DCCT/EDIC 20th Anniversary Symposium
Metabolic Imprinting and the Long-Term Complications of Diabetes Mellitus: Bench to Bedside and Back

April 10–11, 2003

Lister Hill Auditorium, NIH Campus
Bethesda, Maryland

Welcome and Purpose

Dr. Allen M. Spiegel, Director, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), opened the symposium by welcoming attendees to the meeting and expressing anticipation that the ensuing interaction would promote a better understanding of the phenomenon currently termed "metabolic imprinting." Dr. Spiegel noted that the bipartisan support from the 107th Congress in appropriation of special funding for type 1 diabetes research is an indication of the continuing importance and timeliness of the Diabetes Control and Complication Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) Study, as its 20th anniversary is being celebrated.

Dr. Judith E. Fradkin, Director, Division of Diabetes, Endocrinology, and Metabolic Diseases (DDEM), NIDDK, thanked the symposium's organizers: Dr. Saul Genuth, Professor of Medicine, Division of Clinical and Molecular Endocrinology, Case Western Reserve University; Dr. David M. Nathan, Professor of Medicine, Harvard Medical School and Massachusetts General Hospital; and Dr. Catherine Cowie, Director, Type 1 Diabetes Clinical Trials Program, DDEM, NIDDK. Dr. Fradkin then outlined the conference goals:

- To celebrate and commemorate the accomplishments of the DCCT/EDIC on its 20th anniversary;
- To explore the possible mechanistic basis for what has been tentatively termed "metabolic memory" or "imprinting"; and
- To generate plans for the fostering of research in developing new theories for the complications of type 1 diabetes.

Dr. Fradkin stated that the DCCT/EDIC study resoundingly answered its seminal question with regard to diabetes research and is widely recognized as a well-designed and implemented study. She also acknowledged that although tremendous progress has been made toward improving the lives of people with type 1 diabetes, premature death from complications remains an issue of great concern. With representatives of multiple Institutes and organizations in attendance at the meeting, Dr. Fradkin stated that she looked forward to the exchange of ideas and advice for the development of programs to be supported by the $150 million budgeted for special funding for type 1 diabetes research during FY 2004–2008.

Session 1: Glycemia and Vascular Complications: DCCT/EDIC Findings

Overview of the DCCT/EDIC Research Study

Dr. Saul Genuth, Professor of Medicine, Division of Clinical and Molecular Endocrinology, Case Western Reserve University

Dr. Genuth offered an overview of the DCCT/EDIC, beginning with the recruitment of DCCT's 1,441 participants in 1983, and continuing through EDIC's 2002 data reports. Dr. Genuth explained that the study consisted of a first phase involving a randomized clinical trial of intensive versus conventional treatments, and a second, follow-up phase that examined long-term consequences to determine the risk factors of complications influenced by treatment.

Dr. Genuth summarized the DCCT/EDIC research study results:

- Intensive treatment to hemoglobin A1c (HbA1c) of 7.2 percent reduced the cumulative incidences of diabetic retinopathy, nephropathy, and neuropathy by up to 75 percent, compared to conventional treatment at HbA1c of 9.0 percent. This result indicates that a large reduction in glycemic levels for an extended period of time can eventually overcome the influence of prior hyperglycemia.

- The prior history of an individual with diabetes clearly influences the future course of the disease, since the beneficial effect of intensive treatment was not apparent until 3 to 4 years of DCCT follow-up time had elapsed, and the benefits were greater in those participants with shorter duration of diabetes.

- The beneficial effect of intensive treatment on retinopathy, nephropathy, and neuropathy during the DCCT has persisted for 8 years, despite the near equalization of glycemia during that time period.

- Intensive treatment with its lower HbA1c level during the DCCT also reduced the risk of subsequent atherosclerosis in EDIC as assessed by measuring via computed tomography the increase in carotid artery intimal medial thickness (IMT) and accumulation of calcium in the coronary artery bed, with the glycemic effect of conventional treatment widening and becoming more significant as individuals age.

- DCCT measurements show hyperglycemia has a strong influence regarding the mechanisms of tissue damage.

- Advanced glycation endproducts (AGEs) measured in skin collagen near DCCT closeout were lower in the intensive group than in the conventional group and correlated cross-sectionally with diabetic complications, independent of HbA1c.

- The AGEs, in particular furosine and carboxymethyl-lysine (CML), also predicted progression of retinopathy assessed 4 years later during EDIC, again independent of HbA1c.

Dr. Genuth stated that the method currently used to minimize complications from diabetes is to maintain normal glycemic levels by providing insulin therapy, which entails the risk of hypoglycemia. The challenge is for researchers to achieve a better understanding of macro- and microvascular complications and to develop alternative treatments that prevent complications even in the presence of hyperglycemia.

The Effect of Glycemic Exposure on Microvascular Complications During the DCCT and Its Persistent Long-Term Effects in EDIC

Dr. John M. Lachin, Principal Investigator of the DCCT/EDIC Data Coordinating Center, The Biostatistics Center, The George Washington University

Dr. Lachin discussed the relationship between glycemia and complications in the DCCT and its persistent effects during the EDIC. Statistical analyses from the DCCT show that current mean HbA1c within each treatment group is the dominant factor associated with the risk of microvascular complications during the study, even after adjusting for baseline and current values during follow-up of insulin dose and C-peptide, lipids, blood pressure, smoking, body mass, obesity, and dietary factors. No threshold or breakpoint short of normoglycemia exists in the relationship between HbA1c and complications of diabetes. Furthermore, HbA1c levels are intrinsically related to the level and duration of glycemia, and the effects of hyperglycemia are long-lasting.

Analysis also showed that the risk of complications increases with time, exponentially with higher levels of blood glucose, and the effects of prior hyperglycemia are not wholly reversible, emphasizing the finding that lifetime glycemic exposure is clearly the predominant risk factor for long-term microvascular complications from diabetes.

Dr. Lachin then showed that the beneficial effects of DCCT intensive therapy have persisted for the subsequent 8 years of follow-up during EDIC, despite nearly equivalent levels of HbA1c in the former intensive and conventional treatment groups. Virtually all of the reduction in risk of retinopathy progression during EDIC with former intensive versus conventional therapy is due to the difference in HbA1c levels during DCCT, with only a negligible fraction explained by the differences during EDIC. Similar relationships applied to progression of nephropathy, although the EDIC HbA1c explained a somewhat larger, but still minority, of the difference in risk.

Dr. Lachin showed that a projection of the relationships between level of HbA1c and time observed during the DCCT predicts persistence of effects on retinopathy during the subsequent 8 years of EDIC. This suggests that hyperglycemia leads to metabolic or other changes that in turn determine microvascular risk. This is also consistent with initial DCCT findings where the beneficial effects of intensive therapy during the DCCT are not manifest until 3 to 4 years after the initiation of therapy.

Dr. Lachin emphasized that these EDIC results reaffirm the original DCCT recommendation that intensive therapy with the goal of achieving normal glycemia should be implemented as early as possible in patients with type 1 diabetes mellitus.

Metabolic Memory in the Development and Progression of Long-Term Diabetic Complications: Is Imprinting Present?

Dr. David M. Nathan, Professor of Medicine, Harvard Medical School and Massachusetts General Hospital

Dr. Nathan presented a synthesis of the preceding presentations to support the phenomenon of "metabolic memory" or "imprinting" and explain the persistent effects of glycemia on long-term diabetic complications. He began by describing "the glucose hypothesis," which states that treatment that normalizes glucose levels will prevent or delay the long-term complications of diabetes. Support for the glucose hypothesis existed in animal models and epidemiologic studies before clinical trials were performed. The DCCT provided definitive proof of the effects of intensive therapy, implemented over a mean of 6.5 years and aimed at achieving near-normal glycemia, on the long-term complications of type 1 diabetes, including retinopathy, nephropathy, and neuropathy. Subsequent analyses, including those presented by Dr. Lachin, convincingly show that chronic glucose control, measured by HbA1c, is the major contributor to the effects of intensive therapy on the long-term diabetic complications, accounting for more than 95 percent of intensive therapy's effect.

During the almost 10 years of observation of the DCCT cohort during EDIC, the original intensive and conventional treatment groups were treated nearly identically, by design, and their HbA1c levels converged and were nearly identical. If chronic glycemia is the major mediator of long-term complications, how do we explain the persistently decreased frequency of complications in the group of DCCT subjects originally assigned to intensive therapy compared with that of the original conventional therapy group? Since intensive therapy and near normal glycemia are not synonymous, some element of intensive therapy other than its effect on glycemia could have been responsible for improved outcomes; however, the differences in the original intensive and conventional therapy groups, such as frequency of glucose monitoring, means and frequency of insulin administration, and other factors, also dissipated during EDIC.

Dr. Nathan described the time-course of intensive and conventional therapy effects on diabetic complications to understand better their persistent long-term impacts. Following DCCT, researchers predicted the original experimental gap between the intensive and conventional treatment groups would close. However, the separation did not narrow or even remain parallel with the rate during DCCT. During the EDIC observation, the difference in rates of complications continued to widen between the original intensive and conventional treatment groups, despite nearly identical glycemic levels. Dr. Nathan called this phenomenon "metabolic memory" or "imprinting."

Dr. Nathan provided a perspective on similar biologic effects where short-term interventions result in long-term outcomes. He described examples of imprinting in animal behavior, *in utero*, and in genetics (epigenetics). Dr. Nathan addressed the issue of whether what was perceived to be imprinting might more accurately be described as delayed effects. He noted that the effects of glycemia on cardiovascular outcomes in the DCCT/EDIC cohort might very well represent a delayed effect, since it takes a long period of time for cardiovascular disease to become manifest. However, the effects of 6.5

years of intensive therapy compared with conventional therapy, presumably mediated by lower glycemia, continued for at least another 8 years during which glycemia was no longer different between the two groups. This phenomenon represents imprinting and not merely a delayed effect.

Dr. Nathan suggested several mechanisms that might account for imprinting, including:

- Clinical effects of hyperglycemia resulting from an accumulation of subclinical damage or changes that amplify over time;
- An effect of glycemia on long-lived processes such as glycation or cellular modeling; or
- Epigenetic effects.

Discussion

Dr. Jerry Palmer, Professor of Medicine, Division of Endocrinology, University of Washington, observed that, given the strength of imprinting that was seen in EDIC, it would seem that it would have impaired the ability to see changes in the DCCT, especially with regard to the secondary intervention group, and expressed surprise that such a strong treatment effect was seen in the secondary intervention group that had been exposed to higher glycemia for 8 years before intensive therapy was applied.

Dr. Nathan responded that there probably was an imprinting effect in the secondary intervention cohort. The limited ability of intensive intervention to decrease the rate of progression of already established complications, compared with the prevention of complications in the primary prevention group, is probably a consequence of imprinting. In addition, baseline HbA1c had an important effect on outcomes (higher baseline HbA1c was associated with worse outcomes), again suggesting a metabolic memory effect.

Dr. Lachin offered the suggestion that the best demonstration of the pervasive effects of prior hyperglycemia is provided by the course of retinopathy progression during the DCCT, since subjects with the longest duration and highest screening HbA1c on entry were at the highest risk for developing complications in both treatment groups, but moreso in the conventional group. In retrospect, the emergence of treatment effects during the DCCT are a direct manifestation of the same physiological processes that are now observed in EDIC.

A clarification of the issue of DCCT intensive treatment individuals who exhibited a rise in HbA1c was requested. The question was posed: Would it be correct to say that people who received intensive therapy and whose EDIC HbA1c rose did not experience an increase in complications at the rate that would have been predicted if those individuals had not been enrolled in the DCCT?

Dr. Lachin answered that in the former intensive group patients one would expect a slight increase in risk, but at a very low level of risk. Those treated conventionally whose HbA1c rose would have a similar proportionate increase in risk, but at a higher level. This is consistent with available models, although it is admittedly difficult to discern the

significance of this finding due to the small rise of only 1 percent in HbA1c during the transition from DCCT to EDIC. Subjects who entered the study with the lowest HbA1c level and duration showed the lowest risk for the development of complications from diabetes.

It was recommended that an analysis of the data be done to identify possible relationships between modified proteins or the formation of antibodies to explain the delayed effect of treatment demonstrated in the EDIC study.

Dr. Rodney Lorenz, Department of Pediatrics, University of Illinois College of Medicine at Peoria, asked the panel to explain how the effect of hyperglycemia in DCCT, while profound, could explain such a dominant proportion of the difference in risk during both DCCT and EDIC when the HbA1c during EDIC explained only 5 percent of the variation in risk during the DCCT. Dr. Lachin answered that the 5 percent variation in risk derives from a prediction model in terms of the ability to predict which subjects would have progression of complications at each successive visit, and in that context 5 percent is high. Dr. Lachin also pointed out that the effect of DCCT intensive versus conventional therapy alone also explains about 5 percent of the variation in risk, so that it is not surprising that 98 percent of the treatment group effect is explained by the difference in the HbA1c levels between the two groups. These figures persist after adjustment for virtually every other factor assessed during the DCCT, which confirmed that HbA1c was the dominant predictor of risk within both treatment groups over time.

Dr. Gayle Lorenzi, Community Health Project Manager and EDIC Coordinator, Department of Medicine, Division of Endocrinology, University of California, raised the issue of glycemic exposure and suggested that researchers consider the different levels at which individuals in the EDIC group experienced tissue exposure to glycemia. Both Dr. Trevor Orchard, Professor of Epidemiology, Medical Director, Nutrition Lipid Program, Department of Epidemiology, University of Pittsburgh and Dr. Lachin also recommended alternative measurement and analysis methods be further explored, to which Dr. David R. Matthews, Professor of Diabetic Medicine, Oxford Centre for Diabetes Endocrinology and Metabolism, The Radcliffe Infirmary, suggested that an updated analysis might be done using individual rates of change overt time rather than examining the cohort cross-sectionally. Dr. Lachin responded that another approach might be to look at the rate of change over time within subgroup. This would be applicable to a strictly quantitative measure such as albumin excretion rate (AER), although this method might be inappropriate with regard to retinopathy, since it is measured by ordinal scale.

Pathophysiology of Vascular Complications: Glycemic-Related

Hyperglycemic Vascular Damage

Dr. Michael Brownlee, Anita and Jack Saltz Professor of Diabetes Research, Department of Medicine, Albert Einstein College of Medicine

Dr. Brownlee described the relative risks for the development of vascular complications at different levels of HbA1c and credited the DCCT researchers with identifying hyperglycemia as the dominant risk factor, a discovery that has resulted in the development and use of new and more efficient treatments for diabetic individuals.

