.

Ruggeri, Z.M. Platelet interactions with vessel wall components during thrombogenesis. Blood Cells Mol. Dis. 36:145, 2006.

Ruggeri, Z.M., Orje, J.N., Habermann, R., Federici, A.B., Reininger, A.J. Activation-independent platelet adhesion and aggregation under elevated shear stress. Blood 108:1903, 2006.

Sadler, J.E, Budde, U., Eikenboom, J.C., et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J. Thromb. Haemost. 4:2103, 2006.

Savage, B., Ruggeri, Z.M. Platelet thrombus formation in flowing blood. *In:* Platelets, 2nd ed. Michelson, A.D. (Ed.). Academic Press, San Diego, 2006, p. 359.

Regulation of Allogeneic Immune Responses to Cell Transplants

L. Crisa, R. Prinsen, V. Cirulli,* B.E. Torbett

* Whittier Institute, La Jolla, California

Class I and class II MHC antigens are the primary barrier to acceptance of allografts. However, certain class I MHC antigens may also trigger regulatory immune responses. Thus, in humans, HLA-G, a nonpolymorphic class Ib HLA molecule, may mediate immunologic tolerance at sites of immune privilege, such as the anterior chamber of the eye, the testis, the thymus, and the cytotrophoblast.

Several explanations for the immunoregulatory functions of HLA-G have been considered. The limited polymorphism of HLA-G in humans may allow the recognition of tissues expressing high levels of this molecule as "self," thereby preventing the activation of autoreactive or alloreactive T cells and natural killer cells. Alternatively, HLA-G may foster the development of specific immunoregulatory lymphocytes capable of downregulating alloreactivity. Our previous finding that HLA-G is expressed in the thymic medullary epithelium in humans strongly supports both possibilities. Thus, the purpose of HLA-G expression in the thymic medulla may be to both (1) educate developing T cells to recognize HLA-G as self and (2) induce the selection of HLA-G–specific immunoregulatory T-cell populations.

We are investigating the immune responses elicited by HLA-G in human thymocytes and peripheral T cells. Our goals are to dissect the molecular mechanisms of HLA-G immune functions and then use this information to bioengineer HLA-G expression in tissues suitable for transplantation. Particular emphasis is given to models of pancreatic islet transplantation for the treatment of diabetes. For this purpose, we have generated lines of human pancreatic cells expressing either low or high levels of membrane-bound or soluble recombinant HLA-G. These HLA-G^{low} and HLA-G^{high} cell lines are useful tools for studies of HLA-G functions both in vitro and in vivo in models of cell transplantation.

Another promising line of research for the bioengineering of cells for transplantation was provided by our work on the identification of endothelial cell progenitors in human cord blood. While studying human thymopoiesis in a chimeric mouse model in which mice are reconstituted with human cord blood, we discovered that cord blood hematopoietic stem cells engrafted in these mice not only reconstituted the bone marrow and repopulated the human thymic grafts but also contributed to the formation of new blood vessels at sites of wound healing.

We are characterizing this population of putative endothelial progenitors to be used as another target cell type for transplantation. Specifically, we have defined some of the growth and differentiation signals required for the expansion ex vivo of human bone marrow–derived endothelial progenitors. Currently, using a mouse model of bone marrow–derived vasculogenesis, we are characterizing immunologic and angiogenic properties of bone marrow–derived endothelium. Ultimately, cotransplanting HLA-G–transduced allogeneic tissue along with HLA-G–bioengineered endothelial cell progenitors and/or enhancing recruitment of bone marrow–derived endothelium with intrinsic immunomodulatory properties may endow tissue grafts with an additional level of immunoprotection. This approach may be useful in developing novel strategies for the induction of immunologic tolerance and/or the avoidance of rejection after transplantation.

Mechanisms of Breast Cancer Metastasis

B. Felding-Habermann, J.S. Krueger, D. O'Sullivan, W. Hassenpflug, J.S. Forsyth, M. O'Neal, M. Lorger, G. Cattarossi, E.I. Chen, J.R. Yates III, A. Kralli, K.D. Janda, J.F. Kroener*

* Scripps Clinic, La Jolla, California

Patients with breast cancer often respond well to refined surgery and treatments, but development of metastatic disease is still responsible for most deaths in patients who have this neoplasm. Our goal is to develop new therapies that can eliminate the spread of breast cancer, especially to the brain. We therefore seek to understand mechanisms responsible for dissemi-

nation of breast cancer and to identify functional targets to prevent and eradicate metastasis of this cancer.

HYALURONIDASE EXPRESSION AND METASTATIC PHENOTYPE

Hyaluronic acid, a high molecular weight glycosaminoglycan, is a major component of the extracellular matrix in both normal and tumor tissues. Degradation of stromal hyaluronic acid within a tumor may promote escape of cancer cells from the primary site, and the degradation products strongly promote growth of new blood vessels. We tracked metastatic spreading of human breast cancer cells in immunodeficient mice and compared the gene expression profiles of metastatic lesions from bone, lung, and brain with the profiles of the primary tumors in the mammary fat pad.

With one exception, the gene expression patterns were almost identical. All distant metastases, regardless of the target organ, expressed significantly higher levels of the hyaluronic acid–degrading enzyme hyaluronidase 1 than did their primary tumors. Secretion of the enzyme and biological activity in breast cancer metastases, as well as high serum levels of hyaluronidase 1 in mice with metastases, indicate that hyaluronidase 1 may be predictive of metastatic disease and represent a functional target for the inhibition of metastatic spread.

CHANGES IN ENERGY METABOLISM AND BRAIN METASTASIS

Searching for functional characteristics that allow breast cancer cells to spread to the brain, we used multidimensional proteomic analysis to investigate brain metastatic lesions and compare them with the circulating breast cancer cells from which the lesions were derived. Specific changes in protein expression of brain metastases indicated that spread of breast cancer to the brain is supported by a selection or predisposition of cancer cells that can adapt to the unique energy metabolism of the brain. Specific increase in enzymes controlling glycolysis coupled to mitochondrial tricarboxylic acid cycle and oxidative phosphorylation pathways indicated that brain metastatic cells derive energy from glucose oxidation. Importantly, these specialized tumor cells also had enhanced activation of the pentose phosphate pathway and glutathione system to detoxify reactive oxygen species created by the enhanced oxidative metabolism.

ACTIVATED ADHESION RECEPTOR INTEGRIN $\alpha_v\beta_3$ IN BRAIN METASTASIS

We found that breast cancer cells expressing a specific adhesion receptor, the integrin $\alpha_v\beta_3$, in a high-affinity functional state have an aggressive metastatic phenotype that promotes the spread of breast cancer. Importantly, targeting this activated conformer of the receptor with human antibodies isolated from cancer patients can prevent and inhibit breast cancer metastasis in our mouse model. We found that expression of $\alpha_v\beta_3$, particularly its activated form, is essential for the growth of breast cancer cells in the brain. Direct implantation of tumor cells into the brain of immunodeficient mice and analysis of tumor cell expansion and dissemination by noninvasive bioluminescence imaging and histology indicated that expression of $\alpha_v\beta_3$ and activation of the receptor allow breast cancer cells to survive and proliferate in the growth-restricted microenvironment of the brain.

Thus, we defined functional determinants and molecular markers of human breast cancer metastasis, specifically to the brain. To date, no therapies exist that can effectively combat cerebral breast cancer metastases. Our novel human cell and analytical models (Fig. 1) open the unique opportunity to develop and

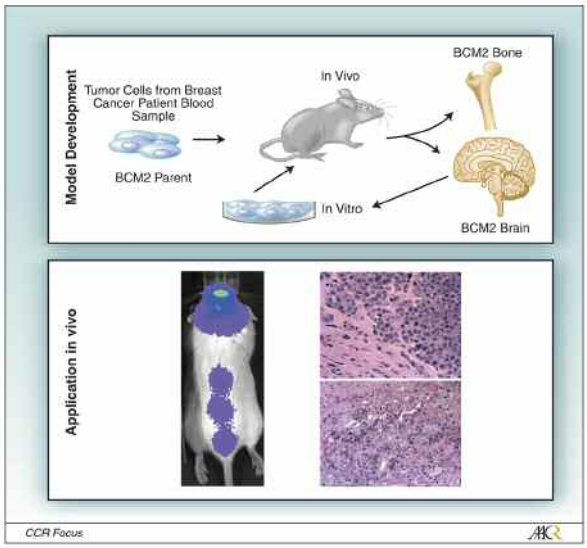

Fig. 1. Development and use of a new human cell model for studies on metastasis of breast cancer to the brain. Top panel, Circulating tumor cells isolated from a breast cancer patient with metastatic disease and established in culture (named BCM2 cells) were injected intravenously into immunodeficient mice. Metastases to the brain and bone were isolated, established in tissue culture, and reinjected into mice. Bottom panel, left, Noninvasive bioluminescence imaging of BCM2 cells labeled with luciferase show the pronounced ability of the cells to colonize the brain of immunodeficient mice and extend down the spine, as often occurs in breast cancer patients with advanced metastatic disease in the brain. Bottom panel, right, Micrographs of metastatic brain lesions from a mouse injected with BCM2 cells (top) and from a breast cancer patient (bottom) show the invasiveness of BCM2 cells and compression of the adjacent, still unaffected, brain tissue. Reprinted from Palmieri, D., Chambers, A.F., Felding-Habermann, B., Huang, S., Steeg, P.S. The biology of metastasis to a sanctuary site. Clin. Cancer Res. 13:1656, 2007.

evaluate new treatment approaches based on molecular mechanisms that we identify as critical for the spread of breast cancer to the brain.

PUBLICATIONS

Chen, E.I., Hewel, J., Krueger, J.S., Tiraby, C., Weber, M.R., Kralli, A., Becker, K., Yates, J.R. III, Felding-Habermann, B. Adaptation of energy metabolism in breast cancer brain metastases. Cancer Res. 67:1472, 2007.

Kim, Y., Lillo, A.M., Steiniger, S.C., Liu, Y., Ballatore, C., Anichini, A., Mortarini, R., Kaufmann, G.F., Zhou, B., Felding-Habermann, B., Janda, K.D. Targeting heat shock proteins on cancer cells: selection, characterization, and cell-penetrating properties of a peptidic GRP78 ligand. Biochemistry 45:9434, 2006.

Liu, Y., Steiniger, S.C., Kim, Y., Kaufmann, G.F., Felding-Habermann, B., Janda, K.D. Mechanistic studies of a peptidic GRP78 ligand for cancer cell-specific drug delivery. Mol. Pharm. 4:435, 2007.

Palmieri, D., Chambers, A.F., Felding-Habermann, B., Huang, S., Steeg, P.S. The biology of metastasis to a sanctuary site. Clin. Cancer Res. 13:1656, 2007.

Molecular Genetics of Hemostasis and Thrombosis

T.J. Kunicki, Y. Cheli, D. Jensen,* P. Marchese, D. Habart, T. Wiltshire,* M. Cooke,* J.A. Fernandez, J. Ware,** Z.M. Ruggeri, S. Kanaji, B. Jacquelin, M. Chang,*** D.J. Nugent,*** E. Trifiro, K. Furihata, F.M. Pulcinelli****

* Genomics Institute of the Novartis Research Foundation, San Diego, California

** University of Arkansas for Medical Sciences, Little Rock, Arkansas

*** Children's Hospital of Orange County, Orange, California

**** University "La Sapienza," Rome, Italy

Klf4 AS A MODIFIER GENE OF HEMOSTASIS

Glycoprotein VI (GPVI) is an important collagen receptor that is expressed by megakaryocytes and platelets. In mice bioengineered to lack the gene for GPVI, the expected abnormal platelet responses to collagen occur in vitro, but a dichotomous bleeding phenotype is evident in vivo. Tail-bleeding times are either prolonged or normal and parallel the ability of the mice to form thrombi in an in vivo model of injury of the carotid artery. The prolonged bleeding time is inherited as an autosomal recessive trait.

We mapped this phenotype to a dominant locus on chromosome 4, and we found a strain-related difference in *Klf4*, the gene for the transcription factor Krüppel-like factor 4 (Klf4), that correlates precisely with the in vivo bleeding phenotype. A cytosine-adenosine repeat polymorphism in the promoter region results in increased transcriptional activity of the 129X1/SvJ haplotype, designated *Klf4B*, relative to that of the C57BL/6J haplotype, designated *Klf4A*. Evidence from several laboratories has established that *Klf4* is an important transcription regulator in cells that are largely responsible for the composition of the extracellular matrix, including endothelial cells, smooth muscle cells, and fibroblasts. Thus, *Klf4* is a legitimate candidate as a modifier gene that regulates the in vivo bleeding phenotype of mice deficient in GPVI.

COREGULATION OF *ITGA2* EXPRESSION

In humans, expression of the integrin $\alpha_2\beta_1$ is controlled predominantly by variations in the transcription of *ITGA2*, the integrin subunit gene, that coincide with a single-nucleotide polymorphism (C-52T) and a unique cytosine-adenine repeat polymorphism in the 5′ regulatory region. This site is involved in coregulation of gene expression, and the longer alleles are specifically bound by members of a transcriptional coregulator complex composed of poly-ADP-ribosyl polymerase 1, topoisomerase IIβ, DNA-dependent protein kinase, Ku80, and Ku70. The association of this complex leads to enhanced histone 3 acetylation and a transcriptionally favorable chromatin structure. The binding of this coactivator complex to the specific-length cytosine-adenine repeat sequence contributes to haplotype-dependent variability of cell $\alpha_2\beta_1$ content.

TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF THE INTEGRIN COLLAGEN RECEPTOR LOCUS

An integrin collagen receptor locus on human chromosome 5q11.2 includes the integrin genes *ITGA1* and *ITGA2* and the cell-cycle regulation gene *PELO* embedded within *ITGA1* intron 1. *ITGA1* transcription is controlled by a CArG box bound by serum response factor; transcriptional regulation of PELO is similar to that of *ITGA2* and is controlled by Sp1. During thrombopoietin-induced in vitro differentiation of primary human cord blood mononuclear cells into megakaryocytes, rapid, progressive CpG methylation of *ITGA1*, but not *PELO* or *ITGA2*, occurs. Thus, selective CpG methylation of the *ITGA1* promoter is a specific feature of $\alpha_1\beta_1$ regulation that coincides with the initiation of megakaryocyte differentiation.

FUNCTIONAL COMPARISON OF PLATELET *GP6* HAPLOTYPES

Two major haplotypes, *GP6a* and *GP6b*, genes for GPVI in humans, influence collagen-related platelet function. The ectodomains of the membrane glycoproteins GPVIa and GPVIb differ at 3 residues, S219P, K237E, and T249A, but these substitutions have no effect on binding of the receptors to type I collagen, collagen-related peptide, or convulxin. Two additional

substitutions are located in the cytoplasmic domain, Q317L and H322N. The presence of leucine at position 317 increases the relative binding of GPVIb to calmodulin in vitro, and platelets from donors who are *GP6b/b* have significantly lower activation-dependent proteolysis of endogenous GPVI than do platelets from donors who are *GP6a/a*.

Thus, the cytoplasmic domain of GPVIb impairs the activation-induced proteolysis of GPVI through the increased binding of the glycoprotein to calmodulin. Our data are consistent with the theory that differences in GPVIa and GPVIb binding to the regulatory protein calmodulin modulate GPVI proteolysis and shedding. Because the soluble form of GPVI attenuates thrombosis in several in vitro and in vivo models, this difference in haplotypes can have an impact on risk for bleeding or thrombosis.

PUBLICATIONS

Kato, K., Furihata, K., Cheli, Y., Radis-Baptista, G., Kunicki, T.J. Effect of multimer size and a natural dimorphism on the binding of convulxin to platelet glycoprotein (GP)VI. J. Thromb. Haemost. 4:1107, 2006.

Regulation of Microglial Activation by Extracellular Matrix Proteins

R. Milner, S.J. Crocker, G.J. del Zoppo, J.C. LaManna*

* Case Western Reserve University, Cleveland, Ohio

Microglia are the primary immune effector cells resident within the CNS. In addition to playing a protective role in host defense, microglia may also be involved in the initiation and maintenance of demyelination in multiple sclerosis. Upon activation, microglia become highly aggressive, migratory phagocytic cells that secrete cytokines, chemokines, and proteases of the matrix metalloproteinase (MMP) family, including MMP-9, which has been implicated in the pathogenesis of multiple sclerosis.

An early event in multiple sclerosis is breakdown of the blood-brain barrier, which leads to deposition of the plasma proteins fibrinogen, fibronectin, and vitronectin within the cerebral parenchyma. Because microglial activation is promoted by fibronectin and vitronectin in vitro, we tested the hypothesis that deposition of fibronectin and vitronectin during demyelinating disease promotes activation of microglia and expression of MMP-9, thus leading to death of oligodendrocytes and demyelination.

Using experimental autoimmune encephalomyelitis in mice as a model of multiple sclerosis, we found that levels of fibronectin and vitronectin were strongly increased and that a close spatial relationship existed between fibronectin/vitronectin deposition and microglial activation and MMP-9 expression. In vitro studies indicated that microglial activation and MMP-9 expression were directly promoted by fibronectin and vitronectin and that these effects were mediated by the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$, respectively. Currently, using microglia from mice that lack the gene for the β_5 subunit of $\alpha_v\beta_5$, we are evaluating the requirement for this integrin in microglial activation and phagocytosis. Early experiments suggest that microglial activation is reduced in the absence of $\alpha_v\beta_5$.

In another project, we are investigating the potential role of $\alpha_5\beta_1$ in cerebral angiogenesis. Because proteins in the extracellular matrix play an important angiogenic role during development and tumor formation, we examined expression of extracellular matrix proteins and β_1 integrins during CNS development. We found that cerebral blood vessels make a switch in expression, from fibronectin and the $\alpha_4\beta_1/\alpha_5\beta_1$ integrins during angiogenesis to laminin and the $\alpha_1\beta_1/\alpha_6\beta_1$ integrins in adults. In vitro studies showed that fibronectin promotes survival and proliferation of brain endothelial cells and that this effect is mediated via the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. Because of the suggested angiogenic role for $\alpha_5\beta_1$ during CNS development, we recently examined whether cerebral hypoxia promotes induction of this integrin on angiogenic vessels in the CNS in adults. Early results indicate that cerebral hypoxia promotes a strong induction of $\alpha_5\beta_1$ on cerebral endothelial cells.