Although DCCT/EDIC data demonstrated that hyperglycemia of long duration and high degree eventually leads to diabetic tissue damage in the sites of microvascular disease, Dr. Brownlee pointed out that genetic determinants of individual susceptibilities have yet to be identified.

Dr. Brownlee outlined the following major mechanisms implicated in hyperglycemia-induced tissue damage:

- Activation of protein kinase C (PKC) isoforms via *de novo* synthesis of the lipid second messenger diacylglycerol (DAG);
- Increased hexosamine-pathway flux;
- Increased advanced glycation endproduct (AGE) formation; and
- Aldose reductase activity and redox changes.

Dr. Brownlee stated that it has been demonstrated that intracellular hyperglycemia can activate various forms of DAG-PKC. Activation of normal isoforms modifies pathologic gene expression, which results in diabetic complications in animal models.

The means by which intracellular production of AGE precursors damages cells include the modification of transcription factors, binding to coactivators and suppressors that activate gene expression, and the diffusion of AGE precursors that modify cell matrix and serum proteins, resulting in altered matrix-cell signaling, pro-inflammatory cytokine release via AGE receptors.

Recently, it has been shown that all of these mechanisms result from a single hyperglycemia-induced process—the over-production of superoxide by the mitochondrial electron transport chain. This highly reactive superoxide inhibits activity of the glycolytic enzyme GAPDH (glyceraldehyde 3-phosphate dehydrogenase), thereby diverting metabolites from glycolysis into the four major metabolic pathways and causing hyperglycemic damage. This superoxide induces DNA strand breakage, which activates poly(ADP-ribose) polymerase (PARP). The activated PARP then polyADP-ribosylates GAPDH, thereby inhibiting its activity."

Prevention of GAPDH inactivation with a PARP inhibitor halts hyperglycemia-induced activation of all of the vascular damaging pathways. Activation of transketolase with benfotiamine can divert glycolytic metabolites from vascular damaging pathways in both

cultured cells and in long-term diabetic animals, preventing activation of these pathways and the resultant development of diabetic microvascular disease.

Glycation and Advanced Glycation Endproducts: Culprits or Just Markers of Diabetic Complications?

Dr. Vincent Monnier, Professor, Department of Pathology and Biochemistry, Case Western Reserve University

Dr. Monnier presented a flowchart of the Maillard Reaction *in vivo* Study (2003), showing results divided into three categories (stressors, propagators, and endpoints (or AGEs)), and identified three pathways of AGE formation from glucose, methylglyoxal, and lipid peroxidation.

Using data from the DCCT/EDIC study, Dr. Monnier concluded that age and diabetes duration affect glycation products and collagen crosslinks, such as CML, pentosidine, fluorescence, and pepsin soluble collagen. Furosine, representing Amadori products, and acid soluble collagen were not associated with duration of diabetes. Long-term intensive treatment in the DCCT resulted in differences in collagen products, where the single major effect was the lowering of furosine by 30 percent, with a lesser but significant effect demonstrated for pentosidine, CML, and collagen solubility. Dr. Monnier pointed out the latter levels were 10 to 25 percent lower in the intensive compared to the conventional treatment group.

Collagen AGE markers were poorly associated with HbA1c at screening, but the association became much stronger with 1-year and multi-year averaged values of HbA1c, indicating an association between tissue changes and cumulative glycemia. Glycated collagen (furosine) was most strongly associated with the 1-year averaged HbA1c value.

Collagen furosine levels were strongly associated with all complications, and all collagen variables were associated with neuropathy. The former association suggested that mean glycemia was an important determinant of complications at the tissue level, while the latter suggested all forms of AGE stress might participate in diabetic neuropathy.

The data also showed an association between AGEs and severity of complication. Most notably, however, collagen AGE products remained highly associated with retinopathy, nephropathy, and neuropathy in spite of statistical adjustment of HbA1c.

Analysis of the EDIC data revealed that those individuals at DCCT closeout with highly elevated furosine levels experienced an increase in retinopathy events. Thus skin furosine was able to predict the risk of developing retinopathy 4 years later. Similar data were obtained with the other markers. Using models for evaluating the risk of a retinopathy event at EDIC Year 4 in the conventionally treated group, Dr. Monnier concluded that the pre-existence of retinopathy does not predict a worsening will occur, and therefore, adjustment for retinopathy at baseline was not necessary.

In contrast, two of the skin collagen measures included in the EDIC study have significant independent predictive value in spite of adjustment for the effect of HbA1c. Dr. Monnier stated that selective skin collagen AGE products (i.e., furosine and carboxymethyl-lysine) might contribute to the memory effect and can be used to predict progression of complications in the EDIC. He added that evidence suggests glycation products may act as culprits in diabetic complications by transmitting unwanted signals to cells.

To conclude, Dr. Monnier diagramed what he described as a vicious cycle between chemical and biological aging in the human extracellular matrix. Central to the cycle is the accumulation of Maillard products that occurs between the chemical and biological components. Dr. Monnier suggested that hyperglycemia may need to be corrected for an extended period of time in order to interrupt the cycle.

RAGE-Dependent Mechanisms and Metabolic Imprinting in the Pathogenesis of Diabetic Complications

Dr. Ann Marie Schmidt, Associate Professor, Chief, Division of Surgical Science, Columbia University

Dr. Schmidt discussed the upregulation of receptors for advanced glycation endproducts (RAGEs) and the vascular complications of diabetes. A long-term consequence of hyperglycemia is the generation of the products of nonenzymatic glycation/oxidation of proteins and the formation of AGEs. In the DCCT/EDIC study, individuals with diabetes experienced an enhanced and increased expansion in muscle and endothelial cells as compared to the non-diabetic control groups. Dr. Schmidt suggested that blockage of this interaction might be a mechanism for therapeutic targeting.

Using animal models, Dr. Schmidt described studies in which diabetic mice with established atherosclerosis that received treatment with RAGE blockage demonstrated stability of the complication, whereas those animals that did not receive treatment exhibited accelerated atherosclerosis. Blockage of RAGE in diabetic atherosclerosis highlights a lipid and glycemic-independent facet in the pathogen of accelerated atherosclerosis, a finding which Dr. Schmidt identified as holding promise for multi-target therapy in diabetic vascular disease.

To extend these concepts to a murine model of type 2 diabetes, apo E null mice were bred into the db/db background to produce what appears to be an exciting new model for microvascular complications. These mice displayed striking upregulations and an overlapping expression of ligand and RAGE, leading to the suggestion that RAGE is at least in some manner an important modulator.

Dr. Schmidt presented these additional significant findings from animal model studies conducted in the area of RAGE and diabetic complications research:

- RAGE and its ligands are expressed in injured vessel walls.
- RAGE blockage suppresses exaggerated neointimal expansion and the expression of

molecules linked to inflammation and extracellular matrix expansion after femoral arterial injury.
- The JAK/Stat 3 pathway in the injured vessel wall is dependent on RAGE. Multiple signaling pathways are associated with RAGE activation; the specific RAGE-dependent pathways clearly are associated with cellular sites and time points after acute versus chronic injury.

Dr. Schmidt suggested pro-inflammatory mechanisms linked to the pathogenesis of diabetic nephropathy as an area where further exploration is warranted, since RAGE expressed in podocytes in the human kidney is enhanced in diabetic individuals. She also recommended a closer examination of albuminuria as a functional endpoint and the consideration of RAGE as a contributor to the inflammatory component in the diabetic kidney.

The Role of Advanced Lipoxidation Endproducts

Dr. John W. Baynes, Carolina Distinguished Professor, Department of Chemistry and Biochemistry, University of South Carolina

Dr. Baynes discussed the role of lipids in the chemical modification of proteins and the development of complications in diabetes. Recent studies in animal models with pyridoxamine (PM) suggest that that PM protects against diabetic complications by both its lipid-lowering effect and by trapping reactive intermediates in the formation of advanced glycoxidation and lipoxidation endproducts (AGE/ALEs). PM has been shown to inhibit or limit the development of early nephropathy, retinopathy, and neuropathy in diabetic rats and also to decrease levels of some AGE/ALEs in skin collagen.

While recognizing the benefits of PM in retarding development of diabetic complications and decreasing chemical modification of tissue proteins in STZ-diabetic rats, Dr. Baynes acknowledged several limitations of its use, including:

- PM did not decrease levels of the AGE pentosidine.
- PM also inhibited hyperlipidemia and formation of the ALE, malondialdehyde-lysine.

Strong correlations existed between plasma triglycerides and CML and between triglycerides and albuminuria. Further studies showed that levels of AGE/ALEs were increased in obese, hyperlipidemic, but non-nondiabetic Zucker rats, and that development of nephropathy in these rats was also inhibited by PM.

Dr. Baynes stated that markers and indicators are required to assess and integrate lipoxidative damage to protein in diabetes. Overall, study data support the conclusion that lipids are an important source of chemical modification of proteins in diabetes, that hyperglycemia may exacerbate lipoxidative damage to protein, and that PM inhibits lipoxidative chemical modification of proteins, trapping intermediates *in vitro* and *in vivo*. Dr. Baynes expressed the opinion that lipids are perhaps more important than carbohydrates in the chemical modification of proteins in diabetes. They are certainly

more oxidizable, and oxidation is, as was suggested by Dr. Brownlee, an important contributor to tissue damage in diabetes.

Dr. Baynes noted that what is currently termed "metabolic memory" was in the past called "glycemic memory." He is encouraged by this change in terminology, because it has broadened the scope to recognize the importance of dyslipidemia in diabetes.

Dr. Baynes suggested that examination of the mechanism of metabolic memory or metabolic imprinting in diabetes requires several understandings: (1) that the most significant damage is oxidative in nature; (2) that protein is a useful sensor, but relevant damage occurs in genetic material; and (3) that the genetic damage is observable in the form of insertions, deletions, transpositions, substitutions, and other DNA modifications, but the injury within the cell from oxidative stress to the genome remains unseen.

Cell damage is propagated by cell division, and damage is more significant at early ages because of the greater replicative capacity of younger cells. The earlier and more severe the period of poor control, the more severe the long-term consequences. On the other hand, rigorous metabolic control at an early age yields apparent protection during later periods of poorer control. Given the fact that the onset of diabetes and obesity is occurring at earlier ages in the human population, Dr. Baynes stressed that early intervention is crucial for the mitigation or prevention of long-term complications from diabetes.

Results of Clinical Trials Using Antioxidants for Treatment of Diabetic Complications

Dr. George L. King, Research Director, Professor of Medicine, Section on Vascular Cell Biology and Complications, Joslin Diabetes Center, Harvard Medical School

Dr. King conducted a recent PubMed search that revealed overwhelming evidence in published data that oxidative stress is significantly increased in neurological and vascular tissues in diabetic and insulin-resistant individuals and in vascular cells exposed to high levels of glucose. Several potential mechanisms have been identified for the effects of hyperglycemia:

- Sorbitol-myoinositol osmolarity changes (via aldose reductase pathway);
- Oxidative-redox stress;
- Non-enzymatic glycation reactions (AGEs);
- Activation of protein kinase C (the diacylglycerol (DAG) pathway); and
- Hexosamine pathway.

However, the data seem to break down when oxidants are examined as the cause for specific complications of diabetes *in vivo*. In cultured cells or tissues, H_2O_2, AGE, and other oxidants can mimic many abnormalities induced by high glucose levels. Further transgenic animal models with increased oxidant productions do not develop vascular pathologies that are similar to diabetes without diabetes. Various types of oxidative stress

indicators have been characterized in plasma and tissues that are common between diabetic and insulin-resistant states.
Since both free fatty acids (FFA) and hyperglycemia can increase oxidative stress, Dr. King considers it unlikely that the specific pathologies in the microvessels of diabetes are mainly due to or initiated by oxidative stress. This conclusion is based on the clinical observation that insulin-resistant people who are euglycemic do not have microvascular pathologies like diabetic patients.

Antioxidant trials for diabetic complications have produced good results, at least in animal models, and there have been a number of clinical studies conducted in this area of research in which some of the early surrogate endpoints have been shown to be positive or reversed by antioxidant therapy. For example, Dr. King cited the vitamin E study results and identified use of vitamin E as a potentially promising therapy due to its ability to inhibit multiple pathways. However, large clinical trials with pathological endpoints, such as the SPACE (Stentgeschützte Perkutane Angioplastie der Carotis vs. Endarterektomie) and HOPE (Heart Outcomes Prevention Evaluation) trials, have failed to show that antioxidant therapy has a positive effect on diabetic complications. These studies suggest that new multi-functional antioxidants are needed to inhibit several hyperglycemic pathways in order to mediate adverse effects. Dr. King proposed that a higher antioxidant dosage and/or longer duration of therapy may be necessary to demonstrate a positive effect. It may also be that antioxidants prevent oxidative stress, but if hyperglycemia and its products, such as AGE, cause genomic expressions, antioxidant therapy will be unable to reverse the complications of diabetes.

Dr. King suggested that oxidants are formed in diabetes by multiple pathways and means. He recommended specific tissue markers for oxidative stress be identified, especially for human studies. Lastly, new antioxidants that have the capability to normalize multiple pathways that are adversely affected by hyperglycemia need to be designed and studied.

Discussion

Dr. Brownlee commented that investigators ought to keep in mind the differences between intracellular and extracellular glycation and lipoxidation. He asked for clarification in terms of a general model, stating that one would expect that if genetic imprinting occurs on gene expression of lipoxidation, it would be reasonable to expect expression of increased neoplasms in hyperlipidemic animals as well. Dr. Baynes responded that the extracellular environment is the one that is most easily sensed when using plasma as the analyte, and that it is important to develop a means similar to glycated hemoglobin for understanding intracellular occurrences. Regarding the frequency of neoplasms, Dr. Baynes acknowledged a lack of hard data, but explained it is difficult to find any evidence for increased frequency of neoplasms in diabetic populations or in persons with other systemic inflammatory diseases, such as arthritis or macrovascular disease. Certainly, evidence exists for increased frequency of congenital malformation in children of poorly controlled diabetic mothers, which might suggest that the condition causes damage in the genome of the child.