PUBLICATIONS

Crocker, S.J., Milner, R., Pham-Mitchell, N., Campbell, I.L. Cell and agonist-specific regulation of genes for matrix metalloproteinases and their tissue inhibitors by primary glial cells. J. Neurochem. 98:812, 2006.

del Zoppo, G.J., Milner, R. Integrin-matrix interactions in the cerebral microvasculature. Arterioscler. Thromb. Vasc. Biol. 26:1966, 2006.

del Zoppo, G.J., Milner, R., Mabuchi, T., Hung, S., Wang, X., Berg, G.I., Koziol, J.A. Microglial activation and matrix protease generation during focal cerebral ischemia. Stroke. 38(2 Suppl.):646, 2007.

Milner, R. A novel three-dimensional system to study interactions between endothelial cells and neural cells of the developing central nervous system. BMC Neurosci. 8:3, 2007.

Milner, R., Campbell, I.L. Increased expression of the β_4 and α_5 integrin subunits in cerebral blood vessels of transgenic mice chronically producing the pro-inflammatory cytokines IL-6 or IFN-α in the central nervous system. Mol. Cell. Neurosci. 33:429, 2006.

Milner, R., Crocker S.J., Hung, S., Wang, X., Frausto, R.F., del Zoppo G.J. Fibronectin- and vitronectin-induced microglial activation and matrix metalloproteinase-9 expression is mediated by integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$. J. Immunol. 178:8158, 2007.

Wang, J., Milner, R. Fibronectin promotes brain capillary endothelial cell survival and proliferation through $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins via MAP kinase signaling. J. Neurochem. 96:148, 2006.

Functional Genomics, Angiogenesis, and Gene Therapy in Transplantation and Retroviral Risks in Xenotransplantation

D.R. Salomon, S.M. Kurian, S. Cherqui, M. Auerbach, K. Marcucci, Y. Grigorev, J. Yang, D. Campbell, C. Marsh, S. Head,* J.R. Yates,** A. Nakorchevskiy,** P.Y. Kwok,*** J. Warrington,**** S. Horvath,***** J. Papp,***** C.A. Wilson†

* DNA Microarray Core, Scripps Research

** Department of Cell Biology, Scripps Research

*** University of California, San Francisco, California

**** Affymetrix, Santa Clara, California

***** University of California, Los Angeles, California

† Food and Drug Administration, Bethesda, Maryland

Successful transplantation requires the orchestration of complex mechanisms set in motion by surgical implantation of cells or organs into a patient. Regulation of the immune response with immunosuppressive drugs has received the most attention. But equally important is the unique cell biology of the transplanted tissue that evolves under stress after transplantation and ultimately determines the function of the transplant.

We use functional genomics to understand the expression and function of genes and proteins after transplantation. How do immunosuppressive drugs work at this fundamental level? What is the difference between a successful and an unsuccessful transplant? Another challenge is to develop an unlimited supply of healthy tissues for transplantation, for example, pancreatic islets to cure diabetes. Progenitor cells, including stem cells from adults, could be used to enhance angiogenesis, the formation of new blood vessels. Revascularization of cell transplants is a critical step in successful engraftment and function. We are showing how revascularization can be enhanced by gene therapy.

Animals could be used as donors, called xenotransplantation, although the potential risks for infectious disease inherent in using animal donors must be better understood so that this method can be used safely. In studies on xenotransplantation, we have focused on the risk associated with porcine endogenous retrovirus (PERV) in pig tissues.

FUNCTIONAL GENOMICS IN ORGAN TRANSPLANTATION

We are using high-density gene chip arrays, tandem mass spectrometry proteomics, and complex trait genetics based on single nucleotide polymorphisms to establish profiles to diagnose acute and chronic transplant rejection. These studies include patients with both kidney and liver transplants. A major objective is to identify new pathways that drive the immune response and cell biology of organ transplants that might be used as the next generation of targets for therapy. For example, with all current drug therapies, the target is the patient's immune response; none target the transplant itself, even though the function of the transplant is the ultimate determinant of success or failure.

We would like to test the hypothesis that gene expression profiles can be used to create a metric or simple diagnostic test for adequate immunosuppression. Physicians could then adjust a patient's drugs on the basis of an objective measure. Our long-term goal is to identify genes, proteins, and genetic polymorphisms that determine the outcome of a transplant to create a systems biology–based understanding of immunity at the molecular level.

ANGIOGENESIS AND GENE THERAPY

A major unmet medical need is a treatment for ischemic vascular diseases of the peripheral vasculature and the heart. We are using retroviral gene therapy to deliver novel proangiogenic molecules to endothelial progenitors that significantly enhance revascularization of transplanted islet cells. We have also created models for ischemia in the leg and in the heart and are testing the effect of transplanting a novel population of human stem cells from adults. In another project, we are using gene therapy and stem cells from adults to treat a congenital form of kidney failure.

XENOTRANSPLANTATION AND RISK FOR INFECTIOUS DISEASE

Although xenotransplantation is a logical strategy to address current shortages of human donor organs, a critical concern is the potential of moving infections from the animals to humans. We established a new mouse model for pig islet xenotransplantation, showed that multiple tissues become infected with PERV, identified the human receptors for this retrovirus, identified functional defects in nonhuman primate cells for viral infection, and continued to refine our understanding of the viral biology and potential risks in a new model of mice transgenic for the human receptor for PERV. The results of our studies in

the transgenic mice have provided the first evidence of a productive PERV infection in any animal (Fig. 1). We are now using this model to study the immune response and develop a vaccine. Our objective is to develop a vaccine to eliminate the risk of PERV and thus safely advance clinical islet xenotransplantation.

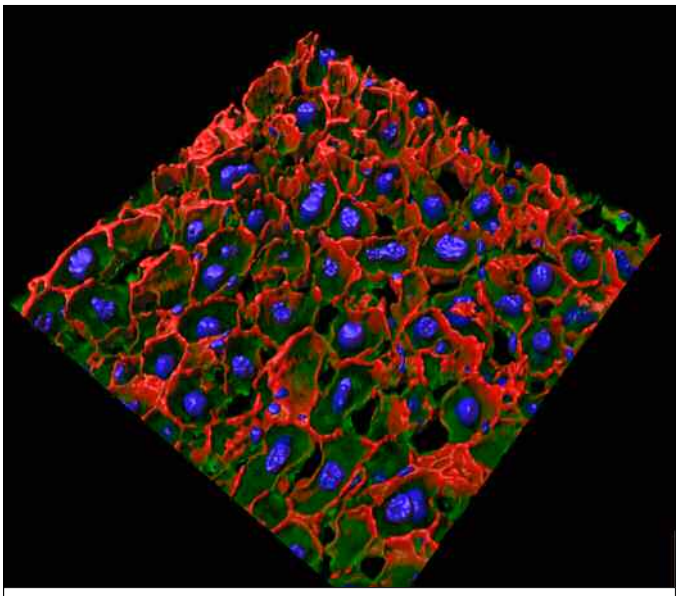

Fig. 1. Infection of the liver with PERV. Mice transgenic for the human receptor for PERV were exposed to PERV produced by pig cells, including via transplantation of pig islets. The micrograph shows widespread infection of hepatocytes. Infected cells are green, cell membranes are red, and nuclei are blue.

PUBLICATIONS

Cherqui, S., Kingdon, K.M.,Thorpe, C., Kurian, S.M., Salomon, D.R. Lentiviral gene delivery of vMIP-II to transplanted endothelial cells and endothelial progenitors is proangiogenic in vivo. Mol. Ther. 15:1264, 2007.

de Jonge, J., Kurian, S., Shaked, A., Salomon, D.R., Olthoff, K.M. Molecular networks of hepatic regeneration and metabolism in human living and deceased donor liver transplants. Hepatology, *in press*.

Flechner, S.M., Godfarb, D., Solez, K., Modlin, C.S., Mastroianni, B., Savas, K., Babineau, D., Kurian, S.M., Salomon, D.R., Novick, A.C., Cook, D.J. Kidney transplantation with sirolimus and mycophenolate mofetil-based immunosuppression: 5-year results of a randomized prospective trial compared to calcineurin inhibitor drugs. Transplantation 83:883, 2007.

Hermida-Prieto, M., Doménech, N., Moscoso, I., Diaz, T., Ishii, J., Salomon, D.R., Mañez, R. Lack of cross-species transmission of porcine endogenous retrovirus (PERV) to transplant recipients and abattoir workers in contact with pigs. Transplantation 84:548, 2007.

Niculescu, A.B., Lulow, L.L., Ogden, C.A., Le-Niculescu, H., Salomon, D.R., Schork, N.J., Caligiuri, M.P., Lohr, J.B. PhenoChipping of psychotic disorders: a novel approach for deconstructing and quantitating psychiatric phenotypes. Am. J. Med. Genet. B Neuropsychiatr. Genet. 141:653, 2006.

Solez, K., Colvin, R.B., Racusen, L.C., Sis, B., Halloran, P.F., Birk, P.E., Campbell, P.M., Cascalho, M., Collins, A.B., Demetris, A.J., Drachenberg, C.B., Gibson, I.W., Grimm, P.C., Haas, M., Lerut, E., Liapis, H., Mannon, R.B., Marcus, P.B., Mengel, M., Mihatsch, M.J., Nankivell, B.J., Nickeleit, V., Papadimitriou, J.C., Platt, J.L., Randhawa, P., Roberts, I., Salinas-Madriga, L., Salomon, D.R., Seron, D., Sheaff, M., Weening, J.J. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am. J. Transplant. 7:518, 2007.

Control of HIV Type 1, Gene Delivery, and Regulation of Hematopoietic Development

B.E. Torbett, G. Cauvi, L. Crisa, K.M. Fischer, G.E. Foos, M.J. Giffin, D. Vanitha John, P.A. McClintock, S. Miller, R.C. Prinsen, J.H. Savage, R. Schrier, C.H. Swan, J.A. Witkowski, A. Brik,* J.H. Elder,** H. Heaslet,** N.L. Letvin,*** Y.-C. Lin,** C.D. Stout,** M.P. Tschan,**** C.-H. Wong*

* Department of Chemistry, Scripps Research

** Department of Molecular Biology, Scripps Research

*** Beth Israel Deaconess Medical Center,, Boston, Massachusetts

**** University of Bern, Bern, Switzerland

Our research interests include the structural and biochemical evolution of the resistance of HIV type 1 (HIV-1) proteases, gene delivery strategies to disrupt cellular entry of HIV-1, and normal and abnormal regulation of myeloid development by the transcription factors PU.1 and cyclin D–interacting Myb-like protein (DMP1).

HIV-1 PROTEASE RESISTANCE

In patients infected with HIV-1, treatment with inhibitors of HIV reverse transcriptase, integrase, and protease suppresses replication of the virus. However, in some patients, HIV-1 variants evolve that escape the approved drug treatments by developing a broad-based resistance to the protease inhibitors. A molecular understanding of the resistance to protease inhibitors is needed so that new inhibitors can be developed to that target drug-resistant viruses and, importantly, are less likely to induce inhibitor-resistant viruses.

In collaboration with J.H. Elder, C.D. Stout, and H. Heaslet, Department of Molecular Biology, we showed that evolution of HIV-1 protease from a form susceptible to inhibitors to a form that is broadly resistant resulted in profound changes in the protease structure. Structural changes in the resistant proteases included alterations in the flap and basal regions and alteration from a symmetric to an asymmetric protease. To better understand how selected inhibitors disrupt function of resistant proteases, we used an inhibitor that targets and inhibits a resistant protease to probe the structure of the protease. We determined the necessity for interactions between the inhibitor and the protease backbone in the resistant protease. The structural changes that occur during the development of resistance and

the use of new protease inhibitors as chemical probes provide insight into the biochemical basis for the loss of activity of protease inhibitors. To better understand how structure contributes to the biochemical basis of resistance, we are continuing investigations on the relationship between structure and function in our wild-type proteases and in mutant proteases that are broadly resistant to inhibitors.

HIV-1 VECTOR DELIVERY OF CCR5-INTRABODY GENES TO HUMAN HEMATOPOIETIC CELLS

CXCR4 and CCR5 are the main chemokine receptors for HIV-1 entry into cells, and blocking these receptors limits entry of the virus. Naturally occurring polymorphisms of the gene for CCR5 indicate that disruption of the gene provides protection from viruses that use CCR5 to gain entry. Because polymorphisms are present in healthy persons, the use of genetic intervention strategies that prevent or limit expression of CCR5 may provide protection from initial infection and limit the spread of the virus.

With C.F. Barbas, Department of Molecular Biology, we showed that intracellular expression of a CCR5-specific single-chain antibody (intrabody) efficiently disrupted expression of CCR5 on the T-cell surface and protected cells from HIV-1 infection. Moreover, we found that human stem cells expressing the CCR5-intrabody develop into T cells and that the decreased expression of CCR5 protected cells against HIV-1 challenge and imparted a survival advantage in the presence of HIV-1 infection. Thus, it seems that gene delivery can provide gene programs that will protect and allow expansion of protected cells during HIV-1-infection.

Currently, we are disrupting the function of viruses that use either the CXCR4 or the CCR5 receptor for entry, the so-called R5X4 viruses. To accomplish our goals, we are using combination vectors that genetically target chemokine receptors and viral and cellular pathways critical for viral entry and replication.

MYELOID DIFFERENTIATION

PU.1, a member of the Ets family of transcription factors, is expressed solely in hematopoietic cells and is necessary for directing myeloid development and for regulating genes required for monocyte/macrophage and neutrophil function. PU.1 has 3 major domains: the transactivation, PEST, and Ets/DNA-binding domains. PU.1 interacts with other transcription factors, and domains of PU.1 have been implicated in its function.

Myeloid development is controlled by temporal gene expression of PU.1 and interactions among specific transcription factors. We are addressing which PU.1 domains regulate myeloid lineage–specific commitment, differentiation, and function. To determine which transcription factors interact with PU.1 and direct myeloid development, we use a strategy in which the gene for PU.1 is expressed only under certain conditions and a gene discovery and proteomics approach. These studies are enabling us to identify gene programs regulated by PU.1.

Cancer often originates from inactivation and/or deregulation of the control of gene expression. The transcription factor DMP1 positively regulates expression of human $p14^{ARF}$ and CD13/aminopeptidase N, thus playing a role in cell-cycle control, differentiation, and function of hematopoietic and nonhematopoietic cells. The tumor suppressor ARF is critical for positive regulation of p53, which in turn controls cellular proliferation and modulates apoptosis. We have identified 2 novel and developmentally expressed human DMP1 splice variants: β and γ. We found that the β variant functions as a dominant-negative regulator of the originally reported DMP1 protein. Currently, we are investigating the molecular and biological roles of the various isoforms in the development of normal and leukemic cells.

PUBLICATIONS

Heaslet, H., Lin, Y.-C., Tam, K., Torbett, B.E., Elder, J.H., Stout, C.D. Crystal structure of an FIV/HIV chimeric protease complexed with the broad-based inhibitor, TL-3. Retrovirology 4:1, 2007.

Heaslet, H., Rosenfeld, R., Giffin, M., Lin, Y.C., Tam, K., Torbett, B.E., Elder, J.H., McRee, D.E., Stout, C.D. Conformational flexibility in the flap domains of ligand-free HIV protease. Acta Crystallogr. D Biol. Crystallogr. 63(Pt. 8):866, 2007.

Lin, Y.-C., Brik, A., Parseval, A., Tam, K., Torbett, B.E., Wong, C.-H., Elder, J.H. Altered gag polyprotein cleavage specificity of feline immunodeficiency virus/human immunodeficiency virus mutant proteases as demonstrated in a cell-based expression system. J. Virol. 80:7832, 2006.

Manuel, E.R., Charini W.A., Sen, P., Peyerl, F.W., Kuroda, M.J., Schmitz, J.E., Autissier, P., Sheeter, D.A., Torbett, B.E., Letvin, N.L. Contribution of T-cell receptor repertoire breadth to the dominance of epitope-specific $CD8^+$ T-lymphocyte responses. J. Virol. 80:12032, 2006.

Rizzi, M., Tschan, M.P., Britschgi, C., Britschgi, A., Hügli, B., Grob, T.J., Leupin, N., Mueller, B.U., Simon, H.U., Ziemiecki, A., Torbett, B.E., Fey, M.F., Tobler, A. The death-associated protein kinase 2 is up-regulated during normal myeloid differentiation and enhances neutrophil maturation in myeloid leukemic cells. J. Leukoc. Biol. 81:1599, 2007.

Swan, C.H., Bühler, B., Steinberger, P., Tschan, M.P., Barbas, C.F. III, Torbett, B.E. T-cell protection and enrichment through lentiviral CCR5 intrabody gene delivery [published correction appears in Gene Ther. 14:626, 2007]. Gene Ther. 13:1480, 2006.

Swan, C.H., Torbett, B.E. Can gene delivery close the door to HIV-1 entry after escape? J. Med. Primatol. 35:23, 2006.

Tschan, M.P., Britschgi, C., Moser, B.A., Reddy, V.A., Sheeter, D.A., Fischer, K.M., Sun, P., Torbett, B.E. Human DMP1β is a negative regulator of the $p14^{ARF}$ tumor suppressor and promotes cellular proliferation. J. Biol. Chem., *in press*.

Yeamans, C., Wang, D., Paz-Priel, I., Torbett, B.E., Tenen, D.G., Friedman, A.D. C/EBPα binds and activates the PU.1 distal enhancer to induce monocyte lineage commitment. Blood, *in press*.

Division of Cellular Biology

James A. Hoch, Ph.D., Division Head

Sensor Kinases That Regulate Sporulation and the Synthesis of Toxins

J.A. Hoch, M. Perego, T. Fukushima, F. Scaramozzino, H. Szurmant, C. Bongiorni, A. Wilson

The complex developmental program of sporulation is under the control of the spo0 genes, which control entry of the cell into sporulation and the production of toxins and virulence factors in pathogens such as *Bacillus anthracis*. The transcription factor Spo0A is the key master regulator of the initiation of developmental transcription. The activity of Spo0A is controlled by a reversible phosphorylation-dephosphorylation mechanism.

In *B anthracis*, expression of the anthrax toxin, and presumably the potential of the toxin to cause pathologic changes, requires a low level of phosphorylated Spo0A. We have discovered a unique pathogenesis mechanism associated with the pXO1 plasmid of *B anthracis* that regulates low-level phosphorylation. The pXO1 plasmid encodes 2 genes whose proteins are highly homologous to the sensor domain of the sporulation sensor kinase BA2291. Expression of these proteins from the plasmid titrates the signal to activate BA2291, converting it from a kinase to a phosphatase of phosphorylated Spo0A. This mechanism in conjunction with the Rap phosphatase also encoded by pXO1 prevents high-level phosphorylation of Spo0A and sporulation while maintaining a low level that enables toxin expression.

The target of Spo0A regulation is the transcription factor AtxA, which is a required activator of expression of the genes that encode the anthrax toxin. We initiated transposon mutagenesis studies to identify additional regulators of *atxA* gene expression as well as mechanisms independent of AtxA. These studies have revealed genes for a heme–cytochrome *c* pathway and 2 unique genes encoded on the pXO1 plasmid required for *atxA* expression in addition to Spo0A. The mechanism by which this pathway directly affects *atxA* expression is unknown.

AtxA is a protein composed of a helix-turn-helix DNA recognition domain and 2 phosphenolpyruvate–sugar phosphotransferase system regulation domains (PRDs). Our mutational studies have shown that the phosphorylation of the histidine residues in the PRDs regulates the activity of AtxA; one of the PRDs requires phosphorylation for activity and the other acts as an inhibitor when phosphorylated. This nonclassical activity of PRDs indicates that unique phosphorylation mechanisms may be acting on these domains and that the regulation of AtxA activity may be much more complex than originally imagined. Indeed, the results of our transposon studies have confirmed the complexity of the regulation: we identified a large number of genes in which a mutation prevents toxin synthesis while allowing normal *atxA* gene expression. The complexity of expression of the genes for anthrax toxin and the relationship of the gene expression to normal cellular processes interrupted by pathogenesis plasmid genes is now being revealed.

Computational Analysis of Molecular Specificity in 2-Component Signaling

J.A. Hoch, R.A. White, H. Szurmant, T. Hwa*

* University of California, San Diego, California

In both prokaryotes and eukaryotes, a large number of pathways with proteins with identical structural folds are used to interpret and propagate vastly different signals specific for unique targets. A central question in understanding signal transduction is how does a signaling protein distinguish its true partner from the much larger number of similar partners present in the cell. In collaboration with T. Hwa, University of California, San Diego, we have developed a sequence-based method, independent of structural considerations, for identifying specificity-determining interactions between proteins for which genomic data indicate a large number of examples of functionally coupled pairs. This method was applied to the phosphotransfer domains of 2-component signaling proteins. Using this method, we identified a network of residue-residue interactions and generated a 3-dimensional structure consistent

with the exemplary cocrystal structure obtained for the Spo0B-Spo0F 2-component complex. We also identified an interaction network that links long-distance interactions with pair specificity of 2-component signaling proteins.

The method provides a simple scoring procedure that can be used to identify potential cross-phosphorylation between functional pairs and to assign orphan 2-component signaling proteins with no known mate to their signaling partners. Although currently we are applying the method to 2-component signal transduction systems for which structural and mutational data allow proof of principle, the method may generate interaction structures for less-characterized protein pairs if sufficient functional pairs exist in genomic data.

Molecular Dynamics of Response Regulators

J.A. Hoch, J. Cavanagh*

* North Carolina State University, Raleigh, North Carolina

Recognition specificity by sensor kinases in 2-component signal transduction depends on the composition of amino acid residues in the surface of the response regulator with which the sensor kinase interacts. Part of this surface of response regulators consists of several dynamic loops generated by the folding of strands and helices to form the core of the response regulator. Using nuclear magnetic resonance chemical-shift perturbation experiments with specific mutants of the Spo0F response regulator, we found that the conformation of the β4-β4 loop and the α4 helix dictates kinase specificity. The presentation of the loop, and therefore kinase recognition, can be altered by perturbations of core residues that propagate to the surface. These results further support our earlier hypothesis that molecular recognition processes are significantly influenced by intraprotein communication networks in the core of the response regulator. These networks are critical in providing the precise surface to the appropriate sensor kinase in signal transduction, for which hundreds of structurally similar proteins have evolved from gene duplication to carry out as many different signaling functions within the cell.

Negative Regulation of Development in Bacilli

M. Perego, C. Bongiorni

Initiation of spore formation in gram-positive bacilli is regulated by the phosphorelay signal transduction system. Multiple positive and negative signals are integrated by the phosphorelay through the opposing activities of histidine protein kinases and aspartyl phosphate phosphatases. The phosphatases belong to 2 families: Rap and Spo0E. Rap proteins act as negative regulators of the initiation of sporulation by dephosphorylating the Spo0F response regulator intermediate of the phosphorelay; the Spo0E proteins act as negative regulators of the phosphorelay by dephosphorylating the Spo0A response regulator and transcription factor for the initiation of sporulation.

In previous studies, we identified the Rap and Spo0E proteins that control the phosphorelay in *Bacillus subtilis* and *Bacillus anthracis*. Among the Rap proteins of *B subtilis*, RapA, RapB, and RapE inhibit initiation of sporulation by dephosphorylating the Spo0F protein. RapC and RapF inhibit the initiation of the early competence pathway to DNA transformation by preventing the ComA transcription factor from binding to its target DNA promoters. Recently, we characterized RapH as a dual-specificity protein that regulates both sporulation and competence. In in vivo and in vitro studies, we showed that RapH acts by dephosphorylating Spo0F and by inhibiting the DNA-binding activity of ComA. RapH creates a negative feedback loop that prevents sporulation while competence is in full development but also helps shut down competence to allow resumption of growth. In this way, RapH ensures the temporal separation of the 2 differentiation pathways of *B subtilis*, that is, competence and sporulation. Our current focus is to understand the molecular mechanism of the interactions of Rap proteins with their specific Phr peptide inhibitor or their target Spo0F.

Additionally, we have structurally characterized the Spo0E-family of proteins by determining the 3-dimensional structure of 2 members of the family from *B anthracis*. The BA1655 and BA5174 proteins are each composed of 2 antiparallel α-helices flanked by flexible regions at the termini. BA5174 is a monomer, and BA1655 is a dimer. The signature motif of amino acids (serine–glutamine–glutamic acid–leucine–aspartic

acid) involved in the interaction with the target Spo0A is situated in the middle of helix $\alpha 2$ with its polar residues projecting outward. The role of the signature motif in the phosphatase activity of Spo0E is being investigated.

Signal Transduction in *Enterococcus faecalis*

M. Perego

Enterococci are commensal bacteria within the intestinal tract in mammals but also can cause disease in compromised hosts. The acquisition of resistance to multiple antibiotics by enterococci makes infections caused by these microorganisms clinically challenging. The ability of the bacteria to adapt and respond to different environmental stimuli, including the host environment, led my group to investigate the role of 2-component signal transduction in the physiology and pathogenesis of *Enterococcus faecalis*.

We identified 17 2-component systems consisting of a sensory histidine kinase and a cognate response regulator. We inactivated each nonessential response regulator and tested the effect of the deletions on a number of physiologic conditions. We found defects in growth, antibiotic resistance, stress response, and formation of biofilms.

Analysis of the 2-component system encoded by the gene *fsr* revealed that this system is the only one that affects growth of enterococci as a biofilm on solid surfaces, because the system regulates the transcription of gelatinase, a zinc metalloprotease. In recent studies, we focused on the molecular characterization of the FsrA response regulator and transcription factor. We showed phosphorylation of FsrA by the FsrC histidine kinase in vitro and binding of FsrA to the promoter regions of *fsrC*, *fsrB*, and *gelE*, the gene that encodes gelatinase. Once we identify the conserved sequences for FsrA DNA-binding recognition, we will analyze the *E faecalis* genome sequence to complete the list of genes regulated by this transcription factor.

PUBLICATIONS

Bongiorni, C., Stoessel, R., Perego, M. Negative regulation of *Bacillus anthracis* sporulation by the Spo0E family of phosphatases. J. Bacteriol. 189:2637, 2007.

Grenha, R., Rzechorzek, N.J., Brannigan, J.A., de Jong, R.N., Ab, E., Diercks, T., Truffault, V., Ladds, J.C., Fogg, M.J., Bongiorni, C., Perego, M., Kaptein, R., Wilson, K.S., Folkers, G.E., Wilkinson, A.J. Structural characterization of Spo0E-like protein-aspartic acid phosphatases that regulate sporulation in bacilli. J. Biol. Chem. 281:37993, 2006.

McLaughlin, P.D., Bobay, B.G., Regel, E.J., Thompson, R.J., Hoch, J.A., Cavanagh, J. Predominantly buried residues in the response regulator Spo0F influence specific sensor kinase recognition. FEBS Lett. 581:1425, 2007.

Santelli, E., Liddington, R.C., Mohan, M.A., Hoch, J.A., Szurmant, H. The crystal structure of *Bacillus subtilis* YycI reveals a common fold for two members of an unusual class of sensor histidine kinase regulatory proteins. J. Bacteriol. 189:3290, 2007.

Smits, W.K., Bongiorni, C., Veening, J.W., Hamoen, L.W., Kuipers, O.P., Perego, M. Temporal separation of distinct differentiation pathways by a dual specificity Rap-Phr system in *Bacillus subtilis*. Mol. Microbiol. 65:103, 2007.

Szurmant, H., Mohan, M.A., Imus, P.M., Hoch, J.A. YycH and YycI interact to regulate the essential YycFG two-component system in *Bacillus subtilis*. J. Bacteriol. 189:3280, 2007.

Tsvetanova, B., Wilson, A.C., Bongiorni, C., Chiang, C., Hoch, J.A., Perego, M. Opposing effects of histidine phosphorylation regulate the AtxA virulence transcription factor in *Bacillus anthracis*. Mol. Microbiol. 63:644, 2007.

White, A.K., Hoch, J.A., Grynberg, M., Godzik, A., Perego, M. Sensor domains encoded in *Bacillus anthracis* virulence plasmids prevent sporulation by hijacking a sporulation sensor histidine kinase. J. Bacteriol. 188:6354, 2006.

Division of Experimental Pathology

Francis V. Chisari, M.D., Division Head

Molecular Biology of Hepatitis B and C Viruses and the Immune Response to Their Antigens

Hepatitis B and hepatitis C viruses are noncytopathic DNA and RNA viruses that cause acute and chronic hepatitis and hepatocellular carcinoma. More than 500 million people worldwide are chronically infected, and more than 2 million people die of these infections every year. The focus of our research is to unravel the life cycle of these viruses, discover the roles played by the innate and adaptive immune responses in the control of the infections, and elucidate the mechanisms responsible for viral clearance and disease pathogenesis. Our goal is to devise novel strategies to prevent and cure these infections.

Impact of Intrahepatic Antigen Recognition on Priming of the CD8$^+$ T-Cell Response

M. Isogawa, F.V. Chisari

By adoptively transferring memory CD8$^+$ T cells specific for hepatitis B virus (HBV) into HBV transgenic mice, we showed that the effector functions of memory T cells are induced in an oscillatory manner as a consequence of intrahepatic recognition of antigen. To define the immunologic events that occur during priming of HBV-specific T cells, we generated T-cell receptor transgenic mice that have CD8+ T cells specific for the HBV core and envelope proteins.

Naive T cells from the T-cell receptor transgenic mice were rapidly activated in the liver when transferred into HBV transgenic mice. The intrahepatically activated T cells vigorously proliferated in situ but did not develop antiviral effector functions such as secretion of IFN-γ and cytolytic ability. The results suggest that intrahepatic T-cell priming triggers the expansion of functionally defective T cells. If similar priming events occur during HBV infections in humans, the events could account for the characteristically weak CD8$^+$ T-cell responses in patients with chronic HBV infections.

Immunologic Priming by the Inoculum and Outcome of Hepatitis B Virus Infection

S. Asabe, S.F. Wieland, R.H. Purcell,* F.V. Chisari

* National Institutes of Health, Bethesda, Maryland

We previously showed that low-dose viral inocula lead to persistent hepatitis B virus infection in chimpanzees. To determine the mechanism responsible for persistent infection, we examined the peripheral CD4$^+$ T-cell response at multiple times after infection. Animals that received high-dose inocula and resolved the infection produced early T-cell responses before the appearance of viral antigens in the liver or serum, suggesting that the T cells were primed by noninfectious viral antigens in the inoculum. In contrast, animals that received low-dose inocula and became persistently infected did not have early T-cell responses, suggesting that the T cells had not been primed by the inocula. Analysis of the CD8$^+$ T-cell response in these animals indicated that the lack of early priming results in weak CD8$^+$ T-cell responses. These results suggest that immunologic priming by input antigen during low-dose viral infection determines the outcome of hepatitis B virus infection.

Impact of Chronic Hepatitis C Virus Infection on the Course of Hepatitis B Virus Superinfection

S.F. Wieland, R.H. Purcell,* F.V. Chisari

* National Institutes of Health, Bethesda, Maryland

Hepatitis C virus (HCV) infection induces an innate immune response that is reflected by the induction of many interferon-stimulated genes (ISGs) in the liver, yet the virus persists, presumably by defeating ISG-mediated antiviral functions in infected cells. In contrast, ISGs are not induced in the liver during initiation of hepatitis B virus (HBV) infection. To determine if the HCV-induced ISGs induce an antiviral state in the liver, we compared the kinetics and magnitude of HBV infection in HCV-naive and chronically HCV-infected chimpanzees.

As expected, the HCV-naive animals had typical resolving HBV infections. In contrast, HBV infection was strongly attenuated (4 logs) and delayed (6–15 weeks) in the chronically HCV-infected animals. These results suggest that the HBV replication space is severely limited to the small fraction of HCV-infected (i.e., ISG-resistant) hepatocytes in chronically infected liver.

Hepatitis C Virus Infection and Very Low-Density Lipoprotein

P. Gastaminza, F.V. Chisari

Intracellular infectious particles of hepatitis C virus (HCV) and precursors of very low-density lipoprotein have higher buoyant density than their secreted counterparts outside the cell. These biophysical differences probably reflect different biochemical compositions and suggest that both kinds of intracellular particles acquire lipids while leaving the cell. Synthesis of very low-

density lipoprotein involves the acquisition of triglycerides and cholesteryl esters by intracellular apolipoprotein B in a process catalyzed by microsomal transfer protein.

We found that an inhibitor of microsomal transfer protein and apolipoprotein B–specific short hairpin RNAs prevented the assembly and secretion of low-density HCV particles in infected cells. These findings suggest that assembly and secretion of HCV particles are tightly regulated by the very low-density lipoprotein metabolic machinery and that only mature, low-density HCV particles are secreted. Most newly assembled high-density infectious particles are degraded, suggesting that acquisition of apolipoprotein B and cellular lipids by HCV imparts a selective advantage as the virus adapts to its natural host.

Antiviral Activity of an Amphipathic α-Helical Peptide Derived From Nonstructural Protein 5A of Hepatitis C Virus

G. Cheng, A. Montero,* P. Gastaminza, C. Whitten-Bauer, S.F. Wieland, M. Isogawa, B. Fredericksen,** S. Selvarajah,*** P. Gallay,*** M.R. Ghadiri,* F.V. Chisari

* Department of Chemistry, Scripps Research

** University of Maryland, College Park, Maryland

*** Department of Immunology, Scripps Research

We recently identified a virucidal peptide derived from the membrane anchor domain of nonstructural protein 5A of hepatitis virus C (HCV) that efficiently inhibits both the initiation and the persistence of HCV infection in vitro. Additional studies indicated that the peptide blocks HCV infection by destabilizing HCV virions both extracellularly and intracellularly. The D-amino acid form of the peptide is fully active, and the D and L forms of the peptide have amphipathic α-helical structure and permeabilize artificial liposomes.

Mutational analysis indicated that the antiviral activity of the peptide depends on its membranolytic activity and α-helical structure, that amphipathicity is necessary but not sufficient for antiviral activity, and that antiviral activity depends on the amino acid composition but not the primary sequence of the peptide. Importantly, the antiviral activity of the peptide extends to other members of the Flaviviridae (West Nile virus and Dengue virus) and HIV, but not to numerous other RNA and DNA viruses. Collectively, these data indicate that the peptide may represent a novel therapeutic strategy for HCV, HIV, and other flavivirus infections.

Induction of a Common Antiviral Signaling Pathway in Human Cells by Double-Stranded DNA and Double-Stranded RNA

G. Cheng, J. Zhang, J. Chung, F.V. Chisari

Recent studies in murine systems showed that cytosolic double-stranded DNA triggers a potent type I interferon response that requires downstream components of the double-stranded RNA signaling pathway. The mechanism of cytosolic double-stranded DNA recognition is currently unknown. We found that cytosolic double-stranded DNA is a potent inducer of IFN-β promoter activation in human hepatoma Huh-7 cells and that this activation requires both the intracellular double-stranded RNA sensor retinoic acid–induced gene I and its adaptor molecule mitochondrial antiviral signaling protein, in addition to the downstream mediators TBK-1, IKK-ε, and IRF-3. These findings indicate that the innate immune signaling pathways induced by double-stranded DNA and double-stranded RNA share more components in human cells than originally thought, although double-stranded DNA appears to trigger that pathway upstream of the innate double-stranded RNA–interacting sensor retinoic acid–induced gene I.