Dr. Fred Whitehouse, Principal Investigator, Department of Endocrinology and Metabolism, EDIC #4, Henry Ford Health System, suggested the possibility that people with the same degree of glycated hemoglobin levels might produce different levels of AGEs, since some individuals seem to do very well despite increased glycated hemoglobin. Dr. Monnier agreed that it is a key question that has been often raised. The CML lipid paradigm offers a new dimension, although the data generated on mean level of CML in humans shows that unless some level of nephropathy is present, the levels in the diabetic are normal or subnormal. This does not appear to fit with Dr. Baynes' model, which suggests that the CML comes from lipids, unless the plasma CML is removed at a higher rate by some mechanism such as a RAGE receptor. The general consensus was that a proper model still needs to be developed.

Dr. Brownlee commented that, although there is a lack of microangiopathy in people with insulin-resistant impaired glucose tolerance (IGT), unpublished data does exist to show that while microvascular endothelial cells do not react to increased free fatty acids by overproducing reactive oxygen and activating these pathways, macrovascular cells do so. It is certainly worth entertaining the notion that insulin resistance and IGT, through increased free fatty acid oxidation activation of vascular-damaging pathways in the macrovascular cells, would explain why some individuals have the metabolic syndrome and not microvascular disease, while hyperglycemia is associated with microvascular disease.

Dr. King added that data has been published demonstrating that, in the insulin-resistant state, endothelial dysfunction is present in both large and small vessels. Although the focus of Dr. King's presentation was on microvascular complications, he stated that the effect of fatty acids in both small and large vessels warrants further research.

Dr. Vlassara asked Dr. Schmidt whether the molecule: (1) exerts its activities by itself or by opposing the pro-oxidative activities of AGEs as they continue to accumulate over time as a matter of age or diabetes or (2) is entering the intracellular compartment and exerting its effects in a similar manner. Dr. Vlassara also questioned Dr. Schmidt regarding the long-term action of the molecule.

Dr. Schmidt responded that the soluble receptor was one of the first tools used to dissect the role of ligand-RAGE interaction in complications. She speculated that the interaction of the different ligands with the receptor generates, at least in part, via activation of NAPDH oxidase, an increasing spiral of reactive oxidase species generation that modulates gene expression. When shut down, this may be one mechanism by which to reduce the modulated gene expression, as well as further generation of these products. Dr. Schmidt said it was important to note that it is recognized that the soluble receptor is not a viable therapeutic target because of the production and mode of administration. However, because all ligands appear to cross-compete in V-domain of the extracellular component of RAGE, this has been the target for the generation of small molecules that are non-toxic and orally available and which will be further tested.

In response to Dr. Vlassara's query regarding whether or not these complexes were excreted in urine, Dr. Schmidt answered that they were, but only with the soluble receptors, because in other settings that was not the biochemical target utilized. With

regard to the question of vascular endothelial growth factor (VEGF), Dr. Schmidt said that the apo null db/db model is promising in that some early evidence suggests that VEGF appears to be a pathologic pathway by which the transcription factor regulates modulation of downstream genes. The homozygous RAGE Null mouse is an important tool for examining each aspect of pathways and potential impact of blockage of the pathway.

Session 2: Other Risk Factors for Vascular Complications

Role of Hypertension as a Risk Factor for Vascular Complications

Prof. David R. Matthews, Chairman, Oxford Centre for Diabetes, Endocrinology and Metabolism

Dr. Matthews described the United Kingdom Prospective Diabetes Study (UKPDS), a 10-year glucose study of 5,102 persons with type 2 diabetes that provided trial and epidemiological data linking hypertension and hyperglycemia. While numerous factors contributing to tissue damage in diabetes have been recognized, the UKPDS established the critical role of hypertension as a risk factor for diabetic complications.

The UKPDS study design divided the participants into conventional and intensive treatment groups, which were then divided further into tight and less tightly controlled hypertensive study subgroups. Using the Early Treatment Diabetic Retinopathy Study (ETDRS) grading, greater changes in retinopathy grading were noted after 7.5 years for the less tightly controlled group than for the tightly controlled group, an indication that treating hypertension in type 2 diabetic individuals prevents progression of retinopathy. Statistically, relative risks for microaneurysms and hard and soft exudates related to blood pressure randomization ranged from 0.53 to 0.66 after 7. 5 years. However, hypertension was not shown to cause differential effects on different retinal lesions.

Various blood pressure medications, including angiotensin-converting enzyme (ACE) inhibitors and beta blockers, were evaluated in monotherapy trials. Blood pressure means were compared at baseline and after 9 years and showed virtually no difference in blood pressure changes between these two types of oral hypertension agents.

The study compared the risk level of hypertension and hyperglycemia and concluded that both are risks for the development of diabetic complications. Risk reduction for glycemic control using microvascular endpoints was 25 percent overall over a 9-year period. Endpoints included photocoagulation, vitreous hemorrhage, renal failure, and renal death. For hypertensive controls, risk reduction for microvascular endpoints began after just 3 years, ending at 37 percent after 9 years, a finding concordant with the DCCT data.

Dr. Matthews concluded with the statement that subjects realizing the greatest risk reduction were within the intensive glycemic control and tight blood pressure control group. This analysis of epidemiological and trial data revealed no significant treatment differences between glycemic control versus hypertensive control.

Lipoproteins and Other Lipid Molecules

Dr. Ira Goldberg, Columbia University, College of Physicians and Surgeons

Dr. Goldberg discussed the role of lipoproteins and other lipid molecules as risk factors for diabetic complications, from the perspective of lipid researchers with limited diabetes experience. Major efforts have been aimed at explaining the increase in atherosclerosis in patients with diabetes, identifying hyperglycemia, hyperinsulinemia, hypertension, and lipid abnormalities as possible causes.

Lipid-centered research has focused on the idea that vascular disease in diabetics is due more to lipid abnormalities than to glucose levels. Data has shown that pre-diabetics with normal fasting hemoglobin levels are at increased risk of macrovascular disease, even though they have not yet become hyperglycemic. Indeed, the UKPDS study showed that the number one risk factor for macrovascular disease was not glucose but LDL cholesterol, followed by HDL cholesterol. HbA1c, while important, fell behind these lipid factors as a risk factor. Dr. Goldberg also pointed out that it is important to note the correlation between LDL and HDL as separate measurements, rather than a combined or total cholesterol.

Citing data from a comparison between studies done at Harvard and at the University of Tokyo, Dr. Goldberg stated that the Japanese diabetic populations with no lipid abnormalities developed very little macrovascular disease. At almost every age, people in Japan had an approximately 20 percent risk of dying due to macrovascular disease compared to the Harvard cohort, a finding from which Dr. Goldberg concluded that while diabetes may lead to macrovascular disease, diabetics with low cholesterol have one-fourth to one-fifth the chance of developing the macrovascular complications independent of blood sugar levels.

Dr. Goldberg listed four consequences from lipid abnormalities in type 2 diabetics—hypertriglyceridemia, small dense LDL, reduced HDL, and postprandial lipidemia.

Dr. Goldberg commented that NIH is currently funding a consortium to devise animal models specifically for use in macrovascular disease and diabetes research. Animal models provide a particularly viable means for the examination of increased incidence of macrovascular disease in diabetics. Studies have been conducted in this area over the past 50 years in rabbits, pigs, and mice. There are a number of reasons why it would be preferable to develop models in mice—their size, rapid breeding, and many strains with specific genetic modifications. Dr. Goldberg stated that the current challenge for animal model researchers is to engineer a mouse so that when it becomes diabetic it develops more atherosclerosis but does not become more hypercholesterolemic; this will allow researchers to assess the independent effects of hyperglycemia on the artery.

Dr. Goldberg described the process of formation of fatty acids and lipolysis products. Diabetic individuals exhibit high amounts of fatty acids. Arteries may be exposed to high concentrations of lipolysis products, because fatty acids in triglycerides are approximately ten-fold greater than free acid concentrations. Since lipoprotein/lipids accumulate within the arteries, Dr. Goldberg

recommended that additional studies be conducted to further investigate whether lipolysis increases atherosclerosis.

Dr. Goldberg identified the pathological effects of lipolysis, including increased arterial wall permeability and greater passage of lipoproteins into the intima, alteration of vasoreactivity, and increased expression of inflammatory molecules. He stated that more recent data suggest that fatty acids may be natural PPAR (peroxisome proliferator-activated receptor) agonists and that some investigators have suggested beneficial effects of fatty acids. While it has been shown that the number one risk factor for cardiovascular disease is age, Dr. Goldberg stressed that the effect of lipid abnormalities, as well as glucose level, ought to be considered.

With regard to the imprinting phenomenon, Dr. Goldberg offered the suggestion that since the primary risk factor for the development of cardiovascular disease is age, it may be that even when glycemia in diabetic individuals is treated earlier and more aggressively so that the risk is somewhat mitigated, the difference in risk is still below the level where one can observe clinically apparent markers of the disease at a later time in an individual's history, and this may be a reasonable explanation for the persistent effect evident in the first part of the DCCT/EDIC study.

Glycoxidant Burden: New Risks and Host Defense in Diabetic Complications

Dr. Helen Vlassara, Mount Sinai School of Medicine

Dr. Vlassara began by stating that while evidence clearly shows that an increased glucose level is a major risk factor for the development of diabetic complications, recent environmental changes, over the past 100 years, are likely to contribute significantly to the dramatic increase in incidence of diabetes. Dr. Vlassara also pointed out that little is known about genetic risk factors that render the population increasingly susceptible, not only to diabetes but also to its complications. As greater knowledge is gained in general, new treatments can be developed and put into use.

Dr. Vlassara described different mechanisms by which glycoxidation products, or AGE compounds, impact adversely on key functions such as immune response, growth, and tissue repair, or target various tissues to cause organ damage. Dr. Vlassara discussed several anti-AGE defenses, including antioxidant systems, AGE-receptors and renal clearance. In her opinion, the best means for preventing or delaying progression of diabetic tissue injury, in addition to controlling glycemia, would be by restricting sources of glycoxidants and by enhancing host-defense systems (i.e., AGE-receptors).

Referring to the recent explosion in knowledge regarding AGE receptors as a part of AGE homeostasis, Dr. Vlassara pointed to the lack of adequate understanding of a critical aspect of this system—AGE removal. Unlike oxidant stress-promoting receptors such as RAGE, the clearance-promoting AGE-R1 appears paradoxically weak, particularly under glycoxidant stress-related states (i.e., diabetes or aging). An inverse relationship has been shown to exist between

AGE-R1 expression/function and circulating AGE levels in aging animals and humans, diabetic or not. Dr. Vlassara, who identified this paradox, remarked that very few studies have been successful in examining the reasons beneath it, suggesting that larger sources of glycoxidant stressors, i.e. modern diets, may have a role. This however will require rigorous scientific inquiry.

Dr. Vlassara stressed the need for new research to move beyond glucocentricity and to consider factors independent of diabetes that might contribute to excess of oxidant burden, an obvious one being the increasing availability/consumption of AGE with modern foods. She pointed to a body of evidence indicating that restriction of AGE in foods reduces diabetic atherosclerosis, nephropathy, or impaired wound healing and other pathogenic processes, including autoimmune diabetes or insulin resistance in diabetic animals. In diabetic patients, a similar intervention led to a suppression of markers of inflammation, as well as AGE levels. Dr. Vlassara recommended that this area of research, which may be vastly underestimated, be given more attention in the future.

Discussion

Dr. Goldberg was asked how much, if at all, tighter glycemic control is a factor in terms of clinical trials with triglyceride and HDL changes, and whether or not clinicians should seek increased LpL activity, which seems to cause bad circulating lipids, or decreased LpL activity, which yields greater fat breakdown at the vessel walls.

Dr. Goldberg prefaced his response by stating that patients are very variable, so clinical trial data may not provide the best information from which to draw conclusions. Improved lipids, especially decreases in triglycerides, correlate with glucose control. However, there is a marked variability in the response to glucose control alone. Dr. Goldberg suggested that data from EDIC and UKPDS may provide a better insight into this aspect of glycemic control.

Dr. Goldberg also referenced data from a study at Cornell by Dr. Donald Zilversmit that suggested that lipid particles circulate and come into contact with blood vessels, where the lipoprotein lipase cuts them into smaller remnant lipoprotein lipases, and this is a major aspect of atherosclerosis. The study also assayed lipase in the vessel wall, and it was determined that blood vessels with more lipoprotein lipase had more atherosclerosis. In contrast, greater amounts of lipoprotein lipase in muscle reduce triglyceride levels and raise HDL levels. This is a dichotomy; lipase in muscles is considered to be beneficial as opposed to lipase in vessel walls. Lipoprotein lipase in vessel walls allows for better LDL particle adhesion.

Dr. Matthews added that UKPDS showed moderate hypertriglyceridemia in most patients. However, those individuals with levels above 250 mg/dL did not respond well to amelioration of glycemia.

The comment was made that fairly good evidence exists to suggest that key AGE-clearance mechanisms operate sub-optimally under conditions of increased oxidant stress (i.e., hyperglycemia), a new paradigm worth further exploration, given that it is likely to contribute to AGE retention, pro-atherogenic lipid generation, and vascular deterioration..
Dr. Lorenzi stated that the UKPDS showed that the effect of glycemia and hypertension are multiplicative. However, it is a multiplication that must be initiated by hyperglycemia, because individuals with hyperglycemia and no diabetes do not exhibit the same kind of lesions.
Dr. Lorenzi asked for clarification regarding the proposed mechanism of the interaction of hypertension with hyperglycemia with regard to increased risk.

Dr. Matthews agreed that hypertensive retinopathy does not resemble diabetic retinopathy, since the etiology of hypertension is probably related to autoregulation. Patients with hyperglycemia, probably lose some degree of the autoregulation function, which results in an increase in sheer stress. Diabetes is an initial aspect of hardening of the microvessels and is followed by an increase in sheer stress, a finding demonstrated by animal models. However, this process is not yet fully understood.

Dr. King raised the issue of glycemic memory, in that during intensive care for retinopathy, there is a worsening in both primary and secondary intervention groups, and that this occurrence is presumably an acute injury phenomenon. However, Dr. King asked whether or not patients recovered from that experience, and if so, did researchers observe a similar delay in both groups? If not, it would seem to argue for the existence of differing mechanisms.

Dr. Matthews responded that it is well-known that rapidly improved glycemia results in increased levels of cotton wool spots. In terms of long-term treatment to the eye, no effect is observed. Apparently, the phenomenon occurs only in that single subset of retinal lesions.

Regarding visual acuity as an endpoint, Dr. Nathan suggested that the lack of analysis of data on visual endpoints was due to a paucity of events at this stage of the currently ongoing research studies, a point with which Dr. Matthews concurred, stating that while the UKPDS patients exhibited a small number of endpoints, the trends which were observed nonetheless ran in the same direction.