PUBLICATIONS

Cheng, G., Zhong, J., Chung, J., Chisari, F.V. Double-stranded DNA and double-stranded RNA induce a common antiviral signaling pathway in human cells. Proc. Natl. Acad. Sci. U. S. A. 104:9035, 2007.

Dryden, K.A., Wieland, S.F., Whitten-Bauer, C., Gerin, J.L., Chisari, F.V., Yeager, M. Native hepatitis B virions and capsids visualized by electron cryomicroscopy. Mol. Cell 22:843, 2006.

Gastaminza, P., Kapadia, S.B., Chisari, F.V. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. J. Virol. 80:11074, 2006.

Kapadia, S.B., Barth, H., Baumert, T., McKeating, J.A., Chisari, F.V. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J. Virol. 81:374, 2007.

Maier, H., Isogawa, M., Freeman, G.J., Chisari, F.V. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific $CD8^+$ T lymphocytes in the liver. J. Immunol. 178:2714, 2007.

Robek, M.D., Garcia, M.L., Boyd, B.S., Chisari, F.V. Role of immunoproteasome catalytic subunits in the immune response to hepatitis B virus. J. Virol. 81:483, 2007.

Sainz, B., Jr., Chisari, F.V. Production of infectious hepatitis C virus by well-differentiated, growth-arrested human hepatoma-derived cells. J. Virol. 80:10253, 2006.

Sakai, A., Takikawa, S., Thimme, R., Meunier J.-C, Spangenberg, H.C., Govindarajan, S., Farci, P., Emerson, S.U., Chisari, F.V., Purcell, R.H., Bukh, J. In vivo study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone (pHC-TN) are infectious in chimpanzees but not in Huh7.5 cells. J. Virol. 81:7208, 2007.

Sidney, J., Asabe, S., Peters, B., Purton, K.A., Chung, J., Pencille, T.J., Purcell, R., Walker, C.M., Chisari, F.V., Sette, A. Detailed characterization of the peptide binding specificity of five common Patr class I MHC molecules. Immunogenetics 58:559, 2006.

Uprichard, S.L., Chung, J., Chisari, F.V., Wakita, T. Replication of a hepatitis C virus replicon clone in mouse cells. Virol. J. 3:89, 2006.

Zhong, J., Gastaminza, P., Chung, J., Stamataki, J., Isogawa, M., Cheng, G., McKeating, J.A., Chisari, F.V. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. 80:11082, 2006.

Reduction in the Severity of Liver Disease by Antiplatelet Therapy in a Mouse Model of Acute Viral Hepatitis

L.G. Guidotti, M. Iannacone, G. Sitia, Z.M. Ruggeri

Using mice acutely infected with RAd35, a hepatotropic, replication-deficient, lacZ-expressing adenovirus, we recently showed that platelets accumulate within the inflamed liver and that their depletion diminishes disease severity by reducing the intrahepatic number of both RAd35-specific cytotoxic T lymphocytes and RAd35-nonspecific inflammatory lymphocytes that the T cells help recruit. Transfusion of normal, but not activation-blocked, platelets into thrombocytopenic animals restored these events, indicating that platelet activation plays a crucial pathogenetic role in this model.

In other studies, aspirin and clopidogrel, 2 drugs that target the proactivating functions of the platelet agonists thromboxane A_2 and ADP, respectively, profoundly reduced both liver injury and recruitment of cytotoxic T lymphocytes and inflammatory intrahepatic lymphocytes when the drugs were administered together to RAd35-infected mice previously immunized with lacZ. Besides impairing platelet function, aspirin and clopidogrel also prevented viral clearance from the liver and caused neither bleeding nor anemia. These same drugs were somewhat less efficient at diminishing liver injury when administered separately.

Thus, antiplatelet therapy diminishes immune-mediated liver disease in a model of acute viral hepatitis. This notion may help in devising new approaches to limit excessive liver immunopathologic changes (as during fulminant hepatitis in humans) or delay viral clearance (as demanded by gene therapy–based procedures with these and other hepatotropic viral vectors).

Platelet-Mediated Viral Clearance and Protection From Lethal Hemorrhage in Mice Infected With Lymphocytic Choriomeningitis Virus

L.G. Guidotti, M. Iannacone, G. Sitia, M. Isogawa, J.K. Whitmire,* P. Marchese, F.V. Chisari, Z.M. Ruggeri

* Department of Molecular and Integrative Neurosciences, Scripps Research

We found that infection of mice with different isolates of lymphocytic choriomeningitis virus (LCMV) induced a mild hemorrhagic anemia, which became severe and eventually lethal in animals depleted of platelets or lacking integrin β_3. Lethal hemorrhagic anemia is mediated by virus-induced IFN-α/β that causes platelet dysfunction, mucocutaneous blood loss, and suppression of erythropoiesis. In addition, platelet-depleted mice did not mount an efficient cytotoxic T-lymphocyte response and did not clear LCMV from liver, spleen, and other infected organs, including the brain. Transfusion of functional platelets into these animals reduced hemorrhage, thereby preventing death, and restored viral clearance induced by cytotoxic T lymphocytes in a manner partially dependent on CD40 ligand. These results indicate that upon activation, platelets expressing integrin β_3 and CD40 ligand are required for protecting the host against the induction of an IFN-α/β–dependent lethal hemorrhagic diathesis and for clearing LCMV infection via cytotoxic T lymphocytes.

PUBLICATIONS

Brown, B.D., Sitia, G., Annoni, A., Hauben, E., Sergi, L.S., Zingale, A., Roncarolo, M.G., Guidotti, L.G., Naldini, L. In vivo administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. Blood 109:2797, 2007.

Iannacone, M., Sitia, G., Ruggeri, Z.M., Guidotti, L.G. HBV pathogenesis in animal models: recent advances on the role of platelets. J. Hepatol. 46:719, 2007.

Pulitanò, C., Sitia, G., Aldrighetti, L., Finazzi, R., Arru, M., Catena, M., Guidotti, L.G., Ferla, G. Reduced severity of liver ischemia/reperfusion injury following hepatic resection in humans is associated with enhanced intrahepatic expression of Th2 cytokines. Hepatol. Res. 36:20, 2006.

Sitia, G., De Bona, A., Bagaglio, S., Galli, L., Paties, C., Uberti-Foppa, C., Guidotti, L.G., Lazzarin, A., Morsica, G. Naive HIV/HCV-coinfected patients have higher intrahepatic pro-inflammatory cytokines than coinfected patients treated with antiretroviral therapy. Antivir. Ther. 11:385, 2006.

Sitia, G., Iannacone, M., Müller, S., Bianchi, M.E., Guidotti, L.G. Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice. J. Leukoc. Biol. 81:100, 2007.

Trifari, S., Sitia, G., Aiuti, A., Scaramuzza, S., Marangoni, F., Guidotti, L.G., Martino, S., Saracco, P., Notarangelo, L.D., Roncarolo, M.G., Dupré, L. Defective Th1 cytokine gene transcription in $CD4^+$ and $CD8^+$ T cells from Wiskott-Aldrich syndrome patients. J. Immunol. 177:7451, 2006.

DIVISION OF HEMATOLOGY AND GENETICS

Ernest Beutler, M.D., Division Head

Regulation of Hepcidin

E. Beutler, K. Crain, J. Flanagan, T. Gelbart, P. Lee, H. Peng, J. Truksa, J. Waalen

It has been known for many years that body iron content is normally tightly regulated. Iron deficiency increases iron absorption, and iron loading decreases iron absorption. However, the basis of this "mucosal intelligence" remained entirely obscure. Hereditary hemochromatosis is a disorder in which the regulatory system does not function normally, and large amounts of iron accumulate, causing tissue damage. The discovery of the role of hepcidin, a 25 amino acid peptide, has greatly furthered our understanding of the regulation of iron homeostasis, and we now realize that it is dysregulation of hepcidin transcription that is the cause of most forms of hemochromatosis. But how is this transcription regulated? The short answer is that we do not know.

In intact animals, and to the extent that studies have been performed in humans, hepcidin is upregulated by iron and by inflammatory cytokines induced by endotoxin and is downregulated by anemia and by hypoxia. Although in primary hepatocytes and in many cells lines hepcidin transcription is regulated in a similar manner by the inflammatory cytokines IL-6, IL-1α, and IL-1β and by hypoxia as in intact animals, the effect of iron on hepcidin transcription in cell cultures or primary hepatocytes is the opposite of that in intact animals. This paradoxical effect is unexplained. Apparently an iron-sensing and signaling mechanism exists in vivo that does not operate in the in vitro systems. In an effort to identify the components of a signaling mechanism, we have performed many studies in which we have coincubated primary murine hepatocytes with a variety of tissues, including macrophages, marrow stromal cells, and marrow hematopoietic cells, without being able to recapitulate the course of events observed when iron is administered in vivo.

Because transcription of hepcidin in HepG2 cells responds to IL-6 and to bone morphogenetic proteins (BMPs), it is possible to identify sequences in the promoter that are required for these responses. We have performed such studies by using the conventional approach of attaching promoters of various lengths to a firefly luciferase reporter and comparing the light output with a cotransfected renilla luciferase control. It had been shown by others that the proximal part of the promoter contains elements important for the response of hepcidin transcription to IL-6, but apparently further upstream sequences increase the IL-6 response, and the response to BMP depends strongly on far-upstream elements that we are currently defining (Fig. 1). We

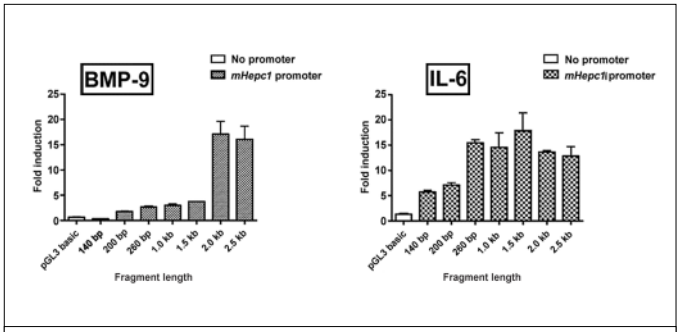

Fig. 1. Induction of a luciferase reporter with *mHepc1* promoter fragments of different lengths transfected into HepG2 cells. Even short promoter fragments support the induction of luciferase expression when IL-6 is used as a stimulus (right panel), but only fragments longer than 1.5 kb are effective when BMP-9 is the stimulus.

were also able to demonstrate that no downstream elements were located in introns or 3′ or 5′ untranslated regions that affected hepcidin expression.

Nuclear extracts have been prepared from mice fed high- and low-iron diets. Attention has been focused on certain candidate areas that are of interest because of a high level of homology between human and murine hepcidin genes and because of results obtained with footprinting.

To narrow the regions that are critical for iron responsiveness in the hepcidin promoter, we have made probes that corresponded to conserved regions between human and murine hepcidin genes. By gel-shift analyses, we have been able to determine which probes are differ-

entially bound by proteins in iron-deficient and high-iron nuclear extracts. For example, we found differential binding of nuclear factors to probes hybridizing with 2 regions: –1797 to –1754 and –1680 to –1641 (Fig. 2).

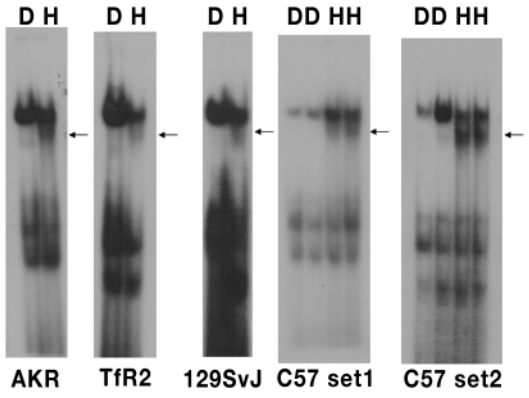

Fig. 2. Gel shift analysis of a probe composed of nucleotides –1797 to –1754 with nuclear extracts from a pool of 4–8 mice fed an iron-deficient diet (D) or 4–8 mice fed a high-iron diet (H) for more than 4 weeks. Mice from 4 strains, as indicated at the bottom of each gel, were investigated.

To identify provisionally iron-responsive transcription factors that regulate hepcidin transcription, we determined which liver-specific transcription factors are differentially expressed between iron-deficient and iron-loaded mice (>4 weeks). We used a protein/DNA array method to identify both transcriptional activators and repressors (Fig. 3). This method also permits identification of coactivators or corepressors that may

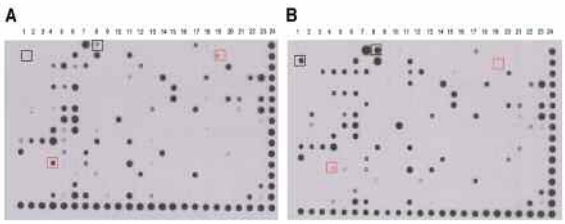

Fig. 3. Protein/DNA array of transcription factors. Nuclear extracts (pool of 8 mice) from 129 strain mice fed an iron-deficient (A) or a high-iron (B) diet for 4 weeks were used to select specific biotinylated probes that were then used to bind to the array membrane. Red squares indicate examples of transcription factors that are upregulated in iron-deficient nuclear extracts; black squares, examples of transcription factors upregulated in iron overload. The chemiluminescent exposure was 10 minutes.

not directly bind to the DNA but may associate with the DNA-binding proteins. Several exposures were obtained in order to identify high- and low-abundance transcription factors within the factors' dynamic range. In this particular experiment, we identified 43 and 39 transcription factors that were upregulated in iron-overload and iron-deficient nuclear extracts, respectively. Of these, 10 and 4 transcription factors from iron-overload and iron-deficient nuclear extracts, respectively, have corresponding consensus motifs in the hepcidin promoter.

Because hepcidin transcription is not stimulated by iron in ex vivo systems, we have devised a method for measuring the response of the hepcidin promoter in vivo. We hydrodynamically transfect mice with constructs containing fragments of the hepcidin promoter fused to a firefly luciferase (*luc*) reporter. To elicit a response to iron, we maintained mice for at least 2 weeks on an iron-poor diet containing only 2–5 ppm of iron and then fed them a diet containing 2 x 10^4 ppm iron for 24 hours. Luciferase expression driven by the different hepcidin promoter regions was measured in the intact animals by using a live imaging instrument.

The results of our studies are shown in Figures 4 and 5. It is apparent that a region of the promoter

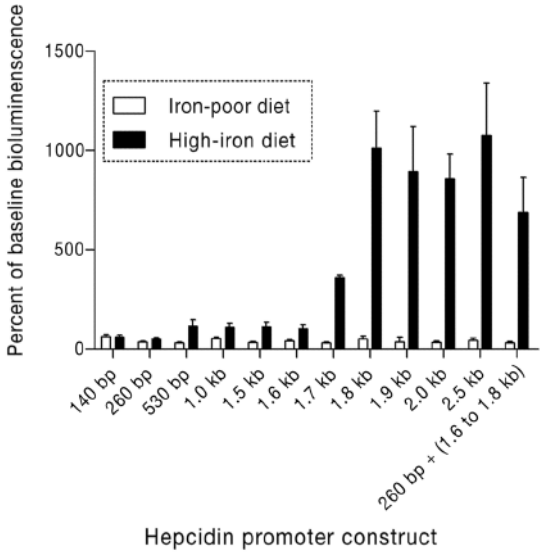

Fig. 4. Location of the iron-responsive element in vivo. Mice fed an iron-poor diet (2–5 ppm) were hydrodynamically transfected with a pGL3 reporter plasmid containing the firefly luciferase gene (*luc*) under the control of various lengths of the murine hepcidin 1 (*mHepc1*) promoter. After 3 days, the basal level of bioluminescence was determined, and mice were divided into 2 groups. The first group received a high-iron diet (2 x 10^4 ppm); the second group remained on the iron-poor diet. After 24 hours, bioluminescence was measured; the results were expressed as a percentage of the basal level bioluminescence. The number of base pairs upstream of the start of translation is given for each promoter construct. The construct designated 260 bp + (1.6 to 1.8 kb) contains the first 260 bp and the part of the promoter between 1.6 and 1.8 kb after the start of translation. Each group consisted of at least 5 animals. The error bars represent 1 SE of the mean.

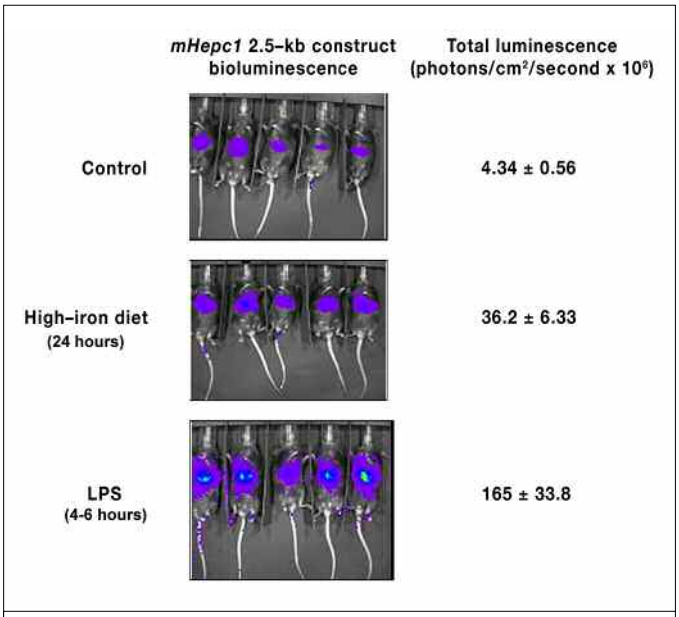

Fig. 5. Bioluminescence in mice hydrodynamically transfected with a hepcidin promoter fused to firefly luciferase. LPS = lipopolysaccharide.

between 1.6 and 1.8 kb upstream from the start of translation is required for the response to iron. This region, together with the first 260 bp of the promoter, is sufficient to provide a near-maximal response to iron stimulation. Interestingly, this region is the same one required for the in vitro response of HepG2 cells to stimulation with BMP-4 or BMP-9 (Fig. 2).