Session 3: Possible Mechanisms for Metabolic Memory/Imprinting

Epigenetic Basis of Human Disease

Dr. Benjamin Tycko, Associate Professor of Pathology, Institute for Cancer Genetics, Columbia University

Dr. Tycko offered a general overview of what he described as classical genomic imprinting, including the physiological functions of imprinted genes. Epigenetics and cell memory have been highlighted recently by mammalian cloning, which has produced animals with identical DNA sequences, but differing phenotypes. Dr. Tycko suggested that diabetes researchers might want to further examine the concept of clonality with regard to resetting genomic imprints. Using recent knockout studies conducted with the Ipl/Tssc3 imprinted gene and the important historical chapter of classical embryological experiments in mice, Dr. Tycko discussed the consequences of monoallelic gene expression due to imprinting.

Theories of imprinting can be divided into two categories: structural and functional. Structurally, the mechanism is based on two very well-documented observations:

- Multiple imprinted genes are clustered in megabase-scale regions (although some details differ between regions, some common themes exist, such as non-translated RNA production and genomic insulator elements); and
- Imprinting is determined by allele-specific DNA methylation at critical sties.

Additional research has focused on the biological function theory, originated by D. Haig and colleagues, which suggests a conflict between maternal and paternal "drives" for reproductive success and predicts that paternally silenced genes retard growth of the conceptus, while maternally silenced genes promote growth and increase nutritional demands on the mother. Dr. Tycko recommended further research into possible explanations for how imprinting might control the dosage of this minor subset of mammalian genes. This subset of genes is small, as indicated by the fact that most genes behave in a Mendelian fashion, while imprinted genes do not. Nonetheless, imprinted genes may have a disproportionate influence on particular biological functions.

Dr. Tycko described results from several *in vivo* studies of imprinted genes conducted in mice, in which researchers concluded that imprinted genes seem to be inordinately devoted functionally to the processes of growth and behavior, both of which are readily accommodated in the conflict model. Mouse knockout experiments and genetic evidence in humans already show that a high percentage of imprinted genes are involved in growth or behavior, and Dr. Tycko stated that the direction of imprinting seems to correlate with the effect on growth that is predicted by conflict. He emphasized that imprinting as a dosage regulation mechanism does control growth, but not through a single biochemical pathway.

Most recently, his lab has looked at the effect of one imprinted gene, called Ipl/Tssc3, on placental growth and its expression in the parts of the mouse and human placenta that mediates feto-maternal exchange, an area of research that may have relevance to susceptibility to type 2

diabetes later in life. In the mouse model, Ipl-positive cells disappear at mid- to late gestation; it is significant to note that the expression of Ipl does not decline progressively, but that the cells that are Ipl-expressing disappear. Dr. Tycko cited this as an example of a maternally expressed imprinted gene that restrains the growth of the placenta and prevents an undue strain on maternal resources, highlighting the potential stem cell-like role of the Ipl-expressing cells. Human Ipl is expressed throughout gestation in the placenta, and this correlates with the fact that human placenta grows progressively through gestation, while the mouse placenta, in which Ipl-expressing genes disappear at mid-gestation, plateaus at day 16 and subsequently declines before birth at day 20.

Based on new subcellular localization data, Dr. Tycko also suggested that Ipl might be restraining placental grown in a non-cell autonomous way, perhaps by regulating secretion of trophic factors, and stressed that imprinting as a gene dosage-regulating mechanism controls growth through multiple and independent biochemical pathways. He stated that further research is warranted in the area of imprinting regulatory sequences with regard to interuterine growth retardation, since growth retardation may underlie some aspects of the metabolic syndrome.

Dr. Nathan asked about the effects of post-translational modification with glucose on the enzymes, such as those that affect methylation and acetylation, which might not be considered part of the imprinting phenomenon per se, but might affect other expression in a wider manner. Dr. Tycko responded that hyperglycemia may affect DNA and suggested that methylation be further examined, since evidence exists to show that culturing early embryos in high or low glucose media, or culturing nuclear transplantation clones in that type of media, affects DNA methylation and the efficiency of mammalian cloning.

Role of Glycemia on Vascular Integrity

Dr. Mara Lorenzi, Senior Scientist, Associate Professor, Department of Ophthalmology, Schepens Eye Research Institute and Harvard Medical School

Dr. Lorenzi suggested that the way in which cells and tissues react to protracted hyperglycemia may be a mechanism for the memory phenomenon under discussion. There are vascular changes in diabetic retinopathy that are early and yet irreversible and remain both histologically and clinically undetectable. Cumulatively, these changes have consequences that alter the anatomy of retinal vessels, adding another level of irreversibility. Changes in vessel anatomy are measured clinically on a discontinuous scale, and the clinical staging may thus not reflect accurately the degree and progression of vascular damage. These factors can account for the inertia of appearance of lesions as well as for the acceleration of their progression once events begin accumulating.

Summarizing data from studies of human diabetic retinopathy performed in retinas obtained postmortem from type 2 diabetic patients, Dr. Lorenzi illustrated how the death of vascular cells occurs early and is already the result of multiple events. TUNEL-positive pericytes are detected in vessels with normal appearance. Prior to death of the pericytes, there had been an upregulation

of Bax, a pro-apoptotic protein, clearly detectable in the TUNEL-positive pericytes. This was in turn probably preceded by activation of NF-κB.

Of great interest, in the human retinal vessels NF-κB-positive cells were almost exclusively pericytes. This was puzzling because literature from *in vitro* studies primarily reports high glucose inducing NF-κB activation in endothelial cells. It appears that microvascular retinal endothelial cells respond in a different manner than do macrovascular endothelial cells. Furthermore, following exposure to high glucose, apoptosis of pericytes occurred, but not apoptosis of the endothelial cells. Dr. Lorenzi suggested that retinal pericytes and endothelial cells sense and/or react to glucose differently. In pericytes high glucose may cause oxidative stress, perhaps mediated by activation of the polyol pathway, inducing NF-κB activation, which in turn induces Bax overexpression and apoptosis. It has been generally assumed that microaneurysms evolve from the loss of pericytes. One may envision that when several pericytes will have died in the same area of the vessel, the development of one or more microaneurysms will make clinically detectable a process that had been ongoing for months or years.

Dr. Lorenzi recommended that further investigation be devoted to endothelial cells, which may be exposed in diabetes to events and processes more damaging to them than high glucose per se. One possibility may be microthrombosis, since data show that the number of microthrombi and total area of thrombi are from two- to four-fold greater in the retinal vessels of diabetic patients than in non-diabetic individuals. Even if microthrombi were to cause only transient occlusion in the early stages, the event would leave an imprint on the endothelial cells, having exposed them to some hypoxia, and therefore to other adaptive changes. Another process that may affect survival and function of endothelial cells in the long term is complement activation, demonstrated to occur, probably through the alternative pathway, in retinal vessels of diabetic patients.

However caused, apoptosis of endothelial cells and subsequent increased turnover can be a mechanism whereby the vascular wall is progressively altered in the absence of clinical signs, at least for a while.

A type of "memory" induced by high glucose is also the persistence of abnormal gene expression when high glucose is removed, noted for genes encoding extracellular matrix proteins.
Dr. Lorenzi cautioned, however, that while abnormal regulation of gene expression may explain some persistent effects of hyperglycemia, the longer term memory that dictates the rate of progression of retinopathy is more likely to reside in the degree by which the retinal vessels have changed in response to diabetes and have distanced themselves from the physiological state.

Augmentation and Sensitization of Calcium Fluxes in Simple and Complex Forms of Memory

Dr. Howard Schulman, Vice President of Research and Development, SurroMed, Inc.

Dr. Schulman prefaced his presentation by stating that while many models do not fully explain the imprinting phenomenon, they can be useful in demonstrating how molecular memory can be

generated from “common” signaling molecules. While examples from these signal transduction systems, primarily neuronal, may not be specifically applicable to the metabolic “imprinting” associated with long-term complications of diabetes, they may offer a broader perspective of possible mechanisms associated with such imprinting. Dr. Schulman’s examples involved enhanced synaptic transmission via calcium ion flux, using the “cognitive” kinases cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase (CaM kinase II). Each of these kinase pathways eventually lead to changes in gene expression that maintain the synaptic change for days, months, or years. Other enzymes may be capable of producing similar self-perpetuating changes.

Citing studies done with Aplysia, Dr. Schulman explained that a biochemical and synopsis analysis following a combination of tactile stimulus and tail shock showed long-term habitualization or sensitization of gill-withdrawal reflex. The combination of these initial stimuli involved Adenylate cyclastic AMP (cAMP), which does not respond well to either calcium or g-protein alone, but when both were used, they produced a much higher response to cAMP, resulting in increased transmitter release and greater synoptic strength.

Dr. Schulman stated that, under normal hormonal stimulation, the cAMP system involves the removal of an inhibitory regulatory subunit, which allows the catalytic subunits to go into an “on state.” Under normal circumstances, these subunits eventually revert to the deactive state. However, as shown in the Aplysia model in which training stimuli are strong and repetitive, the condition is prolonged when test stimuli are resumed.

Following prolonged activation, selective degradation of these regulator subunits occurs, with a resulting excess of constitutive catalytic for the lifetime of the subunit or until more regulatory subunits can be synthesized. Dr. Schulman pointed out the importance of this finding, in that genetically-enhanced degradation allows the system to remain in its active state for longer periods of time. This is, therefore, a self-propagating cyclic phenomenon involving synaptic memory. Prolonged combined stimuli consisting of both tail shock and siphon stimulus, leads to a degradation of regulatory or auto-inhibitory subunits and the production of an excess of catalytic subunits, resulting in a prolonged reduction of inhibitory regulators. Eventually, the normal course of events would show a dissipation of these inhibitors and degradation of catalytic subunits. However, because the catalytic subunits are activated for a longer period of time, they are able to enter the nucleus and induce the expression of a gene that promotes further degradation of the regulatory subunits. This self-propagating process results in a long-term excess of active catalytic subunits, which increases transmission at synapses by phosphorylating and inhibiting a potassium channel and thereby prolonging the state of depolarization with every stimulus.

As a second example of the impact of neurons on genetic memory, Dr. Schulman offered a study of hippocampal long-term potentiation (LTP) in mammals. Measuring synaptic strength following a high frequency stimulus revealed an enhancement to test stimuli in terms of duration of response in addition to level of activity. Changing a single amino acid in the endogenous gene for CaM kinase II, so that autophosphorylation could not occur, resulted in an animal unable to learn spatial cues. This phosphorylation is critical because it disables the built-in inhibitory region of the kinase and allows the kinase to be persistently in the “on” state until

dephosphorylated. Translocation to and binding of CaM kinase II to the NMDA receptor also locked the kinase in an "on" state. The active kinase increases the activity of a critical receptor (AMPA glutamate receptor) for memory as well as increasing the insertion of the receptor in the membrane, similar to insertion of glucose transporters by insulin. Since synaptic memory depends on conditions that allow receptors to be placed in an active state, CaM kinase II is important in synaptic memory because it allows the active state to be maintained for longer durations, thereby enhancing the total number of receptors and synaptic strength.

Dr. Schulman suggested that some shift occurs in sensitivity following activation of the cAMP protein kinase enzyme, so that the same stimulus no longer yields the same output. This might translate to diabetes research in that if the amount of glycated hemoglobin is considered as an index of glycation and peripheral retinopathy or neuropathy, it might be possible to shift the sensitivity of the system to the appropriate stimulus, thereby creating a memory in response.

Dr. Schulman recommended that enzymes such as CaM kinase II be further examined, since their ability to place and maintain the system in an active state might be useful with regard to their examination of and application to genetic memory and diabetes.

Changes in Renal Structure

Dr. Timothy M. Meyer, Professor of Medicine, Department of Nephrology, Stanford University

Dr. Meyer opened by noting that a car that is well maintained initially will last longer than one that receives good maintenance later on after it has begun to wear and deteriorate. Much evidence exists from diabetic nephropathy studies that renal injury often progresses even after the initial cause of the injury has been removed. Patients with diabetic renal injury and falling GFR (glomerular filtration rate) who had HbA1c rendered normal continued to decline, suggesting that once renal injury has occurred, like the car, the kidney continues to decline, despite removal of the original stimulus.

A surprising finding in the DCCT/EDIC study was that the kidney seemed doomed to progressive injury at very early stages, even when little evidence of injury existed at the onset. At the DCCT close-out, GFR was normal and median values for albumin excretion rate (AER) were low. Dr. Meyer pointed out that the identical mean levels of AER between the two treatment groups, however, was not an indication that the populations were themselves identical, and that more patients in the conventional treatment group had perhaps sustained previous serious injury and experienced higher levels of albuminuria than in the intensive treatment group. In fact, some patients who fared poorly during EDIC were members of a group that had less than 40 mg/day of albumin at DCCT close-out. Dr. Meyer suggested it is possible that a portion of these patients possessed clinically damaged kidneys at the end of DCCT, and that the damage progressed during EDIC, even though they exhibited normal GFR and AER rates.

Given these study findings, Dr. Meyer presumed that something occurred to the kidneys, and more particularly the glomeruli, of these patients, that rendered those structures liable to future

damage, although their filtration function and ability to retain albumin in circulation remained within normal ranges. He identified three observations of structural anatomy during the early stage of injury that may give insight into the cause for predisposition to progressive renal injury in diabetic patients: (1) glomerular volume is slightly and variably increased; (2) thickness of basement membranes between epithelial and endothelial cells are markedly increased; and (3) the mesangial matrix volume is increased.

Dr. Meyer stated that good evidence exists in both animal models and human studies to show that structural changes persist, even when glucose is brought back to normal or near-normal levels. It is hypothesized that long-sustained alterations in the collagenous framework of the glomerulus may make it susceptible to albumin leakage and to ultimate deterioration. Insofar as collagenous alterations found in the conventional control predisposed those patients to later injury, this may explain similar observations in the EDIC cohorts.

Dr. Meyer identified visceral epithelial cells or podocytes situated on the outside of glomerular capillaries as another structural candidate for the EDIC findings. These cells cannot undergo mitosis and, once lost, cannot be replaced. Therefore, their loss during poor control represents a structural change that cannot be reversed by later improved control. He described the increase in glomeruli size and maintenance or decrease in epithelial cell numbers observed in animal models and in long-term diabetic patients who experienced no increase in albumin excretion, microalbuminurea, and clinical nephropathy. The cause of epithelial cell loss in these patients is as yet unknown.