Our database of 41,000 subjects participating in the hemochromatosis screening study with Kaiser-Permanente has facilitated epidemiologic investigation of fundamental questions in clinical hematology, including one even as basic as what is the definition of anemia. For most epidemiologic studies, the answer has been assumed to be simple: on the basis of recommendations from the World Health Organization, anemia is defined as hemoglobin levels less than 13 g/dL for men and less than 12 g/dL for women. However, analysis of our data has shown that the distribution of hemoglobin values varies significantly, not only between men and women but also between subjects of different ethnic origins; African Americans have hemoglobin values that are, on average, 0.6 g/dL lower than those of whites. In addition, hemoglobin values decline significantly with age among men, but not among women, and in all ethnic groups. We have validated these findings by using data from the National Health and Nutrition Examination Survey, a population-based, representative sample of the U.S. population. The work has established new sex-, age-, and ethnicity-specific reference values for the definition of anemia. The studies have provided the basis for further investigation into the biological mechanisms underlying ethnic differences in hemoglobin levels and the anemia of aging.

PUBLICATIONS

Bartfai, T., Waalen, J., Buxbaum, J.N. Adipose tissue as a modulator of clinical inflammation: does obesity reduce the prevalence of rheumatoid arthritis? J. Rheumatol. 34:488, 2007.

Barton, J.C., Lee, P.L., West, C., Bottomley, S.S. Iron overload and prolonged ingestion of iron supplements: clinical features and mutation analysis of hemochromatosis-associated genes in four cases. Am. J. Hematol. 81:760, 2006.

Beutler, E. Hematopoietic cell transplantation in the future. *In*: Thomas' Hematopoietic Cell Transplantation, 4th ed. Forman, S.J., Negrin, R.S., Blume, K. (Eds.). Blackwell Science, Boston, *in press*.

Beutler, E. Hemochromatosis. *In:* Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine. Ganten, D., Ruckpaul, K. (Eds.). Springer, New York, 2006, p. 758.

Beutler, E. Hepcidin mimetics from microorganisms? A possible explanation for the effect of *Helicobacter pylori* on iron homeostasis. Blood Cells Mol. Dis. 38:54, 2007.

Beutler, E. PGK deficiency. Br. J. Haematol. 136:3, 2007.

Beutler, E., Gelbart, T. *Helicobacter pylori* infection and *HFE* hemochromatosis. Blood Cells Mol. Dis. 37:188, 2006.

Flanagan, J.M., Beutler, E. The genetic basis of human erythrocyte pyridoxal kinase activity variation. Haematologica 91:801, 2006.

Flanagan, J.M., Gerber, A.L., Cadet, J.L., Beutler, E., Sipe, J.C. The fatty acid amide hydrolase 385 A/A (P129T) variant: haplotype analysis of an ancient missense mutation and validation of risk for drug addiction. Hum. Genet. 120:581, 2006.

Flanagan, J.M., Peng, H., Beutler, E. Effects of alcohol consumption on iron metabolism in mice with hemochromatosis mutations. Alcohol. Clin. Exp. Res. 31:138, 2007.

Flanagan, J.M., Peng, H., Wang, L., Gelbart, T., Lee, P., Sasu, B.J., Beutler, E. Soluble transferrin receptor-1 levels in mice do not affect iron absorption. Acta Haematol. 116:249, 2006.

Flanagan, J.M., Rhodes, M., Wilson, M., Beutler, E. The identification of a recurrent phosphoglycerate kinase mutation associated with chronic haemolytic anaemia and neurological dysfunction in a family from USA. Br. J. Haematol. 134:233, 2006.

Flanagan, J.M., Truksa, J., Peng, H., Lee, P., Beutler, E. In vivo imaging of hepcidin promoter stimulation by iron and inflammation. Blood Cells Mol. Dis. 38:253, 2007.

Lichtman, M.A., Beutler, E., Kaushansky, K., Kipps, T.J., Seligsohn, U., Prchal, J. (Eds.). Williams Hematology, 7th ed. McGraw-Hill, New York, 2006.

Rana, B.K., Insel, P.A., Payne, S.H., Abel, K., Beutler, E., Ziegler, M.G., Schork, N.J., O'Connor, D.T. Population-based sample reveals gene-gender interactions in blood pressure in white Americans. Hypertension 49:96, 2007.

Repiso, A., Oliva, B., Vives-Corrons, J.-L., Beutler, E., Carreras, J., Climent, F. Red cell glucose phosphate isomerase (GPI): a molecular study of three novel mutations associated with hereditary nonspherocytic hemolytic anemia. Hum. Mutat. 27:1159, 2006.

Truksa, J., Peng, H., Gelbart, T., Lee, P., Beutler, E. Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. Proc. Natl. Acad. Sci. U. S. A. 103:10289, 2006.

Zimran, A., Elstein, D., Beutler, E. Low-dose therapy trumps high-dose therapy again in the treatment of Gaucher disease. Blood 108:802, 2006.

DIVISION OF MOLECULAR ONCOLOGY

Thomas F. Deuel, M.D., Division Head

Pleiotrophin: A Cytokine With Critical Roles in Growth and in Development and Progression of Human Neoplasms

T.F. Deuel, Y. Chang, P. Perez-Pinera, W. Zhang

We previously identified and cloned pleiotrophin, an 18-kD cytokine with diverse roles in normal growth and in the development and progression of malignant tumors. Pleiotrophin signals by inactivating the receptor protein tyrosine phosphatase (RPTP) β/ζ. Inactivation of RPTPβ/ζ leads to increased levels of tyrosine phosphorylation of the substrates of RPTPβ/ζ by unknown tyrosine kinases that phosphorylate the same sites that normally are dephosphorylated by RPTPβ/ζ in cells not stimulated with pleiotrophin. Known substrates of RPTPβ/ζ include β-catenin, β-adducin, Fyn, histone deacetylase 2, anaplastic lymphoma kinase (ALK), and TrkA, the receptor of nerve growth factor. Through this unique signaling mechanism, pleiotrophin regulates levels of tyrosine phosphorylation of important proteins in different cellular systems.

AN ALTERNATIVE MECHANISM OF RECEPTOR TYROSINE KINASE ACTIVATION

Receptor protein tyrosine kinases such as the platelet-derived growth factor (PDGF) receptor undergo ligand-enforced dimerization and induce conformational changes in the active-site domains of the receptor that initiate autophosphorylation and autoactivation. In the past year, we found that pleiotrophin stimulates tyrosine phosphorylation of ALK through a mechanism entirely different from that of PDGF with the PDGF receptor; the pleiotrophin-stimulated tyrosine phosphorylation and activation of ALK are the consequence of the pleiotrophin-enforced dimerization of its cognate receptor, RPTPβ/ζ, and occurs without a direct interaction of pleiotrophin with the extracellular domain of ALK. We found that chemically enforced dimerization of RPTPβ/ζ alone is sufficient to stimulate tyrosine phosphorylation of ALK, that ALK is a substrate of RPTPβ/ζ, and that ALK phosphorylated through enforced dimerization of RPTPβ/ζ is dephosphorylated by RPTPβ/ζ at the same site in ALK that is phosphorylated when RPTPβ/ζ is inactivated through enforced dimerization of RPTPβ/ζ. We also showed that pleiotrophin activates the tyrosine kinase activity of ALK in pleiotrophin-stimulated cells and that the activated ALK is the kinase that phosphorylates β-catenin in pleiotrophin-stimulated cells. The site phosphorylated in β-catenin by ALK furthermore is recognized and dephosphorylated by RPTPβ/ζ.

These studies suggest a model in which in unstimulated cells, the tyrosine phosphatase activity of RPTPβ/ζ regulates the steady-state levels of tyrosine phosphorylation of ALK. The levels of expression of pleiotrophin thus critically regulate and determine the relative catalytic activity of RPTPβ/ζ through the degree to which pleiotrophin enforces dimerization and thus inactivation of RPTPβ/ζ. In pleiotrophin-stimulated cells, inactivation of RPTPβ/ζ permits increased tyrosine phosphorylation of ALK that is not restrained by the tyrosine phosphatase activity of RPTPβ/ζ. These findings thus suggest that the levels of expression of pleiotrophin regulate the steady-state levels of the tyrosine phosphatase activity of RPTPβ/ζ, which, in turn, regulates its steady-state levels of tyrosine phosphorylation of ALK and, presumably, regulates the steady-state catalytic activity. We have also identified an alternative mechanism of activation of receptor protein tyrosine kinases.

Our results also indicate that β-catenin is a downstream target of ALK. This tyrosine phosphorylation site in β-catenin is potentially important, because when it is phosphorylated in pleiotrophin-stimulated cells, it disrupts the association of β-catenin with N-cadherin needed for cells to adhere to each other. Because disruption of homophilic cell-cell adhesion is characteristic of highly malignant cells that express *Ptn*, the gene for pleiotrophin, our data suggest that one mechanism through which pleiotrophin stimulates a more aggressive phenotype in malignant cells is disruption of normal cytoskeletal architecture.

BREAST CANCER

In previous studies of pleiotrophin in neoplasia, we used a dominant-negative *Ptn* and found that it reversed the malignant phenotype of human breast cancer cells in vitro and in vivo. We have now found that pleiotrophin signaling cooperates with the signaling pathways stimulated by *PyMT*, the gene for polyoma virus middle

T antigen, driven by the mouse mammary tumor virus (MMTV) promoter in a well-established mammary tumor model. MMTV-*Ptn* expression in MMTV-PyMT-*Ptn* transgenic mice induced rapid growth of morphologically identified foci of "scirrhous" carcinoma; tumor angiogenesis; remodeling of the microenvironment; and striking increases in mouse protocollagens Iα2, IVα5, and XIα1 and in tropoelastin in the breast cancers of MMTV-PyMT-*Ptn* mice compared with breast cancers of MMTV-PyMT mice. The data establish that inappropriate expression of *Ptn* promotes breast cancer progression to a tumor stage with characteristics of the most advanced and dangerous of human breast cancers. These findings add to the growing recognition of the roles of stromal cells and paracrine signaling in the progression of tumors.

Midkine also signals through RPTPβ/ζ. To establish the relevance of the studies in MMTV-PyMT-*Ptn* transgenic mice to human breast cancer, we showed that each of the components of the pleiotrophin-midkine/RPTPβ/ζ-ALK signaling pathway, that is, pleiotrophin, midkine, RPTPβ/ζ, and ALK, is expressed in human breast cancers, suggesting this pathway may be relevant in the pathogenesis of human breast cancer. Surprisingly, the immunohistochemical patterns of RPTPβ/ζ and ALK in breast cancers relative to normal breast tissue were different in each of the breast cancers studied, supporting the possibility that this pathway may be important in the pathogenesis of breast cancer. Although the basis of the different patterns of RPTPβ/ζ and ALK are unknown, the data suggest that ALK may be constitutively activated through RPTPβ/ζ and thus may be a major factor in the pathogenesis of human breast cancer.

PUBLICATIONS

Chang, Y., Berenson, J.R., Wang, Z., Deuel, T.F. Dominant negative pleiotrophin induces tetraploidy and aneuploidy in U87MG human glioblastoma cells. Biochem. Biophys. Res. Commun. 351:336, 2006.

Chang, Y., Zuka, M., Perez-Pinera, P., Astudillo, A., Mortimer, J., Berenson, J.R., Deuel, T.F. Secretion of pleiotrophin stimulates breast cancer progression through remodeling of the tumor microenvironment. Proc. Natl. Acad. Sci. U. S. A. 104:10888, 2007.

Chen, H., Gordon, M.S., Campbell, R.A., Li, M., Wang, C.S., Lee, H.J., Sanchez, E., Manyak, S.J., Gui, D., Shalitin, D., Said, J., Chang, Y., Deuel, T.F., Baritaki, S., Bonavida, B., Berenson, J.R. Pleiotrophin is highly expressed by myeloma cells and promotes myeloma tumor growth. Blood 110:287, 2007.

Deuel, T.F., Chang, Y. Growth factors. *In:* Principles of Tissue Engineering, 3rd ed. Lanza, R., Langer, R., Vacanti, J.P. (Eds.). Elsevier, Philadelphia, 2007, p. 193.

Ezquerra, L., Perez-Garcia, C., Garrido, E., Diez-Fernandez, C., Deuel, T.F., Alguacil, L.F., Herradon, G. Morphine and yohimbine regulate midkine gene expression in the rat hippocampus. Eur. J. Pharmacol. 557:147, 2007.

Perez-Pinera, P., Alcantara, S., Dimitrov, T., Vega, J.A., Deuel, T.F. Pleiotrophin disrupts calcium-dependent homophilic cell-cell adhesion and initiates an epithelial-mesenchymal transition. Proc. Natl. Acad. Sci. U. S. A. 103:17795, 2006.

Perez-Pinera, P., Chang, Y., Astudillo, A., Mortimer, J., Deuel, T.F. Anaplastic lymphoma kinase is expressed in different subtypes of human breast cancer. Biochem. Biophys. Res. Commun. 358:399, 2007.

Perez-Pinera, P., Garcia-Suarez, O., Menendez-Rodriguez, P., Mortimer, J., Chang, Y., Astudillo, A., Deuel, T.F. The receptor protein tyrosine phosphatase (RPTP)β/ζ is expressed in different subtypes of human breast cancer. Biochem. Biophys. Res. Commun. 362:5, 2007.

Perez-Pinera, P., Zhang, W., Chang, Y., Vega, J.A., Deuel, T.F. Anaplastic lymphoma kinase (ALK) is activated through the pleiotrophin (PTN)/receptor protein tyrosine phosphatase (RPTP)β/ζ signaling pathway: an "alternative mechanism of receptor tyrosine kinase (RTK) activation." J. Biol. Chem., *in press*.

Checking on DNA During Replication

X. Wu, A.Y.-L. Lee, C. Nievera, E. Olson, T. Chiba

Genome instability is a hallmark of the malignant phenotype and a driving force for tumorigenesis. S phase is genetically the most vulnerable period of the cell cycle. In this phase, DNA must be replicated faithfully in a timely fashion, and the entire genome must be duplicated exactly once per cell cycle. Cell-cycle checkpoints have evolved to monitor the integrity of the eukaryotic genome and ensure the completion of DNA replication and the repair of DNA damage before progression of the cycle.

In one area of our research, we focus on a disease-linked complex termed Mre11/Rad50/Nbs1 (MRN), which participates in multiple pathways to maintain genome stability. In humans, hypomorphic mutations in the genes *NBS1* and *MRE11* lead to Nijmegen breakage syndrome and ataxia-telangiectasia–like disorder, respectively. Cells from patients with Nijmegen breakage syndrome or ataxia-telangiectasia–like disorder have a defect in the intra-S-phase checkpoint, but the molecular mechanisms are unclear.

Recently, we found that MRN directly interacts with replication protein A in unperturbed cells and that this interaction is needed for MRN to correctly localize to replication centers. Abolishing the interaction of Mre11 with replication protein A leads to pronounced radioresistant DNA synthesis, without affecting phosphorylation of Nbs1 or structural maintenance of chromosomes protein 1 after ionizing radiation. Moreover, MRN is recruited to sites at or adjacent to replication origins by replication protein A and acts there to inhibit new origin firing upon ionizing radiation. These findings suggest that MRN at sites proximal to the origin of chromosomal replication has a direct role in controlling the initiation

of DNA replication in response to DNA damage, thereby providing an important mechanism underlying the intra-S-phase checkpoint in mammalian cells.

The second focus of our research is understanding how DNA replication is controlled so that DNA is replicated once and only once per cell cycle. Rereplication of the genome, or even a segment of it, could lead to genome instability. We found that the S-phase checkpoint mediated by the ataxia telangiectasia–mutated and Rad3-related (ATR) pathway acts as a surveillance mechanism to prevent rereplication, so that disruption of licensing control by the overexpression of the licensing factor Cdt1 does not induce significant rereplication in mammalian cells when the ATR checkpoint is intact. Single-stranded DNA accumulated by uncontrolled DNA unwinding mediated by mini-chromosome maintenance due to Cdt1 overexpression is the initial signal to activate the checkpoint. Our studies reveal the molecular mechanisms by which the ATR-mediated S-phase checkpoint pathway prevents DNA rereplication and thus increases our understanding of how rereplication is prevented in mammalian cells.

PUBLICATIONS

Olson, E., Nievera, C.J., Klimovich, V., Fanning, E., Wu, X. RPA2 is a direct downstream target for ATR to regulate the S-phase checkpoint. J. Biol. Chem. 281:39517, 2006.

Olson, E., Nievera, C.J., Liu, E., Lee, A.Y., Chen, L., Wu, X. The Mre11 complex mediates the S-phase checkpoint through an interaction with RPA. Mol. Cell. Biol., *in press*.

Olson, E., Nievera, C.J., Liu, E., Lee, A.Y., Chen, L., Wu, X. The Mre11/Rad50/Nbs1 complex acts both upstream and downstream of ATR to regulate the S-phase checkpoint following UV treatment. J. Biol. Chem., *in press*.

DIVISION OF ONCOVIROLOGY

Peter K. Vogt, Ph.D., Division Head

Molecular Genetics of Cancer

P.K. Vogt, A. Bader, D. Bai, A. Denley, A. Galkin, M. Gymnopoulos, J. Hart, F. Hosp, H. Jiang, P. Pavlickova, J. Shi, L. Zhao

The focus of our research is molecular mechanisms of carcinogenesis. We study viral and cellular oncoproteins and tumor suppressors, defining their functions in oncogenesis and identifying molecular targets for therapeutic intervention. In high-throughput screens, we look for small molecules that can interact with these targets and inhibit or reverse oncogenic cellular transformation.

ONCOGENIC TRANSFORMATION

Oncogenic transformation of cells requires changes in gene activities, regulated at the level of transcription, translation, or posttranslational modification. These changes result in a gain of function for specific growth-promoting genes and a loss of function for growth-attenuating genes.