Dr. Meyer pointed out several areas that warrant further research, including:

- Development of improved measurement systems to replace the crude method currently used to measure glomerular injuries early in diabetes.
- Identification of a better index of early injury than albuminurea.
- More accurate markers of injury in order to suggest pathways of injury, to sort out patients undergoing injury, and to aid in future trials.

Early DES Exposure: A Prototype for a Delayed Pathological Outcome

Dr. David Sassoon, Associate Professor, Brookdale Department of Molecular, Cell and Developmental Biology, Mount Sinai Medical School

Dr. Sassoon discussed the role of patterning genes in the female reproductive tract. Tissues from the female reproductive tract and breast and the male prostate maintain very high levels of gene expression, a phenomenon normally associated with embryological processes. As such, these tissues are effective models for delayed pathological outcomes related to vascular tissue, which retains a high degree of plasticity in the adult. Dr. Sassoon suggested that tissues that are highly plastic probably remain very vulnerable to insult, and the results of injury may not become apparent until many years after occurrence.

Uterine analyses are very useful because these tissues have the high degree of plasticity necessary for remodeling. It has been observed that certain developmental pathways, which were previously believed to be predominantly an embryonic property, remain in certain adult tissues, and these tissues are particularly sensitive to steroid hormones. Dr. Sassoon described the Wnt gene history and explained that Wnt is a secreted glycoprotein that acts in a manner similar to a growth fat factor by binding to a receptor. One response to engagement of the ligand by the receptor is the translocation of a complex into the nucleus, resulting in a transcriptional outcome.

Dr. Sassoon cited studies of female reproductive tract development showing that the mesenchym provides a signal that initiates cell differentiation. However, it is important to note that while there is a two-way communication between these two tissue types, it is the mesenchym that dictates epithelial outcome. Dr. Sassoon stated that Wnt genes are good candidates to guide these epithelial-mesenchymal interactions in the female reproductive tract during development.

Research with DES, a synthetic estrogen that was administered to pregnant women from the 1940s through 1974, disclosed that all phenotypes in mouse models were seen as line-by-line items in women or daughters born to mothers who took the drug. Dr. Sassoon described the normal lifetime variation of Wnt7a levels and summarized data that suggest that critical and irreversible changes occur at birth for those mice exposed to DES *in utero.* Studies predominantly focused on mice that were 1 month old and sexually mature, and no signs of cancer were observed. However, by the time the female mutant mouse was 6 months old, a delayed pathological outcome had become apparent. Although the mouse survived, it could not reproduce.

Dr. Sassoon identified the delayed pathological outcome as an important aspect of the memory process. Recent research data show that changes in gene expression and morphological abnormalities in the mouse models appear as early as 1 month post-natal. However, cancerous and pre-cancerous states do not become observable in mice for several months, and in women these changes are not detectable for several years, when these DES-exposed women tend to have an increased rate of uterine and cervical cancers in adult life.

Wnt7a, has been identified as a target in DES-mediated endocrine disruption, and Dr. Sassoon emphasized that a critical window for intervention exists, since Win7a levels return to normal within 5 days following *in utero* exposure in mouse models.

Commenting briefly on extracellular matrix proteins that are currently being investigated with regard to their profound effect on cell differentiation, Dr. Sassoon proposed that Wnt genes may target extracellular matrix components and subsequent cytoarchitecture. He shared recent research in this area indicating that these subtle changes in cytoarchitecture brought about by drug exposure can have long-term effects that are not immediately observable.

In conclusion, Dr. Sassoon stated that Wnt genes are potent morphogens in the female reproductive tract and that the female reproductive tract (and probably by extension any other extremely plastic systems) are vulnerable to pathological outcomes, even after a very brief interruption in the normal signaling processes that are required not only to direct the appropriate embryogenesis, but also to maintain them in the proper form. Tumorigenic outcomes may reflect

both direct gene targets in the Wnt pathway or be a result of a more subtle disruption of early cytological architecture. Extracellular matrix components are altered by Wnt signaling.

Discussion

Dr. Paul Robertson, Pacific Northwest Research Institute, Seattle, offered an alternate hypothesis to imprinting by suggesting that results in DCCT are due to the patients' behavior being affected, since some of the variables in the study involved reinforcement by healthcare professionals in addition to good glucose control. It may be that those patients have simply learned to be healthier in ways that are unappreciated because researchers have yet to ask the right questions, and that improvement might be a result of improved behavior rather than cellular imprinting.

Dr. Schulman referred to a previous speaker who addressed the issue and concluded that it did not seem to be a confounding feature of the study. However, he acknowledged that the possibility certainly exists that behavior modification occurred and suggested that it might be prudent to examine whether those patients who improved could have done so based solely on behavioral changes.

Dr. Monnier pointed out that animal models are an exception to the behavior paradigm.
Dr. Sassoon added that patients with poorer glucose levels, once informed of the study outcomes, then exhibited more controlled glucose levels themselves. Dr. Genuth stated that there were clearly behavioral changes, but as far as blood glucose fluctuations throughout the day are concerned, no specific analyses have been conducted as yet, although preliminary data suggest that mean HbA1c was the only correlate with retinopathy.

Dr. Nathan commented that Dr. John Lachlin's results suggesting that a large fraction of the difference between interventions is explained by glycemia are new data, generated for this meeting, and expressed his interest in exploring other areas of research presented during the symposium.

Several participants offered remarks regarding the behavioral changes affected by patients in the DCCT/EDIC, and the comment was made that paradigms beyond the environmental and genetic as presented during the symposium were appreciated.

Dr. Paul Beisswenger, Dartmouth Medical School, spoke of the lack of a clear correlation between albumin excretion and the microhistology of the kidney, leading to the assumption that some patients have damage to kidneys that is not evident in a clinical sense.

Dr. Vlassara addressed an issue raised by Dr. Meyer, stating that before structural exchanges are expressed, it is known that changes in the expressions of genes are apparent in the extracellular matrix. However, calculations of creatinine versus carboxymethyl-lysine (CML) have been conducted that reveal that even at the beginning stages of diabetes, absent evidence of renal abnormalities, the overall excretion rate of CML is one-seventh of the creatinine clearance, indicating a tremendous amount of reabsorption taking place at the tubular cell layer, four-fold lower at the onset of diabetes as compared to a normal individual that has been age-matched. Dr.

Vlassara suggested that CML or a related test might actually be a more sensitive marker for assessing renal function in diabetes than GFF (glomerular filtration fraction) or creatinine.

Dr. Meyer answered by identifying two aspects to the problem: (1) the kidney can be injured by a variety of things that circulate in diabetes, since it serves as a sort of a "molecular garbage disposal," and (2) these things may themselves be a marker of injury. Both of these aspects argue for further examination of DCCT/EDIC specimens with regard to a variety of biochemical measurements that will ultimately predict later clinically important injury.

Dr. Eva Feldman, University of Michigan, Ann Arbor, asked Dr. Schulman whether or not a true learning phenomenon might have occurred that would have altered behavior and whether neurons in the peripheral neural system "learn" metabolically how to handle substrates differently if they are normally glycemic for an extended period of time. Dr. Schulman agreed that a placebo effect is certainly demonstrable by imaging and that it was a difficult question to answer, since physiological measures become neuro-protective following controlled glycemia.

Dr. Matthews described a model in a trial using glycoside to lower glycemia in humans to prevent onset of diabetes in which withdrawal of glycoside resulted in worsening scores, which demonstrated that lowering glycemic load in order to retrain beta cells was unsuccessful.

In terms of complications, Dr. Nathan felt it fair to say that researchers have very little information with regard to protective mechanisms and their loss, as well as reparative mechanisms, and concluded that, at least from a pathophysiological point of view, very little is known about this area. Dr. Genuth added that the same is true from the physiological point of view.

Dr. King asked the panel to offer their opinions regarding whether or not new techniques for mass screening (either proteins (proteomics) or DNA (genomics)) would pick up the genes or proteins that have been examined thus far. Dr. Tycko said that such investigation deals with changes in nuclear gene expression and, given results from studies in this area, encouraged Dr. King to move in that direction with respect to his own research. Dr. Sassoon added that the histology in his studies clearly indicated a multitude of gene expression differences. He identified two areas of critical research that need to be explored further: (1) the minimal time for rigorous treatment in order to achieve long-term beneficial effects and the earliest initiator response times and (2) methods preceding overt morphological alterations.

Dr. Schulman suggested that broad analysis of metabolites, proteins, and peptides would be beneficial in understanding whether control based on glycated hemoglobin would be applicable to other glycated molecules with different time courses. In addition, Dr. Schulman agreed with Dr. Meyer that there is a need for biomarkers at all levels that probably exist in the fluids that are being stored or collected in ongoing studies and trials.

Dr. Robert E. Silverman, Senior Director of Regulatory Affairs, Merck Research Laboratories, summarized the general acknowledgment that the conventional group might have problems as a result of damages and identified the more difficult issue as the search for an explanation for the maintenance of benefit in the intensively treated group. He then asked the panel to compare the

rate of the intensive group's progression versus newly diagnosed diabetics, since it appears that what has been seen is a manifestation that, after 5 years of control, homeostatic mechanisms have been re-established, repairs have been made, and individuals approach a non-diabetic state, followed by new onset non-severe diabetes at the time these patients move from DCCT to EDIC. Dr. Sassoon responded with the comment that a catch-up in the next 5 years will be elucidating.

Dr. Monnier mentioned an experiment that examined dietary restrictions in rats and longevity, data very important concerning the question of importance of early intervention, which showed that the total duration of dietary restriction was more important than dietary restriction of the first 6 months. However, the animals died following consumption of the same number of calories, indicating a connection between caloric intake and lifespan, which may be a useful paradigm for the complications of diabetes.

Dr. Nathan posed the session's final question, stating that hardly a tissue exists that is as plastic as the female reproductive tract and asked Dr. Sassoon his assessment of the similarity of the plasticity of microvascular tissue such as that of the kidney, as well as those of the nerves and the eyes. Dr. Sassoon responded with his belief that the plasticity might be underappreciated.

Session 4: Developing New Therapies for Vascular Complications

Animal Models of Diabetic Complications

Dr. Timothy S. Kern, Director, Center for Diabetes Research, Department of Medicine and Endocrinology, Case Western Reserve University

Dr. Kern presented evidence from several studies indicating that animal models are generally useful tools for the examination of diabetes-induced complications that develop in humans. Although most animals do reproduce a variety of the background changes of diabetic retinopathy, to date none have gone on to reproducibly develop the advanced characteristically clinically observed lesions. However, they do develop capillary non-perfusion, which is, in Dr. Kern's opinion, the single most important lesion that is causally related to the ultimate development of neovascularization.

Animal models have recently been used to study critical events in the pathogenesis of diabetic complications. They provide valuable insight into the role of specific biochemical pathways or specific cell types in the pathogenesis of the early stages of complications. Dr. Kern cited data from published studies in which investigators examined the role of hyperglycemia in nondiabetic animals using a galactose-feeding model in dogs, rats, and mice. The model showed that non-diabetic animals with normal insulin, protein metabolism, and lipid metabolism nevertheless developed a retinopathy that was morphologically indistinguishable from that which occurs in diabetes, providing what may be strong evidence that it is the sugar itself that at least initiates retinopathy in diabetes, as opposed to insulin-deficiency or alterations in lipids.

Dr. Kern pointed out some interesting differences with regard to the galactosemic model: (1) the lesions of retinopathy are inhibited by aminoguanidine in diabetic animals, but no beneficial effect of aminoguanidine has been observed in galactosemic animals; and (2) unlike the retina, the kidney of animals that are fed aminoguanidine exhibit very little renal pathology, in contrast to what is seen in diabetes. Dr. Kern cautioned that the galactosemic model, when used appropriately, yields good data, but cannot be considered to be a wholesale marker for diabetic complications.

Dr. Kern summarized data from an unpublished study in which animal models were used to identify specific chemical alterations that are important in the pathogenesis of retinopathy. Mice in whom ICAM or its ligand, CD–18, had been knocked out were made diabetic for 11 months or experimentally galactosemic up to 22 months. When leukostasis, vascular permeability, cell death, and development of lesions of retinopathy were assessed, results showed that ICAM–1 and CD–18 play critical roles in the death of retinal capillary cells and the development of acellular capillaries in diabetes. As expected, a diabetes-induced increase in the number of acellular capillaries occurred, which was essentially totally eliminated by knocking out a single molecule, either ICAM or CD–18. Dr. Kern stated that although the mechanism by which this is occurring is not entirely clear right now, it is important to note that in these animals the death of pericytes was largely eliminated by the modification of a single molecule, the knockout of ICAM or CD–18.

While the previously described studies are examples by which local blockage of retinal blood vessels could cause death of capillary cells and nonvascular cells downstream, Dr. Kern identified apoptotic death of vascular endothelial cells and pericytes as a second method for development of acellular (nonperfused) capillaries in the retina.. This apoptosis is strongly associated with the development of retinopathy, but more importantly, therapies that significantly inhibit the development of acellular capillaries (such as aminoguanidine, vitamin E, and nerve growth factor, among others) have all been shown to inhibit the apoptosis of the retinal capillaries. These two seemingly unrelated pathways, the leukostasis model and the apoptotic model, are the types of experiments that make good use of animal models to explore the pathogenesis and treatment of diabetic retinopathy.

Animal models also have identified a number of other beneficial therapies for the inhibition of retinopathy in diabetic animals, including aminoguanidine, aspirin, antioxidants (including α-tocopherol), nerve growth factor, *bcl*-2 overexpression, pyridoxamine, and benfotiamine.

Dr. Kern stated that animal models have also provided unexpected results, such as resistance of retinopathy to arrest after re-institution of normal glycemic control on a morphological level (evidenced by 5-year diabetic dog studies by Drs. Engerman and Kern) and on a biochemical level (such as lipid peroxides and caspase-3 activity in diabetic rats as in Dr. R. Kowluru's studies). These particular biochemical abnormalities may not be causally involved in the development of progression of retinopathy, but demonstrate that, contrary to expectation, retinal biochemistry is not rapidly normalized after elimination of hyperglycemia. This data provides strong evidence that there are biochemical operations that remain perturbed for very long periods even following intervention to improve glycemic control and likely contribute to the continued progression of retinopathy even after intervention with good glycemic control.

ACE inhibitors inhibit the development of diabetic retinopathy, but when researcherslooked at glucose accumulation in retinal cells in animal models in order to explain why this phenomenon occurs, another unanticipated result came to light in which the mechanism of beneficial effect of the ACE inhibitor captopril on retinopathy was demonstrated. Captopril was found to significantly inhibit glucose accumulation within retinal cells (*in vivo* and *in vitro*), with the implication that if glucose does not accumulate intracellularly in diabetes, the biochemical abnormalities postulated to cause diabetic complications will not develop.

Dr. Kern stated that recent studies have lead to the recognition of a variety of biochemical changes observed in the retina in diabetes that are consistent with retinopathy being a chronic inflammatory disease. Work is currently being conducted with animal models to identify therapies to treat diabetic retinopathy and kidney disease from an inflammatory standpoint.

Dr. Kern concluded by saying that animal models provide much information about the early stages in the pathogenesis of diabetic complications, the stages that precede and apparently contribute to the development of the later stages of the disease. More information will become available when animals are manipulated with reference to a specific biochemical abnormality or specific cell type involved in the development of diabetic retinopathy. Dr. Kern pointed out the Animal Models of Diabetic Complications Consortium (AMDCC) is attempting to develop and characterize new animal models and providing assistance to other investigators to help assess

their animal models. Future studies with animal models may investigate why diabetic retinopathy resists arrest after intervention with improved glycemic control and may identify genes that influence the rate at which complications of diabetes develop.

Endogenous Inhibitors of Angiogenesis

Dr. Raghu Kalluri, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School

Dr. Kalluri identified endogenous inhibitors of angiogenesis as a very promising area of research leading to tissue-specific targeted angiogenesis therapy. Studies conducted over the past 6 years using basement membrane components have prompted the discovery of several protein fragments suspected of anti-angiogenic activity, all of which were determined to be internal fragments of larger proteins, which, when in the larger protein context do not have that property. Following screening of 400 to 500 protein fragments, 7 were found to have potent anti-angiogenic activity, which for the purposes of Dr. Kalluri's research, is defined as having endothelial cell specificity.

Angiogenesis, the formation of new capillaries from pre-established blood vessels, is necessary in growing tissue for adequate blood flow and oxygenation during embryonic development, wound healing, menstruation, and tumor growth. Dr. Kalluri said that adverse long-term effects in diabetes include abnormal angiogenesis, which emerging evidence is indicating contributes to vasculopathy.

Dr. Kalluri cited a number of widely conducted studies that demonstrated that tumor growth accelerated in the absence of tumstatin, its receptor $\alpha V\beta 3$, and the enzyme that generates tumstatin. Proteolytic reactions release tumstatin, a protein fragment and an endogenous suppressor of pathological angiogenesis. Dr. Kalluri explained that tumstatin's anti-angiogenesic activity is dependent on the presence of $\alpha V\beta 3$ integrin. Endothelial cells from knockout mice showed that tumstatin-active receptors inhibit proliferation of wild-type endothelial cells, whereas in contrast, absence of integrin disables proliferation inhibition. $\alpha V\beta 3$ integrin expression was not demonstrated until tumors reach 500 mm^3, suggesting hypoxia or other mechanisms may be involved in integrin switching. Dr. Kalluri hypothesized that the binding of tumstatin and $\alpha V\beta 3$ integrin suppresses angiogenesis by reversing active signaling.

Dr. Kalluri identified the mechanism for the tumstatin molecule action within epithelial cells, stating that tumstatin acts to arrest Cap-dependent translation.

Evidence has recently been published to show that arresten, endostatin, and canstatin act similarly with $\alpha 1\beta 1$, $\alpha 5\beta 1$, and $\alpha 3\beta 1$ integrin receptors, respectively, which is pertinent to diabetes research in that they provide the delicate balance required to prevent acceleration of angiogenesis. Although both diabetes and cancer are systemic diseases, unlike cancer, diabetic complications are associated with both excessive and deficient angiogenesis, which argues in favor of local environmental control, not systemic control, as a key determinant of angiogenesis potential in diabetes mellitus. In diabetes, excessive angiogenesis leads to retinopathy and

possibly nephropathy, whereas insufficient angiogenesis results in poor wound healing and coronary collateral vessel development. This makes understanding the angiogenesis in the individual organ sites and specific tissues involved in diabetic complications necessary, as well as developing targeted angiogenesis and anti-angiogenesis therapies.

Discussion

Dr. Robertson questioned the use of rapamycin to support pancreatic islet transplantation, because of the risk it might inhibit host-induced angiogenesis to reach out to the new islets. The drug is a mainstay in protocols for islet transplantation. Dr. Kalluri agreed that the drug has an anti-angiogenic component to it, but it is not alone among chemotherapeutics in this regard. However, every drug that affects every cell will affect endothelial cells, and clinicians must weigh the benefit of any drug with its lack of benefit or adverse effects.

Dr. Sassoon asked about the detection of circulating levels of proteins in the blood, and whether it was wise to globally control what may very well be a local process. Dr. Kalluri differentiated between his specific area of research in basement membrane and matrix-derived inhibitors and the general field of angiogenesis and explained that the information can nonetheless be a valuable insight for diabetic complication research once the circulating levels are more fully understood.

Dr. Lorenzi requested that Dr. Kalluri present available evidence for neovascularization in the kidney. Dr. Kalluri responded with the speculation that the endothelial cells in the glomeruli are in flux and tend to have a greater proliferative index than those in a normal kidney. However, they are not acting in such a manner that, within the context of glomeruli, would indicate the presence of greater angiogenesis levels. The contribution and its effect of enhanced angiogenesis in this regard is not yet known.

Dr. Monnier asked why, if neovascularization is present in the retina, animal models do not reflect it. Dr. Nathan reiterated Dr. Kern's earlier comment that one of the deficiencies of retinopathy in animal models was that the final expression is non-existent. Dr. Kalluri suggested it might be due to a feature of genetic controls, since even different mice presented different neovascularization levels. Dr. Kern stated that, in comparison to cancer, the duration of diabetes is short and the VEGF induction is very minimal as opposed to observations made in tumors.

Dr. Brownlee requested Dr. Kern's opinion regarding the mechanism for the ACE-inhibitor inhibition of glucose transport in the retinal cells. Dr. Kern stated that, in a variety of preliminary experiments, a modest effect on glucose transport has been observed, but that no change in glucose transporter levels has been detected.

Dr. Feldman asked Dr. Kern whether changes in protein expression were observed that could then be ameliorated with therapy, similar to the changes noted in the eye-specific gene array that were subsequently reversed. Dr. Kern's proteomic work has primarily been nitration-based, and although extensive changes have been observed in terms of nitration of proteins that are inhibited by these therapies, his research has not looked at all proteins that are present.

New Investigative Tools in Retinopathy, Nephropathy, and Neuropathy

Dr. Nathan introduced the portion of the meeting dedicated to the investigation of new investigative tools in diabetic complications and the perceived need for new biomarkers, surrogates, and tools that can be used both to judge the efficacy and effectiveness of new interventions.

Early Assessment of Diabetic Retinopathy Treatment Response Using Functional Magnetic Resonance Imaging (MRI)

Dr. Bruce Berkowitz, Professor, Department of Anatomy and Cell Biology, and Ophthalmology, Wayne State University, School of Medicine

Dr. Berkowitz began by describing the retinal capillary damage caused by diabetes and clinical methods currently used to detect diabetic retinopathy. He pointed out that a number of physiological parameters are thought to change prior to observation of histopathological changes, including changes in vascular reactivity and breakdown of the blood-retinal barrier.

Current clinical detection of retinopathy is focused on end-stage histopathology, and these techniques, while useful and necessary, are not optimal for the development of treatment strategies or drug development processes. Dr. Berkowitz stated that determining a patient's response to therapy earlier than is currently possible will increase the likelihood of preventing long-term complications. Application of this research to-date has been limited to basic science studies and to clinical trials.

Magnetic resonance imaging (MRI) is a useful tool for this area of research because (1) it provides a full field of view rather than a limited field of view around the optic nerve, (2) it is possible to obtain two and three dimensional images of the eye, (3) the procedure is not inhibited by media opacities, and (4) the information available from the MRI experiment is translatable both from bench-to-bedside and from animal models to humans. Nonetheless, Dr. Berkowitz identified drawbacks to the procedure as well. The resolution is not clear enough for observation of individual vessels or identification of retinal from coroidal circulation, and it is not an in-office procedure.

MRI is a valuable resource for the study of vascular reactivity through observation of the oxygenation response to an inhalation challenge. During a 2-minute oxygen challenge, oxygen is delivered to the retina through the retinal circulation and diffuses from that point into the retina and the vitreous near the retina. On an appropriately weighted MRI, an increase in signal intensity in the pre-retinal vitreous space indicates the retinal oxygenation response. Dr. Berkowitz explained that oxygen changes in the pre-retinal vitreous space reflect oxygen supply during the challenge and because this is a well-defined contrast agent in a clean system with no flow, the signal intensity changes in the pre-retinal vitreous space are a linear function of oxygen levels. This technique works quite well as an accurate measurement of oxygen delivery to the retina in that a comparison between ΔPO_2 in an adult rat during oxygen breathing during MRI and with an oxygen electrode at different locations (i.e., pre-retinal or mid-vitreous) were in agreement.

Dr. Berkowitz outlined several preclinical studies using MRI as a measurement technique. Researchers have examined conditions with short- and long-term diabetic and glactose-fed rats and mice. In a summary of ΔPO_2 data, Dr. Berkowitz stated that the ability of the inferior portion of the retina to oxygenate between 2 to 4 months was no different in the diabetic mice from the control group; however, the superior segment of the retina showed a subnormal response in the diabetic animals compared to the controls, a pattern repeated in all preclinical models. By 15 to 18 months of galactose feeding, the superior hemiretina remained subnormal, but the inferior hemiretina had reached equally subnormal levels, from which Dr. Berkowitz concluded that the superior can be considered an early warning that a problem exists, even one which occurs prior to evidence of histopathology.

The subnormal superior hemiretinal change in oxygenation response appears to be a very early event associated with the histopathology of diabetes. Using data from studies conducted on aminoguanidine, Dr. Berkowitz suggested that MRI can also be used to assess treatment efficacy and that correction of the early subnormal retinal ΔPO_2 seems to be very promising as a predictor of therapeutic efficiency. Studies done with the iNOS and PKC knockouts in mice made diabetic for 4 months highlighted both as potential therapeutic targets, but further research is necessary in this area. This is a particularly appealing aspect, since any institution with MRI capabilities should be able to implement the procedure as part of a diabetes research program.

Dr. Berkowitz demonstrated that dynamic contrast-enhanced MRI studies are a powerful method for the study of damage to the blood-retinal barrier in the rat and in patients with diabetes. He identified a subnormal retinal ΔPO_2 as the more sensitive indicator because it appeared to precede evidence of passive transport through the damaged blood-retinal barrier. Studies using MRI also underscore the potential to rapidly evaluate treatment efficiency for diabetic macular edema both in the clinic and using an experimental VEGF model.

Dr. Berkowitz concluded that MRI provides early, powerful, and translational functional markers (ΔPO_2 and blood-retinal barrier damage) of pathophysiology associated with diabetic retinopathy, with the ΔPO_2 being more sensitive to early damage. These markers appear to be useful for the early monitoring of treatment and/or drug discovery and have high clinical potential.

Endogenous and Exogenous Glycation Control and Diabetic Nephropathy

Dr. Paul Beisswenger and Dr. Benjamin Szwergold, Department of Medicine, Division of Endocrinology, Dartmouth Medical School

The fundamental concept underpinning Drs. Beisswenger's and Szwergold's research is that humans have mechanisms to control the damage caused by unavoidable nonenzymatic glycation. These protective mechanisms are determined by genetically encoded enzymes that, in turn, determine the concentrations of reactive glycating agents. In diabetes, these protective mechanisms are important due to increased glycation stress caused by the hyperglycemia, and they may be further impaired by metabolic perturbations produced by the diabetic state itself.

Drs. Beisswenger and Szwergold have focused their research in this area on direct and indirect glucose toxicity, using the term not as increasing insulin resistance, but as being toxic to tissue. In particular, their efforts have centered on the identification of factors that control the reduction of toxic glycose by-products, identification of agents that can inactivate glucose by-products, and identification of processes preventing formation of precursors of toxic advanced glycation endproducts (AGEs). Dr. Beisswenger pointed out that the structure of many AGEs has been defined and stressed the importance of identifying the specific AGEs to gain information with regard to the precursors and pathways involved that lead to diabetic complications.

With a focus on methylglyoxal (MG) and 3-deoxyglucosone (3DG), Dr. Beisswenger explained how these carbonyls lead to specific AGEs. The major source of MG is dihydroxy acetone phosphate. GAPDH, the immediate downstream enzyme, is important by virtue of its sensitivity to changes by a number of factors, including oxidative stress, glycation, genetic factors, and decreased levels of NAD+ . Specific mechanisms exist that degrade MG, particularly glyoxalase, which is dependent on an adequate level of reduced glutathione.

Dr. Beisswenger stressed that GAPDH activity is relevant to more than MG production. Dihydroxyacetone phosphate also leads to DAG production, which in turn activates PKC; it may also lead to activation of the upstream hexosamine and polyol pathways, amplifying its importance beyond overproduction of MG.

Multiple studies have shown that diabetic kidney disease is largely determined by genetic and ethnic predisposition. Dr. Beisswenger hypothesized that increased susceptibility to diabetic kidney disease is closely related to the increase in MG generated on exposure to high glucose levels, while lower MG production may be protective. Three studies were summarized: a group with accelerated nephropathy; one involved with the natural history of nephropathy; and a third examining the degree of nephropathy in a Pima Indian study population. The largest "Natural History" study group involved 110 subjects with type 1 diabetes whose degree of kidney damage had been measured via kidney biopsies. In each person, MG production and GAPDH activity was measured by blood cell response to high glucose incubation. Subjects with rapid and slow progression of kidney damage were identified and studied. Data from these studies showed:

- MG levels are significantly elevated in subjects with diabetes who show more rapid progression of kidney damage.
- Red blood cells from rapid progressors produce more MG when exposed to high glucose levels.
- Increased MG levels are related to the degree of reduction in GAPDH activity.

Dr. Beisswenger stated that further studies will be conducted in the areas of GAPDH-specific activity, GAPDH mutations, and other aspects of GAPDH in the progressor and non-progressor populations.

Agents that can inactivate glucose by-products include aminoguanidine and metformin, and a direct correlation has been found between MG and triazepinone in type 2 diabetes. Clinical studies have been conducted comparing urinary MG and triazepinone during metformin therapy that show a direct correlation between MG and triazepinone. While not the only condensation

product between these two compounds, it is certainly, in Dr. Beisswenger's opinion, an important marker.