PHOSPHATIDYLINOSITOL-3′-KINASE IN CANCER

The discovery of cancer-specific mutations in *PIK3CA*, the gene that encodes the catalytic subunit p110α of phosphatidylinositol-3′-kinase (PI3K), was a breakthrough in cancer research. The finding that these mutants are highly restricted to 3 narrowly defined hot spots in the gene immediately suggested that the mutated p110α plays a causative role in cancer. We showed that the 3 hot-spot mutations, introduced individually into wild-type p110α, confer oncogenicity to the protein, making it capable of transforming cells in culture and inducing tumors in vivo. This gain of function is accompanied by enhanced enzymatic activity, constitutive activation of signaling by Akt/protein kinase B, and essential involvement of the target of rapamycin kinase in the oncogenic signaling pathway.

RARE CANCER-SPECIFIC MUTATIONS IN P110α

In addition to the hot-spot mutations, which account for about 80% of all cancer-mutated p110α, numerous rare mutations have been identified in diverse cancers. These rare mutations are distributed over the entire coding region of *PIK3CA*. We examined 15 of the rare mutations and found that 14 induce a gain of function that results in oncogenic transformation when the mutant protein is expressed in normal cells. The rare mutants also have increased catalytic activity and constitutively activate the Akt pathway. Rare mutants are, however, at least 10 times less oncogenic (as measured by the number of cell-transforming events per nanogram of DNA) than the hot-spot mutants. This reduced potency accounts for the rare occurrence of these mutants.

MULTIPLE MOLECULAR MECHANISMS FOR MUTATION-INDUCED GAIN OF FUNCTION IN P110α

The protein p110α has several distinct structure-function domains: an N-terminal domain that binds the regulatory subunit, a Ras-binding domain, a C2 domain, a helical domain, and a C-terminal kinase domain. We have mapped hot-spot and rare mutations on a model structure of p110α. The locations of the

mutations in the functional domains suggest at least 3 different molecular mechanisms for the gain of function. Mutations in the C2 domain increase the positive surface charge and thereby enhance recruitment to the plasma membrane. Mutations in the helical domain affect the interaction with a regulatory protein, probably p85. By interfering with the p85 interactions, these mutations relieve the inhibitory actions of p85 on p110α. Mutations in the kinase domain affect the position and the movement of the activation loop. They may lock the activation loop in the "on" position.

PI3K is an exceedingly attractive target for cancer therapy. Inhibitors specific to the cancer-derived mutations of PI3K would not affect normal PI3K signaling. The fact that PI3K is an enzyme and that the cancer-specific mutations result in gain of function greatly facilitates the design of effective inhibitors.

SMALL-MOLECULE REGULATORS OF THE MYC NETWORK

Increased levels and enhanced function of the transcriptional regulator Myc are common in cancer. They result from gene amplification, elevated levels of transcription, and activated translation. In many cancers, a correlation exists between the gain of function in Myc, tumor grade, and poor prognosis, suggesting that Myc plays an important role in the causation and progression of cancer.

Myc is a transcription factor that functions only as a dimer with another protein, Max. The structure of the Myc-Max dimerization interface is known; single amino acid substitutions at critical sites can break or stabilize dimerization. In collaboration with D.L. Boger and K.D. Janda, Department of Chemistry, we have isolated several small molecules that interfere with the dimerization of Myc and Max. As a consequence, these molecules also prevent Myc DNA binding, Myc-dependent transcriptional activation, and Myc-induced oncogenic transformation.

The Myc-Max dimer belongs to a complex network that includes activators as well as repressors of transcription. All of the activators and repressors function as dimers with the Max protein, making Max the common denominator of the network. Max is also the only component of the network that can form homodimers, albeit weak and transcriptionally inactive homodimers. Small molecules that specifically stabilize the Max homodimer would trap this essential partner and make it unavailable for heterodimerization and for transcriptional regulatory activities. Such compounds would downregulate the entire network.

We have searched for small molecules that could bind specifically to Max and stabilize the Max homodimer while leaving Myc-Max dimerization unaffected. The search was performed in silico with the helix-loop-helix leucine zipper dimerization domain of Max and the National Cancer Institute diversity set of compounds. We used the software docking program AutoDock developed here at Scripps Research, and the computations were performed on the supercomputer at the University of California, San Diego. The candidates identified in silico were then screened by using fluorescence resonance energy transfer followed by cell-based assays for inhibition of Myc. The final compound that passed all these tests inhibits Myc-dependent cell growth, Myc-mediated transcriptional activation, and Myc-induced oncogenic transformation.

PUBLICATIONS

Bader, A.G., Kang, S., Vogt, P.K. Cancer-specific mutations in *PI3KCA* are oncogenic in vivo. Proc. Natl. Acad. Sci. U. S. A. 103:1475, 2006.

Bader, A.G., Vogt, P.K. Leucine zipper transcription factors: bZIP proteins. *In*: Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine. Ganten, G., Ruckpaul, K. (Eds.). Springer, New York, 2006, p. 964.

Denley, A., Gymnopoulos, M., Hart, J.R., Jiang, H., Zhao, L., Vogt, P.K. Biochemical and biological characterization of tumor-associated mutations of p110α. Methods Enzymol., *in press*.

Denning, G., Jean-Joseph, B., Prince, C., Durden, D.L., Vogt, P.K. A short N-terminal sequence of PTEN controls cytoplasmic localization and is required for suppression of cell growth. Oncogene 26:3930, 2007.

Fang, J., Meng, Q., Vogt, P.K., Zhang, R., Jiang, B.H. A downstream kinase of the mammalian target of rapamycin, p70S6K1, regulates human double minute 2 protein phosphorylation and stability. J. Cell. Physiol. 209:261, 2006.

Gymopoulos, M., Vogt, P.K. Rare, cancer-specific mutations in *PIK3CA* show gain of function. Proc. Natl. Acad. Sci. U. S. A. 104:5569, 2007.

Sawa, M., Hsu, T.-L., Itoh, T., Sugiyama, M., Hanson, S., Vogt, P.K., Wong, C.-H. Glycoproteomic probes for fluorescent imaging of fucosylated glycans in vivo. Proc. Natl. Acad. Sci. U. S. A. 103:12371, 2006.

Vogt, P.K., Kang, S., Elsliger, M.-A., Gymnopoulos, M. Cancer-specific mutations in phosphatidylinositol 3-kinase. Trends Biochem. Sci. 32:342, 2007.

Molecular Mechanisms of Cancer Development and Protein Modification by a Ubiquitin-Like Modifier

D.-E. Zhang, O.A. Malakhova, L.F. Peterson, M. Yan, A. Boyapati, J.-K. Luo, W. Zou, J.R. Biggs, J.-H. Kim, E.-Y. Ahn, J. Wang, A.J. Okumura, F. Okumura, B. Abdulla, X. Yin, M.-C. Lo, Y. Chen, S. Matsuura, W.-J. Shia, C. Burkat, X. Cong, S. Mauen

TRANSCRIPTION FACTOR *AML1* AND ITS FUSION PROTEIN AML1-ETO IN BLOOD CELL DIFFERENTIATION AND CANCER DEVELOPMENT

Acute myeloid leukemia is a major hematopoietic malignant neoplasm characterized by the proliferation of a malignant clone of myeloid progenitor cells. One of the most common targets of chromosomal translocations implicated in this neoplasm is the gene *AML1* (*RUNX1*). The gene was isolated through a study of t(8;21) chromosomal translocation; the results revealed that the runt homology domain of *AML1* is fused to a gene termed *ETO* (*MTG8*) to form a fusion protein called AML1-ETO. Subsequent studies indicated that the protein *AML1* is crucial for normal hematopoiesis. We previously discovered that AML1 synergistically activates the expression of a critical myeloid gene, the gene for the M-SCF receptor, with 2 other important transcription factors, C/EBP and PU.1.

To study the effect of AML1-ETO on hematopoiesis, we produced various mouse models, including homologous recombination, transgenic, and retrovirus-mediated gene expression and bone marrow transplantation, in which wild-type *AML1* was replaced by *AML1-ETO*. Currently, we are identifying the molecular pathways regulated by AML1 in blood stem cells, cofactors involved in the synergy among various transcription factors and AML1-ETO–associated development of leukemia, and critical target genes of AML1 and AML1-ETO in hematopoiesis.

A NOVEL UBIQUITIN-SPECIFIC ENZYME, UBP43

In studying genes differentially expressed in AML1-ETO mice, we isolated the gene for a novel enzyme, UBP43 (USP18), which belongs to a family of ubiquitin-specific proteases. Like phosphorylation and dephosphorylation, ubiquitylation and deubiquitylation are mechanisms for protein modification. Recently, we showed that UBP43 is the only currently known enzyme that removes a ubiquitin-like modifier, ISG15, from ISG15 conjugates. In mice that lacked the gene for UBP43, UBP43-deficient bone marrow cells were hypersensitive to treatment with type I interferon and died via apoptosis in the presence of interferon. Most important, in UBP43-deficient cells, interferon induced a prolonged Stat1 tyrosine phosphorylation, DNA binding, and interferon-mediated gene activation. UBP43-deficient mice are resistant to certain viral and bacterial infections and to the development of leukemia. Currently, we are analyzing molecular pathways affected by UBP43.

ROLE OF ISG15 CONJUGATION IN IMMUNE RESPONSES

The gene for ISG15 was originally cloned as a gene highly upregulated by interferon and encodes a small ubiquitin-like protein. Unlike ubiquitin and other ubiquitin-like modifiers, ISG15 is not present in lower eukaryotes, such as yeast, indicating that it may be associated with specialized functions in higher eukaryotic cells. Upon viral infection, bacterial infection, or other stress stimulation, ISG15 protein can be detected in cells both in free and in conjugated form (ISGylation). Using high-throughput Western blot analysis and mass spectrometry, we have identified ISGylated proteins that are involved in various cellular functions. We also have identified an ISG15-conjugating enzyme and several ISG15 ligases. Regulation of protein ISGylation may provide valuable treatments to control cell function and survival. We are using techniques such as gene depletion, protein interaction, biochemical purification, and gene regulation to study the biological function of this interesting protein modification.

PUBLICATIONS

Biggs, J.R., Peterson, L.F., Zhang, Y., Kraft, A.S., Zhang, D.-E. AML1/RUNX1 phosphorylation by cyclin-dependent kinases regulates the degradation of AML1/RUNX1 by the anaphase-promoting complex. Mol. Cell. Biol. 26:7420, 2006.

Boyapati, A., Yan, M., Peterson, L.F., Biggs, J.R., Le Beau, M.M., Zhang, D.-E. A leukemia fusion protein attenuates the spindle checkpoint and promotes aneuploidy. Blood 109:3963, 2007.

Dao, C.T., Luo, J.K., Zhang, D.-E. Retinoic acid-induced protein ISGylation is dependent on interferon signal transduction. Blood Cells Mol. Dis. 36:406, 2006.

Malakhova, O.A., Kim, K.I., Luo, J.K., Zou, W., Kumar, K.G.S., Fuchs, S.Y., Shuai, K., Zhang, D.-E. UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. EMBO J. 25:2358, 2006.

Okumura, A.J., Peterson, L.F., Lo, M.-C., Zhang, D.-E. Expression of AML/Runx and ETO/MTG family members during hematopoietic differentiation of embryonic stem cells. Exp. Hematol. 35:678, 2007.

Okumura, F., Zou, W., Zhang, D.-E. ISG15 modification of the eIF4E cognate 4EHP enhances cap structure-binding activity of 4EHP. Genes Dev. 21:255, 2007.

Peterson, L.F., Boyapati, A., Ahn, E.-Y., Biggs, J.R., Okumura, A.J., Lo, M.-C., Yan, M., Zhang, D.-E. Acute myeloid leukemia with the 8q22;21q22 translocation: secondary mutational events and alternative t(8;21) transcripts. Blood 110:799, 2007.

Peterson, L.F., Yan, M., Zhang, D.-E. The $p21^{waf1}$ pathway is involved in blocking leukemogenesis by the t(8;21) fusion protein AML1-ETO. Blood 109:4392, 2007.

Rempel, L.A., Austin, K.J., Ritchie, K.J., Yan, M., Shen, M., Zhang, D.-E., Henkes, L.E., Hansen, T.R. Ubp43 gene expression is required for normal Isg15 expression and fetal development. Reprod. Biol. Endocrinol. 5:13, 2007.

Yan, M., Kanbe, E., Peterson, L.F., Boyapati, A., Miao, Y., Wang, Y., Chen, I.M., Chen, Z., Rowley, J.D., Willman, C.L., Zhang, D.-E. A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. Nat. Med. 12:945, 2006.

Yan, M., Luo, J.K., Ritchie, K.J., Sakai, I., Takeuchi, K., Ren, R., Zhang, D.-E. Ubp43 regulates BCR-ABL leukemogenesis via the type I interferon receptor signaling. Blood 110:305, 2007.

Zhou, G.B., Kang, H., Wang, L., Gao, L., Liu, P., Xie, J., Zhang, F.X., Weng, X.Q., Shen, Z.X., Chen, J., Gu, L.J., Yan, M., Zhang, D.-E., Chen, S.J., Wang, Z.Y., Chen, Z. Oridonin, a diterpenoid extracted from medicinal herbs, targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t(8;21) leukemia in vitro and in vivo. Blood 109:3441, 2007.

Zou, W., Kim, J.-H., Handidu, A., Li, X., Kim, K.I., Yan, M., Li, J., Zhang, D.-E. Microarray analysis reveals that type I interferon strongly increases the expression of immune-response related genes in Ubp43 (Usp18) deficient macrophages. Biochem. Biophys. Res. Commun. 356:193, 2007.

Zou, W., Wang, J., Zhang, D.-E. Negative regulation of ISG15 E3 ligase EFP through its autoISGylation. Biochem. Biophys. Res. Commun. 354:321, 2007.

Division of Research Rheumatology

W.M. Keck Autoimmune Disease Center
Joel N. Buxbaum, M.D., Division Head

Pathogenesis of Late-Onset Genetic Diseases Related to Abnormalities of Protein Conformation

J.N. Buxbaum, N. Reixach, Z. Ye, L. Friske, T. Coelho,* D. Jacobson,** G. Gallo,*** C. Tagoe,**** A. Roberts,***** E. Masliah†

* Hospital Geral Santo António, Porto, Portugal

** Boston University School of Medicine, Boston Massachusetts

*** NYU School of Medicine, New York, New York

**** Albert Einstein College of Medicine, Bronx, New York

***** Molecular and Integrative Neuroscienes Department, Scripps Research

† University of California, San Diego, California

We are studying the pathogenesis of a group of hereditary and sporadic human diseases, the transthyretin amyloidoses, that are the result of age-dependent protein misfolding. The misfolded molecules are deposited in the heart, kidneys, and peripheral nerves, producing organ-specific disease. We use 4 major approaches: biophysical analysis of recombinant and naturally occurring murine and human transthyretin, animals transgenic for human transthyretin, cell cultures to determine how the misfolded proteins injure their cellular targets, and genetic epidemiology to identify potential disease carriers and assess the effects of other hereditary and environmental factors on the disease.

Currently, we are completing 2 studies on the clinical impact of the transthyretin mutation Val122Ile, an allele carried by 3%–4% of African Americans. One study, carried out in collaboration with the Cardiovascular Heart Study, a multiinstitutional cooperative study of risk for cariovascular disease in community-dwelling individuals more than 65 years old, indicates that African American carriers of the allele who are more than 70 years old have a higher frequency of new-onset congestive heart failure than do controls matched for age, sex, and ethnic background and also have more features of heart disease consistent with cardiac amyloidosis. In the second study, a case control analysis done in collaboration with D. Jacobson, Boston University of Medicine; G. Gallo, NYU School of Medicine; and C. Tagoe, Albert Einstein College of Medicine, we compared living carriers of the allele with controls matched for age, sex, and ethnic background. We found an increased frequency of cardiac changes associated with cardiac amyloidosis in the carriers more than 65 years old. These data reinforce the notion that this late-onset genetic disorder is a significant health risk for the elderly African Americans who have this gene.

Our animal models continue to give us insights into the biology of these diseases of protein structure. In our continuing collaboration with D.R. Salomon, Department of Molecular and Experimental Medicine, we have shown that pathologic deposition of transthyretin in the hearts and kidneys of the same animals results in different molecular pathways of injury and response. The role of the molecular chaperoning response of the liver in sparing the heart from exposure to damaging protein aggregates appears to be quite clear, although the details remain to be elucidated. In collaboration with Dr. Salomon, J. Kelly, Department of Chemistry, and T. Coelho, Hospital Geral Santo António, we will be extending our observations to patients with the hereditary forms of transthyretin.

In biophysical investigations related to aging, we have explored the effects of oxidation on the stability of transthyretin, a protein known to be more susceptible to in vivo aggregation as humans become older. In this model, we showed that methionine oxidation actually reduces the tendency of transthyretin to form fibrils similar to those seen in the amyloidoses but makes the protein more toxic to cultured cells. These data challenge theories of aging that invoke generalized severe oxidation as an explanation for increased protein aggregation as people become older.

In the course of our studies with transgenic animals, we have examined the transcriptional profiles of various organs during normal aging of the animals. These analyses have revealed that few transcriptional features of aging are shared by different organs. Some organs show relative increases in transcription; others, decreases; and others, no quantitative change. The organs also differ qualitatively, with different degrees and distribution of expression of genes in various functional categories, particularly genes responsible for inflammatory responses in the absence of any apparent inflammatory stimulus apart from aging. These observations are quite consistent with current ideas that aging may represent a chronic low-grade inflammatory state, although perhaps not involving the same cells as conventional inflammatory disorders of infectious or noninfectious etiologies.