In identifying processes leading to removal of toxic glucose products, Dr. Beisswenger stated that to date no defects in glyoxalase activity have been found in diabetic patients, but that further studies of this question are needed. Therapeutic implications include the idea that the degree of glycemic control required to prevent complications may differ among individuals. Inhibitors of AGE formation, including aminoguanidine, pyrodoxiamine, and metformin, may be very useful for removing α-dicarbonyl toxicity. Antioxidants may reduce glycation stress and PKC activity partially through increased GAPDH activity. ARIs may reduce glycation as well as polyol pathway activity through increasing the levels of NAD+. Dr. Beisswenger emphasized the importance of these defense mechanisms and invited Dr. Szwergold to elaborate on their collaborative research.

Dr. Szwergold presented a summary of their research involving basic biochemistry and glucose as it pertains to defenses against AGEs and the early stages of glucose involvement as a potentially toxic agent in diabetic complications. The research is based on the hypothesis that cells have defense mechanisms to protect against this direct glucose toxicity, but in persons with diabetes, the mechanisms are often overwhelmed and result in damage to cells and organs.

Dr. Szwergold described having discovered an enzyme, fructosamine-3-kinase (FN3K), which has been cloned, sequenced, and characterized in terms of its two isoforms. He proposes that the net effect of having this enzyme is an enzymatic deglycation of fructoslysine adducts on proteins, which allows the tissues to rid themselves of this early glycation product.

He reported on results of his recent, preliminary experiments with chemical inhibitors of FN3K, which, when applied to fibroblasts in culture produced cytostatic inhibition of cell growth. Future studies with FN3K will include experiments to knock down FN3K expression in cultured cells by siRNA. If FN3K indeed functions as a deglycating enzyme, suppression of its expression will inhibit cell growth, DNA synthesis, and protein synthesis.

Discovery of FN3K and a pathway for enzymatic deglycation of nonenzymatically glycated proteins represents a potential new paradigm for the understanding of noenzymatic glycation and its relationship to diabetic complications.

In addition to FN3K-mediated deglycation, preliminary results also show evidence for the existence of an alternative deglycation pathway that catalyzes the decomposition of the very first early glycation product—glucoselysine. This second deglycation mechanism may be important because glucoselysine and fructoselysine are sources of free radicals. Therefore, lowering and controlling their levels should be advantageous in terms of reducing oxidative stress

In addition to FN3K related studies, other future directions for research of Drs. Beisswenger and Szwergold include:

- Genetic studies on the kidney and other cells in larger study populations to discover the factors responsible for the observed susceptibility to kidney damage, possibly due to an inherited abnormality in GAPDH or other enzymes.

- Development of markers to determine those at greatest risk for kidney disease.

- Investigation of other products formed by MG and potential binding drugs to use as tools in large study populations with type 2 diabetes in which coronary disease outcomes are being studied.

Neuropathy Assessment in the EDIC Cohort

Dr. Eva Feldman, Professor of Neuropathy, Department of Neuropathy, University of Michigan, Ann Arbor

Dr. Feldman spoke of new clinical tools available to quantitate autonomic clinical neuropathy and understand "metabolic memory" with regard to neuropathy.

Currently available tools, which were not available at the inception of the DCCT, include the following, the first two of which could be easily administered to a large cohort:

- Clinical symptom survey.
- Cardiovascular autonomic function testing using the ANSCORE system.
- Selected EDIC sites, measures of quantitative sudomotor axon reflex testing (QSART).
- Selected EDIC sites, measures of sympathetic cardiac innervation using positron emission scanning (PET).

The clinical symptom survey is a series of questions regarding neurological symptoms the patient may be experiencing, such as lightheadedness, dry mouth or dry eyes, temperature and sweating of the hands or feet, nausea or diarrhea, incontinence, and so forth.

The ANSCORE system quantitatively measures parasympathetic function using (1) the 30:15 ratio, where R-R interval is quantitated upon standing at beat 30 (maximum) versus beat 15 (minimum), (2) the Valsalva maneuver, and (3) the expiration/inspiration (E/I) ratio. According to Dr. Feldman, the ANSCORE is probably the most reliable and sensitive measure of parasympathetic function that can be done easily at the bedside. It is ideally suited for multi-center EDIC trials in addition to the already validated autonomic symptom scores, since it is portable and easily utilized.

Dr. Feldman identified two tests of sympathetic function that could be used at selected EDIC centers: The QSART and the PET scan. QSART is sensitive, reproducible, and relatively easy to administer; however, it is much more expensive to purchase than the ANSCORE system. Two other simple tests of sympathetic function are blood pressure, supine and standing, and the thermoregulatory sweat test.

Perhaps another approach would be to quantitate sympathetic denervation in the heart. Dr. Feldman presented a working hypothesis showing normal vasculature in the heart and normal cardiac innervation, speculating that, similar to peripheral diabetic neuropathy, one would see denervation in the heart with a distal at the apex to proximal loss of cardiac sympathetic innervation. In order to measure this, researchers have used PET scanning, which allows visualization of blood flow using an ammonia tracer (N–13), and permits observation of sympathetic nerve innervation using hydroxyephedrine (C–11 HED) as a tracer. The two tracers allow comparison of blood flow to nerve innervation to understand the function of the heart. Polar maps from patients are subtracted from normal polar maps, and 3-dimensional composites can be compiled from PET scans. Additionally, PET scans can detect progression and regression of cardiovascular autonomic neuropathy over 3 years in type 1 diabetes.

In reference to metabolic memory, Dr. Feldman spoke of evidence that has identified apoptosis in sensory neurons and ensheathing Schwann cells in animal and culture models of diabetes. Currently, researchers are investigating more of the cellular and molecular mechanisms underlying this occurrence. Metabolic memory may also be a factor of growth factor signaling, the extracellular matrix, and changes in focal adhesion complexes in diabetic animal and culture models versus nondiabetic, all of which are the focus of various ongoing studies.

Unpublished work looking at structural changes in nerves has been initiated based on the EDIC data. It is based on previously unconsidered and unmeasured measures of diabetic nerves that may be predisposed to metabolic memory. Dr. Feldman described results of studies using the atomic force microscope (AFM), together with new approaches of nanotechnology, employed in the examination of nerve structure and tensile strength, including the role of collagen. An AFM image of a collagen-ensheathed diabetic rat sciatic nerve axon showed that, at a scale of collagen bundles from 1 to 10 μm, glucose is relatively free to diffuse among the hundreds of available collagen fibrils looking for a binding site. The atomic force microscope can also be used to look at nerve mitochondrial structure in both diabetic and control tissues, a very important concept, since the procedure can easily be done on both human fibroblasts and experimental or control animals. This is, in Dr. Feldman's opinion, a very powerful tool to aid in understanding the experimental occurrences and the metabolic memory that might exist for mitochondrial structure.

Dr. Feldman also recommended the use of nanotechnology to introduce markers targeted toward tissues of interest. She identified this method as a means for encouraging input from individuals beyond the diabetes field. In addition to functional MRIs of peripheral nerves, Dr. Feldman suggested the use of AFM on skin biopsies and nanotechnology on skin fibroblasts and proposed coupling the mechanistic approach of proteomics of sera, urine, and cells using antibody arrays with a discovery strategy using HPLC and flow cytometry.

Discussion

Dr. Nathan asked Dr. Feldman about PET scanning applications with regard to examining sympathetic denervation, wondering whether a parallel manner for looking at parasympathetics is possible. Dr. Feldman stated that it is certainly clinically believed to be true. PET scans done on patients with even early neuropathy revealed 20 percent PET scan defects, indicating that this

is a reliable method for measuring even very early sympathetic denervation. As neuropathy progresses, so does sympathetic denervation, even prior to observation of symptoms. However, Dr. Feldman remarked that no better method for measuring parasympathetic dysfunction exists beyond the ANSCORE.

Dr. Vlassara agreed with the panel's hypotheses that AGEs are present within the peripheral nerves from diabetic animals and humans. However, they have previously been associated solely with the sheath that covers the neuron. She then asked whether the panel has looked at the myelin structure. Dr. Feldman said that primarily what she presented were collagen fibrils from the endoneurium and that researchers have imaged the node of Ranvier. She said the next steps were to image within the axon and the Schwann cells.

Dr. Lorenzi stated that an issue with animal models is the permeability of retinal vessels. She referred to Dr. Berkowitz's statement that with the contrast agent (gadolinium) used by the MRI method, no hints of increased permeability up to 6 months of diabetes was more than what is seen in humans, as opposed to what is often reported in models. Dr. Lorenzi asked how his contrast agent compared with the other markers used by other researchers to test permeability. Dr. Berkowitz cautioned that it is important to remember that this is a passive-restricted diffusion tracer, that to his knowledge, this is the first demonstration of a linear assay, and that no one should do a physiology assay without assessing the linearity of its assay. Without seeing that another investigator's assays were linear, accurate, and agreed with other researcher's results under standard conditions, it is difficult to interpret other data. He also remarked that his data was in very good agreement with radio-labeled sucrose data in diabetic animals, which also shows no leakage of the blood-retinal barrier up to approximately 6 months, demonstrating consistency with more sensitive models.

The feasibility of MRI on peripheral nerves as a substitute or supplement to motor nerves for measuring conduction velocity sounded like an attractive idea to one participant. Dr. Feldman responded that functional MRI with particular emphasis on bloodflow has been conducted, primarily at MIT and Stanford, but the data is relatively new and has focused on normal physiology rather than diseased humans. Dr. Nathan added that plans are in place to examine normal humans in the near future.

Dr. Tom Hohman, Associate Director of Commercial Development at Pharmacia Corporation, stated that data presented indicated an accumulation of AGE products as the initiation of the functional or structural lesion development and asked how this was reconciled with animal data, since most animal experiments in AGE formation blockage are prevention studies in which it is impossible to distinguish formation from accumulation. If accumulation leads to lesion development, unless that accumulation is reversed, it suggests that the lesions are untreatable. Dr. Feldman responded by saying that data show that over time the collagen in the nerve fiber gets bigger, thicker, and stiffer, and is not reversed following the institution of tight control. However, Dr. Feldman said that these are quite new results and require much more investigation. Dr. Beisswenger added that part of the problem is that AGEs have differing degrees of formation and reactivity, and it depends upon what is measured. In general, these products, once formed, are chemically irreversible, but not necessarily biologically irreversible.

Dr. Mark Cooper, Baker Heart Research Institute, Melbourne, Australia, commented that he and Dr. Schmidt have done studies where delayed interventions were administered, and some turnover of AGEs has been demonstrated. It seems as though even blocking formation following AGE formation will result in reduced AGE accumulation due to turnover.

Dr. Josephine Briggs, Director, Division of Kidney, Urologic, and Hematologic Diseases, NIDDK, remarked that a recent interest in the renal field has been the report that loss of nocturnal dipping in blood pressure may be one of the earliest predictions for the subsequent development of renal disease, and this is an additional reason for having more sensitive means of assessing renal function.

Dr. Schmidt added that nerve regeneration in diabetic animals has been shown to be impaired, and that the AFM would be useful for the examination of nerve crush, since it has been suggested that the abnormal collagen creates an inappropriate scaffold for regeneration.

Session 5: Treatment of Vascular Complications by Glycemic and Non-Glycemic Interventions

Lessons Learned About Cardiovascular and Mortality Outcomes After Pancreas Transplantation

Dr. R. Paul Robertson, Scientific Director, Pacific Northwest Research Institute

Dr. Robertson discussed the impact of pancreas transplantation on glycemia, insulin resistance, hypoglycemia, micro- and macrovascular disease, and quality of life. He opened with a slide depicting a simultaneous pancreas and kidney transplant, the most common procedure to normalize fasting and post-prandial glycemia and HbA1c levels in a type 1 diabetic patient. These patients are typically 34 ± 5 years of age, having had the type 1 diabetes for 22 ± 6 years and will have an 80 percent probable organ survival rate of 3 years of more. Forty-four patients followed for 6 years achieved a fasting plasma glucose level of 100 mg/dL or slightly lower after 1 year. Sixteen of these patients were followed for up to 20 years post-transplant, and most maintained levels between 65 to 100 mg/dL, indicating that when the transplantation works, it is an extraordinarily successful procedure. A good part of this success is due to the exuberance with which the transplanted pancreas produces insulin, which may lead to hyperinsulinemia, considered a risk factor for cardiovascular disease. However, this has not proven to be the case for these patients.

On the issue of pancreas transplantation impact on counterregulation of hypoglycemia, Dr. Robertson stated that hypoglycemic clamps have been used to demonstrate normalized glucagon secretion during hypoglycemia. After 5 years of diabetes, patients lose the ability to have glucagon secretion in response to hyperglycemia. Patients are generally hyperinsulinemic after pancreas transplantation because of systemic venous drainage of the allografts. However, they rarely experience significant hypoglycemia due to successful restoration of counterregulation and symptom recognition of hypoglycemia. Patients with a successful pancreas transplantation have a slightly higher glucagon level than normal, but this is a result of the implant rather than immunosuppressive drugs. Similar results occurred with a marked decrease in epinephrine secretion being typical of patients with type 1 diabetes mellitus during hyperglycemia compared to control. After pancreas transplant, epinephrine responses were improved, but not normalized.

Dr. Robertson cited several studies regarding the impact of pancreas transplantation on microvascular disease. In more than half of post-transplant patients (22 of 38), a steady decline in retinopathy occurs until approximately 3 months post-transplantation, at which point it appears that people with successful transplantation have stabilized, whereas those who failed at transplant continued to deteriorate. However, Dr. Robertson pointed out that too few subjects were available to conclude that a statistical difference in retinal disease exists following transplantation. Lesions of diabetic nephropathy were reduced following pancreas transplantation. Renal structure studies have documented normalization of structure, and nerve studies indicate stabilization of motor and nerve conduction after 10 years of normal glycemia.

Dr. Robertson described the effects of the impact of pancreas transplantation on macrovascular disease by showing study results of simultaneous kidney and pancreas transplantation versus kidney transplants alone, in which patient survival rates were increased when the dual transplantation was performed. Doppler and angiographic studies indicate there is less major artery narrowing in patients who have combined pancreas-kidney transplants versus those receiving kidney transplant alone. There does not appear to be an increase in lipid levels following transplantation.

Dr. Robertson also shared data showing that patients with autonomic insufficiency achieve decreased mortality rates following successful pancreas transplantation. At 5 years post-transplant, their 5-year mortality rate was lowered to 20 percent compared to 50 percent in such patients who are not transplanted. The majority of deaths apparently are related to cardiovascular events.