Studies in our murine model of human disease have also revealed, not unexpectedly, that even proteins of similar structure and function in mice and humans may have strikingly different biophysical properties. We have been able to synthesize recombinant mouse transthyretin, which we compared with recombinant human transthyretin. Both types of transthyretin are tetrameric proteins, but mouse transthyretin is more stable than is human transthyretin to chemical denaturants, and it is not amyloidogenic. Furthermore, the incorporation of mouse transthyretin subunits into the human protein results in a stable heterotetramer. These results parallel those obtained in vivo. In animals that have both the human protein and the mouse protein, transthyretin circulates as human-mouse heterotetramers, and this protein is more stable than the human transthyretin circulating in sera from animals in which the gene for mouse transthyretin is inactive. The disease model is apparent only when the human gene is highly expressed in excess of the mouse gene or is minimally or moderately expressed in animals in which the endogenous gene has been molecularly silenced.

A number of studies during the past 10 years have suggested an interaction between transthyretin and the amyloid β-peptide in the pathogenesis of Alzheimer's disease. We have crossed mice that have a human Alzheimer's gene with animals in which human transthyretin was overexpressed or in which the mouse transthyretin gene was silenced. Overexpression of human transthyretin suppressed the behavioral and neuropathologic phenotype usually seen in the mice with the Alzheimer gene, and the absence of the mouse gene was associated with earlier and more severe onset of the neuropathologic changes. We also discovered a specific interaction of purified human and mouse transthyretin with aggregates of the amyloid β-peptide, suggesting that the effects on the phenotype are mediated by an interaction between transthyretin and the peptide in vivo. These data clearly indicate an important effect of transthyretin in the pathogenesis of disease in these animals that may be relevant to the disease in humans. The presence of such a pathway would suggest other potential modes of therapy for the human disease.

PUBLICATIONS

Bartfai, T., Waalen, J., Buxbaum, J.N. Adipose tissue as a modulator of clinical inflammation: does obesity reduce the prevalence of rheumatoid arthritis? J. Rheumatol. 34:488, 2007.

Buxbaum, J.N. The amyloidoses. *In*: Cecil Textbook of Medicine, 23th ed. Goldman L., Ausiello, D. Saunders, Philadelphia, *in press.*

Buxbaum, J.N. The amyloidoses. *In*: Rheumatology, 4th ed. Hochberg, M.C., et al. (Eds.). Mosby, St. Louis, *in press.*

Buxbaum, J.N. The genetics of the amyloidoises: interactions with immunity and inflammation. Genes Immun. 7:439, 2006.

Maleknia, S.D., Reixach, N., Buxbaum, J.N. Oxidation inhibits amyloid fibril formation by transthyretin. FEBS J. 273:5400, 2006.

Reixach, N., Adamski-Werner, S.L., Kelly, J.W., Koziol, J., Buxbaum, J.N. Cell based screening of inhibitors of transthyretin aggregation. Biochem. Biophys. Res. Commun. 348:889, 2006.

Tagoe, C.E., Reixach, N., Friske, L., Mustra, D., French, D., Gallo, G., Buxbaum, J.N. In vivo stabilization of L55P transthyretin (TTR) by murine wild-type TTR and diflunisal in transgenic mice. Amyloid, *in press*.

Protein Oxidation, Oxidative Stress, and Disease

J.S. Friedman, F.M. Martin, A.C. Takeda, J. Yi

Protein oxidative damage, in particular, protein carbonylation, is increased in inflammatory disorders, neurodegenerative processes, and aging. This type of damage is also a prominent feature of loss of superoxide dismutase 2 (SOD2), an endogenous antiox-

idant protein, in mouse blood cells. SOD2 deficiency in murine blood cells results in an anemia similar to the human disorder sideroblastic anemia. Using clinical samples, we found that this type of protein oxidation is also characteristic of bone marrow cells from patients with sideroblastic anemia. The samples were provided by our collaborators J. Nieva, J. Andrey, and A. Saven, Scripps Clinic, La Jolla, California; J.C. Barton, Southern Iron Disorders Center, Birmingham, Alabama; and J. Prchal, University of Utah, Salt Lake City, Utah.

To better understand the role of protein carbonylation in disease processes, we developed 2 novel methods for enriching and identifying oxidized proteins. The first method involves the use of multiple fluorophores (e.g., Cy-2, Cy-3, and Cy-5) that can form derivatives of carbonylated proteins via a hydrazide moiety. Individual samples are labeled with distinct fluorophores and then are combined for comparative 2-dimensional gel analysis (Fig. 1). This method is similar to the comparative proteomic method termed difference gel electrophoresis, or DIGE, and thus we coined the term oxo-DIGE. The second method involves the use of a biotin "hook" to obtain oxidized proteins from more complex protein mixtures. Using these techniques, we can enrich, identify, and quantitatively compare oxidized proteins in experimental samples—and allow for whole proteome comparisons of differential oxidation.

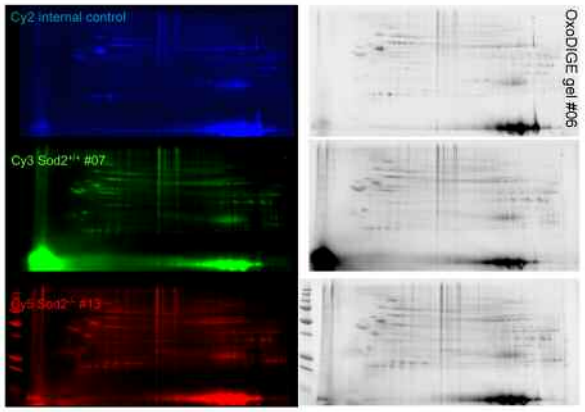

Fig. 1. A multiplex oxo-DIGE gel. In this experiment, 50 µg of each sample (Cy-2, internal control; Cy-3, hydrazide $Sod2^{+/+}$; and Cy-5, hydrazide $Sod2^{-/-}$) was mixed after fluorophore labeling, precipitated, washed, resuspended for isoelectric focusing, and separated by size. Images were obtained at 100-µm resolution with excitation and emission filter sets specific for each dye. Both pseudocolor and gray-scale images are presented. The Cy-3 and Cy-5 channels identify protein carbonyls; the Cy-2 channel labels all proteins in a pooled control sample to serve as an internal reference for comparison and matching of multiple gels.

The National Center for Research Resources has just awarded us a grant to set up a DIGE facility in the Core Proteomics laboratory of the Department of Molecular and Experimental Medicine. In the next year, we will use the facility and the methods described in new collaborative research with J. Waalen and E. Beutler, Department of Molecular and Experimental Medicine, to study anemia of aging and to investigate basic questions about protein oxidation: What is the hierarchy of protein oxidation? Are specific proteins carbonylated first that serve as a buffer against oxidative injury? Does protein oxidation occur at random in susceptible proteins, or do specific residues (e.g., near metal-binding sites) become oxidized first? Carbonylated proteins are subject to degradation in the proteasome—does degradation of "carbonyl sensor" proteins activate cellular responses to oxidative damage? We think that patterns of protein oxidation probably will be found that correspond to normal cellular responses, whereas other patterns will be found that represent the signature of specific disease states.

PUBLICATIONS

Martin, F.M., Bydlon, G., Friedman, J.S. SOD2-deficiency sideroblastic anemia and red blood cell oxidative stress. Antioxid. Redox. Signal. 8:1217, 2006.

Transcriptional Modulation of HIV Type 1 Expression and Latency

K.V. Morris

RNA-mediated transcriptional gene silencing occurs in human cells through the antisense strand of small interfering RNAs and the protein Argonaute 1. The use of RNA to regulate gene expression represents a shift in the current understanding of gene regulation. We wish to understand the mechanism of RNA-mediated regulation of gene expression in human cells and to use the mechanism therapeutically to treat infection with HIV type 1 (HIV-1). We have learned that small interfering RNAs targeted to promoter regions can direct silent-state epigenetic modifications and that DNA methyltransferase 3A and a low-copy RNA spanning the targeted promoter are involved. However, until recently, a biological example of RNA-mediated transcriptional regulation had not been reported. We hypothesized that an HIV-1–expressed microRNA (miR-N367) is used by

the virus to modulate the establishment and maintenance of latency (Fig. 1).

To more clearly assess the role that miR-N367 might be playing in the establishment or maintenance of HIV-1 latency, we generated phosphorothioate oligonucleotides (ODNs) targeted to either the sense or the antisense strand of miR-N367 (Fig. 2A). The ODNs were transfected into ACH-2 cells, which contain a

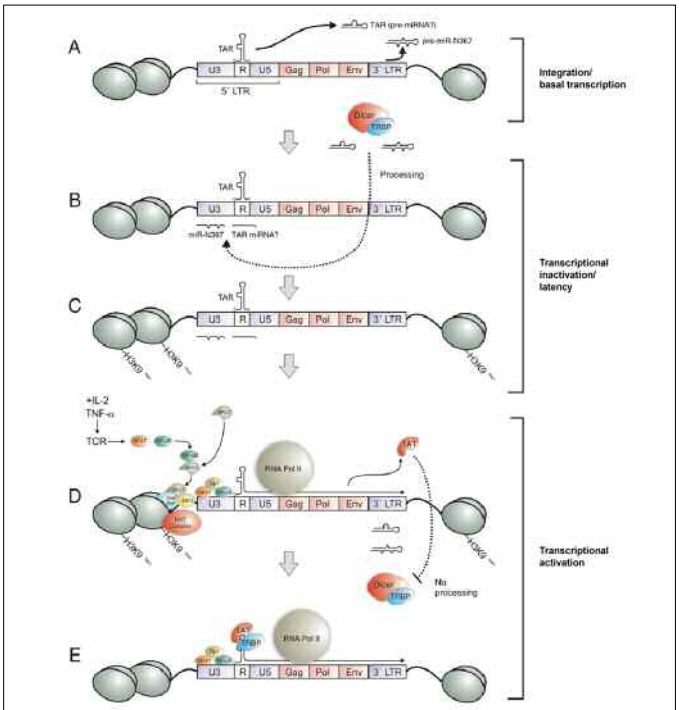

Fig. 1. A putative pathway for the establishment and reactivation of HIV latency. A, The integrated provirus could produce miRNAs either within the viral genome (miR-N367) or from the unelongated transactivating response (TAR) element. B, The miRNAs could be processed by the enzyme Dicer and the TAR RNA binding protein (TRBP) and thus be capable of targeting the LTR in a transcriptional fashion. C, The targeting of the LTR by small interfering RNAs can lead to H3K9^{me+} and subsequent gene silencing (i.e., induction of a latent state). D, When the T-cell receptor (TCR) is stimulated by IL-2 or TNF-α, the nuclear factors NF-AT and NF-κB can become activated. NF-κB can activate BRG-1, a subunit of a complex that can function with other factors to remodel the chromatin at the viral LTR and produce a favorable environment for transcription. Moreover, NF-AT and NF-κB can activate the LTR and induce expression of the viral transactivator Tat, which when present in high concentrations results in feedback to enhance the elongation of viral transcription from the TAR element, as well as inhibit Dicer by competitive interactions with TRBP. E, The result would be a reversal of the silent-state H3K9 methylation (due to the inhibition of Dicer/TRBP by Tat); an induction of viral expression (due to NF-AT, NF-κB, and Tat), which leads to H3K4 and H3K36 methylation; and a transcriptional active chromatin state. This process ultimately results in the production of full-length viral products and the emergence of HIV-1 from latency. Reprinted from Weinberg, M.S., Morris, K.V. Are viral-encoded microRNAs mediating latent HIV-1 infection? DNA Cell Biol. 25:223, 2006.

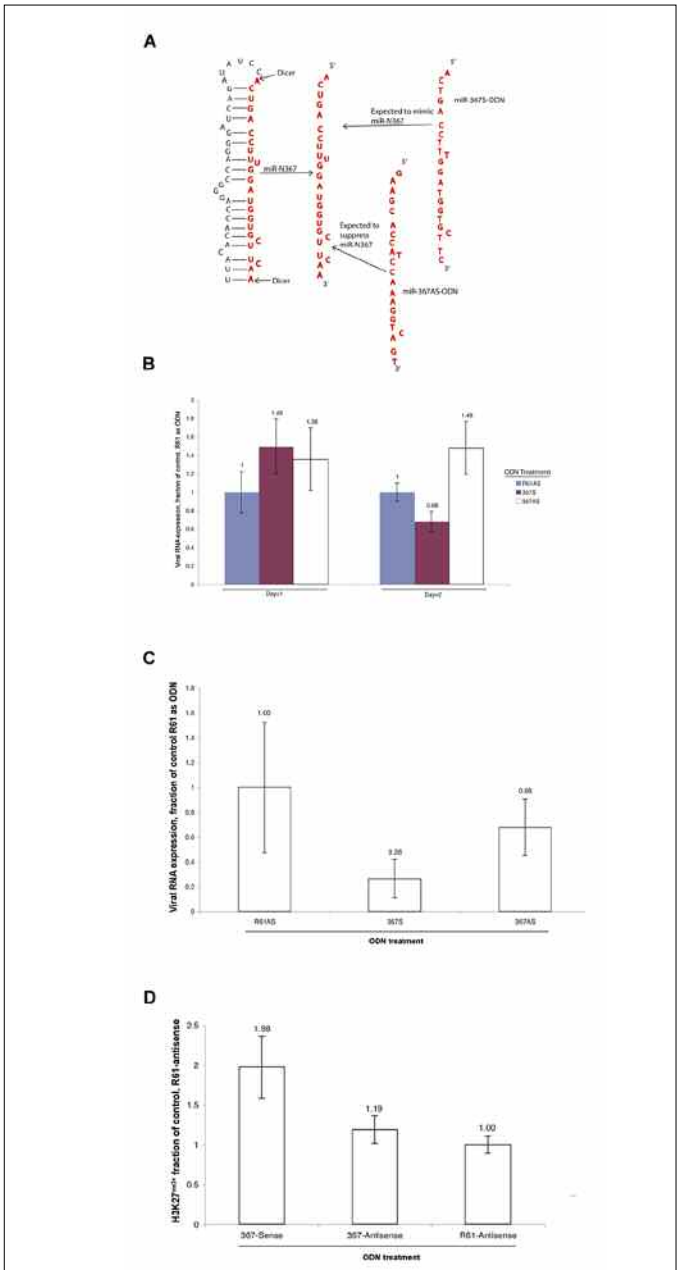

Fig. 2. Phosphorothioate targeting of miR-N367. A, The miR-N367 was generated and processed by Dicer as depicted. In order to determine if miR-N367 is active in ACH-2 cells, antisense ODNs were generated to either mimic miR-N367 (miR-N367S-ODN) or target and degrade miR-N367 (miR-N367AS-ODN) in an RNAse H–dependent manner. B, The effects of miR-N367 sense and antisense ODNs relative to the control R61 as ODN were determined by transfecting the ODNs into ACH-2 cells in triplicate, and viral RNA was assessed 24 and 48 hours later. Triplicate transfections are shown with the standard errors of the means. C, In order to determine the longer term effects of ODN treatment, the respective ODNs were transfected into ACH-2 cells in quadruplicate, and viral RNA was assessed 96 hours later. D, The miR-N367 target site in the HIV-1b LTR of ACH-2 ODN-treated cultures was also assessed by using chromatin immunoprecipitation 96 hours after transfection to determine methylation of histone 3 and lysine 27 (H3K27^{me3+}). Error bars (C and D) indicate the standard deviations.

stable integrated HIV-1 that expresses low levels of miR-N367. Despite a low transfection efficiency, a noticeable and reproducible suppressive trend that would be predicted on the basis of the particular miR-N367 ODN used (Fig. 2A, sense vs antisense) was observed (Fig. 2B). To determine if the observed effects on viral RNA expression were the result of epigenetic modifications at the putative miR-N367 target site in the HIV-1 long terminal repeat (LTR) of ACH-2 cells, we repeated the experiments and assayed the viral RNA at 96 hours. The ODN-treated cultures showed a similar trend in viral RNA expression (Fig. 2C). When these cultures were assessed by using chromatin immunoprecipitation, a distinct silent-state epigenetic methyl-mark ($H3K27^{me3+}$) was observed at the miR-N367 target site in the HIV-1 LTR in cultures treated with the sense ODN and not in those treated with the antisense ODN (Fig. 2D).

These data suggest that an endogenous mechanism involving the miR-N367 sequence is used by HIV-1 to regulate viral expression and latency in an epigenetic manner in ACH-2 cells. The knowledge gained of the mechanism of HIV-1 latency may one day be advantageous in therapeutic strategies to treat HIV-1 infections.

PUBLICATIONS

Han, J., Kim, D., Morris, K.V. Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc. Natl. Acad. Sci. U. S. A. 104:12422, 2007.

Kim, D.H., Villeneuve, L.M., Morris, K.V., Rossi, J.J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. Nat. Struct. Mol. Biol. 13:793, 2006.

Autoimmunity Induced by Xenobiotics

K.M. Pollard, D. Cauvi, P. Hultman*

* Linköping University, Linköping, Sweden

We focus on mechanisms involved in the initiation of autoimmunity and how the interplay between genetics and the environment influences the expression of systemic autoimmune diseases.

ROLE OF DECAY-ACCELERATING FACTOR IN SYSTEMIC AUTOIMMUNITY

Decay-accelerating factor (DAF/CD55) is one of the complement regulatory proteins that protect host cells from damage by autologous complement. Deficiency in DAF1 can exacerbate development of various autoimmune diseases, and this effect may occur because the factor limits T-cell hyperresponsiveness. Some research has suggested that the lack of DAF1 aggravates murine autoimmune disease in a complement-dependent manner, but other results have shown that activation of human T cells by DAF can be complement independent. The contribution of the interaction between DAF1 and complement to lymphocyte activation, cytokine expression, and antibody production in mice that are not DAF1 deficient remains to be determined.

In mercury-induced autoimmunity in mice, we found that an accumulation of $CD44^{high}$ $DAF1^{low}$ $CD4^+$ T cells is associated with the development of autoimmunity. In recent studies, we used cobra venom factor to show that complement depletion does not affect the accumulation of activated $CD4^+$ T cells, elevation of splenic IL-4 expression, or production of autoantibodies in this model. In addition, neither the accumulation of $CD44^{high}$ $DAF1^{low}$ $CD4^+$ T cells nor the downregulation of DAF1 expression on $CD4^+$ T cells was influenced by a lack of complement. These observations indicate that reduction of DAF1 on $CD4^+$ T cells does not enhance the contribution of complement to T-cell activation and autoantibody production during development of autoimmunity.

GOLD-INDUCED AUTOIMMUNE AND IMMUNOSTIMULATORY RESPONSES IN MICE

Natrium aurothiomaleate (GSTM) is a useful drug in patients with rheumatism, but many patients treated with GSTM experience a variety of immune-mediated adverse effects. We used a mouse model to study the effects of GSTM on the immune system, including induction of systemic autoimmunity. Mice were given weekly intramuscular injections of GSTM or equimolar amounts of sodium thiomaleate (controls). The effects of treatment on lymphocyte subpopulations were determined by using flow cytometry. Humoral autoimmunity was measured by using indirect immunofluorescence and immunoblotting, and deposition of immunoglobulin and C3 was used to assess immunopathologic changes.

We found that GSTM stimulated the immune system, causing strain-dependent lymphoproliferation and autoimmunity, including an MHC-restricted autoantibody response against the nucleolar protein fibrillarin. The drug did not cause deposition of immunoglobulin G in the glomeruli or vessel walls, but it did have a strong B cell–stimulating effect, including both type 1 and type 2 T helper cell–dependent isotypes. All of these autoimmune and immunostimulatory responses depended on the MHC genotype, suggesting a strong genetic link for the major adverse immune reactions associated with GSTM treatment.

PUBLICATIONS

Cauvi, D.M., Cauvi, G., Pollard, K.M. Reduced expression of decay-accelerating factor 1 in $CD4^+$ T cells in murine systemic autoimmune disease. Arthritis Rheum. 56:1934, 2007.

Havarinasab, S., Johansson, U., Pollard, K.M., Hultman, P. Gold causes genetically determined autoimmune and immunostimulatory responses in mice. Clin. Exp. Immunol., *in press*.

Pollard, K.M., Hultman, P. Fibrillarin autoantibodies. *In*: Textbook of Autoantibodies. Shoenfeld, Y., Gershwin, M.E., Meroni, P.-L. (Eds.). Elsevier, Philadelphia, 2007, p. 317.

TRANSLATIONAL VASCULAR MEDICINE RESEARCH

John H. Griffin, Ph.D., Division Head

The Antithrombotic, Anti-inflammatory and Antiapoptotic Protein C Pathway

J.H. Griffin, B.N. Bouma, H. Deguchi, D.J. Elias, S. Eichinger,* J.A. Fernández, E. Kerschen,** P. Kyrle,* S. Li, L. Mosnier, S. Navarro, N. Pecheniuk, H. Weiler,** X. Xu, X. Yang, S. Yegneswaran, B.V. Zlokovic***

* Medical University of Vienna, Vienna, Austria

** Milwaukee Blood Center, Milwaukee, Wisconsin

*** University of Rochester, Rochester New York

Various host defense systems act in concert in normal physiology, and their dysfunctioning contributes markedly to pathophysiologic changes. Coagulation pathways, fibrinolysis pathways, and anticoagulant mechanisms prevent bleeding while avoiding harmful blood clots. The protein C pathways provide antithrombotic, anti-inflammatory, and cytoprotective activities and are a major focus of our research.

ANTIAPOPTOTIC AND CYTOPROTECTIVE EFFECTS OF ACTIVATED PROTEIN C

The antiapoptotic activity of activated protein C (APC), first described in 2001, may provide cytoprotective activity that reduces cell death after a variety of cellular injuries. Recombinant APC, a well-defined anticoagulant enzyme, reduced mortality in patients with severe sepsis and multiorgan failure in a large phase 3 clinical trial; the only adverse side effect was serious bleeding. The contribution of distinct APC activities to the overall therapeutic efficacy of the enzyme in patients with sepsis is unknown.

To generate recombinant APC variants that have reduced anticoagulant activity and thus are less likely to cause bleeding, we dissected APC's anticoagulant activity from its cytoprotective activity by using site-directed mutagenesis. Using staurosporine-induced endothelial cell apoptosis assays, we showed that several specific mutations in 2 APC surface loops that severely reduced anticoagulant activity resulted in APC variants that retain normal antiapoptotic activity. Like wild-type APC, these mutants require protease-activated receptor-1 and endothelial cell protein C receptor for cytoprotective activity. In contrast, 2 APC light-chain mutants had about 350% anticoagulant activity but only about 5% cytoprotective activity.

In collaboration with H. Weiler and colleagues, Milwaukee Blood Center, and using a lethal mouse endotoxemia model of sepsis, we showed that the survival benefit conferred by APC was abolished in mice genetically deficient in endothelial protein C receptor (<10% of normal) or lacking protease-activated receptor-1. An APC variant with normal cytoprotective activities but weak anticoagulant activity (<8% of normal) was as effective as wild-type APC in reducing mortality. Our data suggest that the survival-promoting efficacy of APC in this lethal sepsis model requires APC's 2 cellular receptors and its cytoprotective actions. Moreover, these results suggest that minimally anticoagulant APC variants may reduce serious bleeding risks while providing the life-saving beneficial effects of APC in sepsis and other serious injuries.

NEUROPROTECTIVE ACTIVITIES OF APC

Stroke is a major cause of morbidity and mortality, and few therapeutic options are available for ischemic stroke. Thrombolytic therapy with tissue plasminogen activator (tPA) is one option, but use of tPA is problematic because of its neurotoxic effects, including induction of bleeding. Previously, in collaboration with B. Zlokovic, University of Rochester, we showed multiple neuroprotective activities of APC. More recently, we found that APC reduces the neurotoxic effects of tPA and blunts tPA-induced apoptosis of both endothelial cells and neurons.

Remarkably, studies in mouse and rat models of ischemic stroke indicate that recombinant murine APC reduces bleeding induced by tPA. APC stabilizes the blood-brain barrier against bleeding because it blunts the tPA-induced increases in mRNA and protein levels of matrix metalloprotease-9, which causes breakdown of the barrier.

We think that APC merits clinical trials as a neuroprotective agent in patients with ischemic stroke. Fur-

thermore, we speculate that APC may add substantially to the effectiveness of tPA therapy for stroke in humans.

INFLUENCE OF LIPIDS ON BLOOD COAGULATION

Venous thrombosis is clinically distinct from arterial thrombosis; the two differ in thrombus appearance, pathogenic mechanisms, and therapeutic approaches. High-density lipoprotein (HDL) protects against arterial atherothrombosis, but it is not known if HDL protects against recurrent venous thromboembolism. We hypothesized that HDL protects against recurrent venous thrombosis because the lipoprotein has multiple antithrombotic and anti-inflammatory actions. These protective activities include downregulating thrombin generation by acting as an anticoagulant cofactor for APC/protein S, enhancing the protective activity of endothelial nitric oxide synthase, reducing leukocyte adhesion to endothelium, and exerting antiapoptotic effects on endothelium.

To test this hypothesis, in collaboration with S. Eichinger and P. Kyrle, Medical University of Vienna, we studied prospectively 772 patients who had had a first episode of spontaneous venous thromboembolism. During a mean follow-up of 48 months, 100 of the patients had recurrent venous thromboembolism. Compared with patients who had no recurrence, those with recurrence had significantly lower mean levels of apolipoprotein AI but similar levels of apolipoprotein B. For patients with levels of apolipoprotein AI greater than the 67th percentile of the values in the study population, the relative risk of recurrence was 0.51. Comparisons of plasma levels of lipoprotein particles in patients with recurrence vs those without recurrence gave similar results; HDL parameters were lower in patients with recurrence. Patients with high levels of apolipoprotein AI, HDL cholesterol, and large HDL particles had decreased risk of recurrent venous thromboembolism. Thus, HDL appears to protect against recurrent venous thrombosis, leading to speculations that HDL parameters might be predictive of the risk for venous thrombosis and that lipid-altering drugs that increase HDL levels might reduce the risk for first or recurrent venous thrombotic events.

Antithrombotic Mechanisms

M.J. Heeb, B.N. Bouma, A. Gruber,* S.R. Hanson,*
U. Marzec*

* Oregon Health and Sciences University, Portland, Oregon

Natural anticoagulant proteins regulate blood coagulation and can prevent thrombosis. Recently, we focused on protein S, but we also investigated protein Z and protein Z–dependent protease inhibitor, all inhibitors of procoagulant factor Xa and other targets. Deficiency of anticoagulant protein S or protein Z is associated with increased risk of stroke and venous thrombosis, illustrating the importance of these proteins.

PROTEIN S IN A THROMBOSIS MODEL

Protein S is a vitamin K–dependent plasma protein known as a cofactor for the anticoagulant activated protein C. We showed that protein S also has direct anticoagulant (PS-direct) activity independent of activated protein C via inhibition of coagulation factors Xa, Va, and VIIIa. Purified protein S varies in PS-direct activity, depending on the methods used to isolate the protein. We examined whether protein S purified by different methods has PS-direct activity in vivo.

In the Hanson thrombosis model, platelets and fibrinogen are radiolabeled and infused into a baboon. Then saline or a test agent is infused for 1 hour along the wall of a femoral arteriovenous shunt, upstream from a thrombogenic segment of the shunt. The diameters of the segments vary to simulate arterial or venous flow. Deposition of platelets is measured during a 2-hour period by using a gamma camera. Deposition of fibrin is measured after the platelet label has decayed.

Immunoaffinity-purified protein S inhibited platelet deposition 39%–97% in "arterial" segments and 65%–100% in "venous" segments; protein S purified by anion exchange inhibited deposition 5%–34% in arterial segments and 0%–73% in venous segments. Protein S was also infused in the absence or presence of antibody (HPC4) that blocked protein C activation. Protein S suppressed platelet deposition in the presence of HPC4 nearly as well as in its absence (79%–97% vs >95%). Fibrin deposition after 2 hours was suppressed 34%–83% by protein S in the presence of HPC4 and 67%–90% in the absence of the antibody. Protein S significantly depressed formation of thrombin-antithrombin complexes in the presence and absence of HPC4.

Thus, immunoaffinity-purified protein S had PS-direct activity in vivo, and the activity did not depend on activated protein C. These studies suggest potential for protein S as an antithrombotic agent.

ZINC AS ESSENTIAL FOR PS-DIRECT ACTIVITY

Immunoaffinity-purified protein S has good PS-direct activity that matches the PS-direct activity of protein S in plasma, but protein S purified by anion exchange has poor PS-direct activity. We discovered that immunoaffinity-purified protein S contained a mean of 1.4 moles of zinc per mole of protein S, whereas anion exchange–purified protein S contained 0–0.3 moles of zinc per mole of protein S. Zinc correlated with PS-direct activity and with the method used for purification of protein S ($P < .002$).

Active protein S lost PS-direct activity when treated with the zinc chelator phenanthroline and then regained most of the activity when incubated with zinc at pH 7.4. Inactive protein S gained zinc content associated with PS-direct activity when incubated with zinc at pH 2.7 or in 6 M urea but not when incubated with zinc at pH 7–8.

The previously unrecognized role of zinc and of deleterious purification methods has led to confusion about the validity of PS-direct activity. A postulated zinc-binding site was located at the interface of 2 laminin G-type domains in protein S. Zinc may thus be essential for interdomain interactions required for PS-direct activity.

LOW PROTEIN Z LEVELS AND STROKE

Protein Z is a vitamin K–dependent protein that acts as a cofactor for inhibition of factor Xa by protein Z–dependent protease inhibitor. We previously showed an association between low protein Z levels and increased risk for ischemic stroke in men but not in women. We hypothesized that hormonal changes with age in women may play a role in this difference. After dividing the data on the patients in our study to data on those older or younger than the median age of 57 years, we found a similar association between low protein Z levels and stroke in younger women and in younger and older men but not in older women, compared with matched controls.

Structure and Function of Coagulation Cofactors

A.J. Gale, T. Cramer, J. Cruz, D. Rozenshteyn, X. Xu, J.-L. Pellequer*

* CEA Valrhô, DSV/iBEB/SBN/LIRM, Bagnols ser Cèze, Cedex, France

Coagulation factors Va and VIIIa are highly homologous cofactors of the serine proteases factor Xa and factor IXa, respectively. These cofactors are the primary targets of activated protein C (APC) in its downregulation of the procoagulant pathway. In collaboration with J.-L. Pellequer in France, we used homology modeling techniques to model the 3-dimensional structures of these multidomain proteins. We are using the models as guides to create mutants of these cofactors and APC to investigate mechanisms of cofactor function and regulation. For example, we engineered disulfide bonds between domains in both factor Va and factor VIIIa. In factor Va, the disulfide bond facilitated investigation of the mechanisms of inactivation of factor Va by APC cleavage.

Factor VIIIa, however, is inactivated by 2 mechanisms. Thrombin activation of factor VIII results in a heterotrimer that consists of the A1 subunit, the A2 subunit, and the light chain. Both spontaneous dissociation of the A2 subunit and proteolytic cleavage of factor VIIIa by APC inactivate factor VIIIa. Hemophilia A, a deficiency of factor VIII, is treated by infusions of purified recombinant factor VIII. But the usefulness of factor VIII is limited because it is unstable after activation by thrombin as a result of the spontaneous dissociation of the A2 subunit.

We generated 2 mutants of factor VIII in which 2 newly introduced cysteine residues form a de novo disulfide bridge that cross-links the A2 and A3 domains. These interdomain disulfides prevent the spontaneous dissociation of the A2 subunit. These variants may provide a new, improved therapy for hemophilia A. We are using both in in vivo assays in mice and ex vivo assays in whole blood and plasma to evaluate the therapeutic potential of these stabilized variants. One disulfide variant clearly has improved functional properties both ex vivo and in vivo.

We are also studying the mechanisms of inactivation of factor VIIIa and factor Va by APC. In particular, we are investigating the APC-cofactor activity of factor V during APC proteolysis of factors VIIIa and Va. We are using disulfide-stabilized variants as tools to inves-

tigate mechanisms of factor VIIIa inactivation alone and in combination with mutants of APC cleavage sites. In other studies, we are investigating modulation of function of factors VIII and VIIIa by the neutrophil proteases cathepsin G and elastase and of factors VIII and V by the intrinsic pathway of coagulation.

PUBLICATIONS

Bouma, B.N., Mosnier, L.O. Thrombin activatable fibrinolysis inhibitor (TAFI): how does thrombin regulate fibrinolysis? Ann. Med. 38:378, 2006.

Cheng, T., Petraglia, A.L., Li, Z., Thiyagarajan, M., Zhong, Z., Wu, Z., Liu, D., Maggirwar, S.B., Deane, R., Fernández, J.A., LaRue, B., Griffin, J.H., Chopp, M., Zlokovic, B.V. Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. Nat. Med. 22:1278, 2006.

Eichinger, S., Pecheniuk, N.M., Hron, G., Deguchi, H., Schemper, M., Kyrle, P.A., Griffin, J.H. High-density lipoprotein and the risk of recurrent venous thromboembolism. Circulation 115:1609, 2007.

Feistritzer, C., Schuepbach, R.A., Mosnier, L.O., Bush, L.A., Di Cera, E., Griffin, J.H., Riewald, M. Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells. J. Biol. Chem. 281:20077, 2006.

Fernández, J.A., Heeb, M.J. Role of protein C inhibitor and tissue factor in fertilization. Semin. Thromb. Hemost. 33:13, 2007.

Fernández, J.A., Lentz, S.R., Dwyre, D.M., Griffin, J.H. A novel ELISA for mouse activated protein C in plasma. J. Immunol. Methods 314:174, 2006.

Fernández, J.A., Pecheniuk, N.M., Deguchi, H., Elias, D.J., Griffin, J.H. Is adiponectin implicated in venous thromboembolism? J. Thromb. Haemost. 4:1151, 2006.

Fernández, J.A., Vento, A.E., Jormalainen, M., Griffin, J.H., Pesonen, E., Syrjälä, M., Repo, H., Jansson, S.E., Rämö, O.J., Petäjä, J. Activated protein C in the cardioplegic solution on a porcine model of coronary ischemia-reperfusion has deleterious hemodynamic effects. Cardiovasc. Drugs Ther. 20:113, 2006.

Gruber, A., Marzec, U.M., Bush, L., Di Cera, E., Fernández, J.A., Berny, M.A., Tucker, E.I., McCarty, O.J., Griffin, J.H., Hanson, S.R. Relative antithrombotic and antihemostatic effects of protein C activator versus low-molecular-weight heparin in primates. Blood 109:3733, 2007.

Heeb, M.J., Fisher, M., Paganini-Hill, A. Association of low protein Z levels with ischemic stroke in young women. Thromb. Haemost. 97:495, 2007.

Heeb, M.J., Radtke, K.-P., Fernández, J.A., Tonnu, L. Plasma contains protein S monomers and multimers with similar direct anticoagulant activity. J. Thromb. Haemost. 4:2215, 2006.

Mineo, C., Deguchi, H., Griffin, J.H., Shaul, P.W. Endothelial and antithrombotic actions of HDL. Circ. Res. 98:1352, 2006.

Mosnier, L.O., Bouma, B.N. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. Arterioscler. Thromb. Vasc. Biol. 26:2445, 2006.

Mosnier, L.O., Zlokovic, B.V., Griffin, J.H. The cytoprotective protein C pathway. Blood 109:3161, 2007.

Pecheniuk, N.M., Deguchi, H., Elias, D.J., Xu, X., Griffin, J.H. Cholesteryl ester transfer protein genotypes associated with venous thrombosis and dyslipoproteinemia in males. J. Thromb. Haemost. 4:2080, 2006.

Radtke, K.P., Griffin, J.H., Riceberg, J., Gale, A.J. Disulfide bond-stabilized factor VIII has prolonged factor VIIIa activity and improved potency in whole blood clotting assays. J. Thromb. Haemost. 5:102, 2007.

Raivio, P., Fernández, J.A., Kuitunen, A., Griffin, J.H., Lassila, R., Petäjä, J. Activation of protein C and hemodynamic recovery after coronary artery bypass surgery. J. Thorac. Cardiovasc. Surg. 133:44, 2007.