Dr. Robertson suggested further research be conducted in the areas of lipid and protein metabolism, diabetic limb salvage, gastroparesis, and diabetic diarrhea. However, quality of life studies strongly support pancreas transplantation, since it has a high success rate, and when successful, it results in normal glycemia with hyperinsulinism but no adverse outcomes regarding plasma lipids, improved hormonal counterregulation and symptom recognition of hypoglycemia, improved measures of macrovascular disease and improved kidney structure, stabilized nerve function, and a greater quality of life, despite the necessity for immunosuppressive drugs.

Discussion

Dr. Fred Whitehouse, Principal Investigator, EDIC, Henry Ford Health System, asked whether it was known if death among patients who have cardiac autonomic neuropathy experienced sudden death due to v-fibrillation (VF) or due to standstill. Dr. Robertson stated that when sudden death occurs in diabetes, it is assumed to be cardiovascular death, but that the percent that were VF and standstill were not known.

Dr. Feldman added that PET scans correlate with autopsies to show that hyperventilation is present at the base of the heart, leading one to assume that sympathetic hyperinervation causes tachyarrythmias. However, Dr. Nathan remarked that studying sudden death episodes is not clinically feasible. Dr. Whitehouse recommended research be conducted to try to identify whether VF is the reason, since it may be that standstill is more likely the cause of sudden death.

Dr. Brownlee asked for clarification of the studies of reversibility of renal lesions following transplantations. Dr. Robertson responded that it was a 10-year study of patients who had diabetes for 20 years and received a pancreas-only transplantation, in which data showed a great shrinking of the mesangium at 10 years but not 5 years, and a thinning of the basement membrane at 10 years but not 5 years.

Dr. Nathan gave an aside that the tool developed from the DCCT has been used by at least two other investigators to measure quality of life.

Dr. Szwergold asked to what extent the outcomes correlate with the histocompatibility matches. Dr. Robertson said that tissue matching is conducted as well as blood matching prior to transplant, and the better the match, the better the organ will do, in general. Dr. Szwergold also wondered whether any indication of contributing factors could be inferred from the mortality curves shown for individuals with autonomic deficiencies. Dr. Robertson responded that mortality figures are generally thought to be cardiovascular events.

Dr. Matthews asked what markers are currently being used for rejection. Dr. Robertson listed the following markers for rejected pancreas transplantation: (1) using creatinine as a surrogate marker, which is not necessarily a good indicator, since there may be differential rejection of the two different organs; and (2) urinary amylase, which can be used to assess the health of the transplanted pancreas, since the ecocrine drainage of the allograft is hooked up to the urinary bladder. If the pancreas is connected to the gut, that ability is lost. A third marker would be a patient self-administered oral glucose tolerance test with post-prandial glucose levels being reported to surgeons. This would allow treatment for rejection to be begun at earlier stages. Dr. Nathan agreed that urine amylases have not been useful in many people's hands.

Dr. Monnier speculated that it appears that no new imprinting occurs after 10 years of glycemia, in that the renal lesions are reversible. Dr. Robertson concurred that renal lesions return to normal, but it takes a long time. Whether or not that is a test of imprinting is uncertain.

Dr. Jerry Palmer, Professor of Medicine, University of Washington, Seattle, asked whether or not glucagon response has been tested with a protein meal. Dr. Robertson said it has been tested in response to intravenous arginine.

Dr. Beisswenger was interested in the fact that not every transplanted kidney experiences equal amounts of nephropathy, suggesting that there might be some selective factor depending on the genetics of the kidney. He wondered whether, when glucose was normalized, some people normalized their glomerulus at 5 or 10 years. Dr. Robertson responded that none normalized at 5 years, but like most series, the mean remained the same. He cited data from a study that showed people that developed nephropathy with diabetes have a family history of diabetic individuals who developed nephropathy, indicating a gene or genes might determine nephropathy.

Dr. David Robbins, Medical Advisor, Eli Lilly and Company, added that 10 percent of Pima Indians have micro-albuminuria prior to development of diabetes and micro-albuminuria is predictive of diabetes in the Pimas. It is also very highly correlated, even at that point, with subtle changes in the diastolic function by echocardiography. From that perspective, nephropathy is a capillary disorder that probably is widespread throughout the body, with a very high genetic susceptibility that may or may not be related to the dose-response issue of a patient's hyperglycemia.

Dr. Robertson offered an anecdote regarding a patient who is a recipient of a half-pancreas from her sibling. At presentation, she had 4+ proteinuria and creatinines of 2.0. She is now 20 years post-transplant, with the remarkable result of complete disappearance of proteinuria within 2 years of transplantation and a creatinine level of 1.0.

Clinical Trial Results With PKC, AGE, and ACE Inhibitors and Growth Factor Agonists/Antagonists

Dr. Nathan prefaced the afternoon session with the announcement that Dr. John H. Karam, friend, mentor, and teacher to many in attendance, passed away a short time previously and that a memorial service in California was taking place at the same time as the present meeting. In addition, a long-term important contributor to the DCCT/EDIC, Dr. Donnell (Don) Etzwiler, who was one of the original principal investigators, also passed away recently.

Dr. Genuth reminded participants that the DMICC would be holding a meeting immediately following this final session of the symposium and invited attendees to remain for the meeting.

Clinical Trials With a PKC Inhibitor

Dr. Lloyd Paul Aiello, Joslin Diabetes Center and Harvard Medical School, Boston

Dr. Aiello reported on the initial results of the Protein Kinase C β Inhibitor Diabetic Macular Edema Study (PKC-DMES). PKC-DMES was an international, multi-center, double-masked, placebo-controlled clinical trial evaluating 686 patients with either type 1 or type 2 diabetes, DME that was not imminently vision threatening, and mild to moderately severe nonproliferative diabetic retinopathy (NPDR). The overall objective of the study was to determine if inhibition of PKC β would delay or impact the progression of DME. Participants were randomly assigned to receive either a placebo or a 4 mg, 16 mg, or 32 mg daily oral dose of ruboxistaurin (RBX, LY333531) mesylate, a selective PKC β inhibitor. Since activation of PKC β by hyperglycemia is thought to be involved in mediating endothelial damage, angiogenic molecule release, and angiogenic factor signaling, it was postulated that inhibiting PKC β might ameliorate diabetes-induced retinal complications.

Ruboxistaurin is known to inhibit VEGF-induced and diabetes-induced retinal vascular permeability in rats and diabetes-induced retinal blood flow change in rats and patients. It also inhibits ischemia-induced retinal neovascularization in pigs. Results from a phase 1B study of patients with short-term diabetes and mild diabetic retinopathy showed that increasing doses of the compound produced progressive normalization in blood flow.

The primary objective of the PKC-DMES was to determine if ruboxistaurin would delay the development of diabetic macular edema. The outcome was measured by photographically determining if DME had progressed to within 100 microns of the center of the macula or if the patient received application of focal/grid coagulation. The trial design included a 6-week screening phase and a minimum 30-month treatment phase that all the subjects completed. Forty-five percent of the subjects completed 36 months or more of follow-up.

Subjects were 55 ± 10.6 years old, had diabetes for 16 ± 8.5 years, and had HbA1c levels ranging from 5.1 to 13.1 percent. The majority were Caucasian (77 percent), 40 percent were females, and 18 percent had type 1 diabetes. Sixty-five percent were on insulin treatment.

At 36 months, no statistically significant differences were observed between the placebo and three treatment groups in the primary endpoint of vision-threatening DME or photocoagulation. However, 18 percent of the primary study outcomes were the result of the application of focal/grid photocoagulation prior to photographically documented progression of DME to within 100 microns. When the outcomes based on photocoagulation alone were excluded, there was a 23 percent risk reduction in progression to vision-threatening DME in the ruboxistaurin groups.

Analysis of patients by HbA1c levels at baseline demonstrated >35 percent reduction in progression to vision-threatening DME when patients in the highest quartile of HbA1c (>10 percent) were excluded. Results were even more striking when those with the most normal quartile of HbA1c were also excluded.

Treatment with the ruboxistaurin was well tolerated, and there has been no clinically significant difference in adverse effects between the treatment groups, including evaluations of vitals, weight gain, cardiovascular events, hypoglycemic episodes, or development of cataracts.

Dr. Aiello's conclusions drawn from early results of the PKC-DMES were that treatment with ruboxistaurin did not have a statistically significant effect on the primary endpoint of DME progression or the application of focal photocoagulation. Subset analysis of photographically documented DME progression suggests a trend toward a positive effect of the compound. When patients with very poor glucose control were excluded from the analysis, the DME progression risk was reduced 30 to 35 percent at 36 months.

Clinical Trial Results With AGE Inhibitors

Dr. Helen Vlassara, Mount Sinai School of Medicine, New York

Dr. Vlassara listed two classes of potential therapeutic agents to prevent diabetic complications related to AGE: AGE formation inhibitors and cross-link breakers. Aminoguanidine (AG), a hydrazine compound is an AGE inhibitor and inhibitor of diabetic complications. When administered to 690 patients with type 1 diabetes and overt nephropathy, over 36 months, it decreased proteinuria significantly, based on ACTION, a randomized, double-blind, placebo-controlled, multi-center trial, the outcomes of which have not been formally disclosed. AG also decreased the rate of decline of GFR (glomerular filtration rate) by 23 percent, reduced total cholesterol and triglycerides, improved the LDL-HDL ratio and diastolic blood pressure, and significantly slowed progression of diabetic retinopathy, when compared to "placebo" treated patients (treated with ACE inhibitors). There were only minor adverse effects associated with AG.

The second class of AGE interventions includes phenyl-thiazolium bromide (PTB), ALT-711, and others and is shown to exert such effects as cause disaggregation of amyloid fibrils forming in Alzheimer's Disease and to restore large artery dysfunction in diabetic rats. ALT-711, a second-generation compound, has been the one best studied thus far. In addition to demonstrating significant efficacy against vascular stiffness and in improving left ventricular compliance in patients with heart failure, it has the added feature of being rapidly absorbed and,

after an initial cycle of activity, to be regenerated as an active compound, thus limiting the need for repeated or prolonged administration.

A new, potentially therapeutic approach recently developed by Dr. Vlassara's group takes advantage of the soluble AGE-binding peptide lysozyme, a native component of the human immune defense. Lysozyme is a well-known antibacterial protein that contains an 18 amino acid long peptide exhibiting high affinity binding for AGE. Lysozyme enhances serum AGE clearance, blocks AGE-induced cellular activation, and significantly suppresses AGE-promoted TNF-alpha secretion by macrophages. Details of the mechanisms by which AGE compounds, such as methylglyoxal and CML (carboxymethyl-lysine) lead to enhanced oxidant stress are not yet known; however, Dr. Vlassara stated that evidence is available to show that lysozyme blocks the ability of AGE compounds to deplete intracellular antioxidant systems, such as glutathione in endothelial cells.

Lysozyme administration significantly accelerates *in vivo* AGE clearance from plasma. Short-term *in vivo* administration of this peptide in diabetic animals with early albuminuria proved to effectively decrease albuminuria within 2 weeks. While this was admittedly a small effect, it was accompanied by suppression of several early kidney-toxic processes, such as the induction of growth factor and matrix component genes. Taking this into account, Dr. Vlassara's research team is investigating a recombinant gene transfer approach as a working possibility. Using both full-length and specific portions of human lysozyme constructs, lysozyme was transferred by a viral vector into TIB-186 macrophage-like cells. A marked increase in AGE uptake by macrophages containing the active lysozyme constructs was associated with complete inhibition of cell activation (i.e., suppressed TNF-alpha and IGF-1 generation).

Dr. Vlassara concluded that the findings might indicate future development of a therapeutic approach for diabetic complications, since lysozyme not only is a native, immune-protective substance, it can also serve in maintaining AGE homeostasis by enhancing cellular removal and renal clearance of AGE, by blocking AGE-mediated inflammatory events, improving tissue repair and wound healing, and by enhancing anti-bacterial reserves. A combination of AGE-inhibitors and AGE-crosslink breakers, together with hypoglycemic agents, may constitute an optimal therapeutic strategy to counter glycoxidation-related tissue injury in persons with diabetes.

Clinical Trial Results With ACE Inhibitors

Dr. Mark E. Cooper, Professor of Medicine and Head, Vascular Division, Baker Heart Research Institute, Melbourne, Victoria, Australia

Dr. Cooper stated that angiotensin-converting enzyme (ACE) inhibitors ought to be considered first-line treatment for diabetic complications and predicted that all future studies will have to be performed in the context of some effect of ACE inhibition, making some of the power calculations and detection of the benefit of new treatments more difficult.

The Danielle Alberti Memorial Centre for Diabetes Complications, as part of the Baker Heart Research Institute, has been studying the effect of the renin-angiotensin system (RAS), a major hemodynamic pathway, on diabetic complications, particularly diabetic nephropathy. Dr. Cooper described a range of animal studies that indicated that blockade of RAS by the use of ACE inhibitors reduces albuminuria and is useful in preventing progression of nephropathy. Of particular importance, these studies were conducted in animals with normal blood pressure. Agents used included the ACE inhibitor ramipril and the angiotensin II (AII) receptor antagonist valsartan. Nearly all long-term beneficial effects on both albuminuria and structure occurred due to the blockade of angiotensin II action.

Dr. Cooper suggested that AII action accelerates diabetic nephropathy, not only because it influences glomerular capillary pressure, but because of its non-hemodynamic effects as well. AII has been shown to directly affect extracellular matrix accumulation, monocyte and macrophage recruitment, and mesangial cell contraction. Clearly, angiotensin has meaningful actions relevant to the progression of renal disease in the presence or absence of diabetes.

Dr. Cooper emphasized that the unusual structure of the glomerulus should not be underestimated. In his opinion, one of the most promising targets will be the development of agents that reduce glomerular vasomotor tone, including vasopressin and endothelin antagonists.

Meta-analysis of studies done with type 1 diabetic patients experiencing microalbuminuria and normal blood pressure showed ACE inhibition reduces albuminuria and documented beneficial effects on glomerular filtration rate (GFR). One of the most interesting findings to come out of a study done comparing ACE inhibitors to calcium channel blockers was that ACE inhibitors were particularly effective in normotensive people. However, Dr. Cooper identified a major drawback of these studies is that the main endpoint is GFR, and for type 1 diabetic patients with microalbuminuria followed for 5 years, no significance difference on GFR was apparent. Still, these studies did provide data clearly indicating a degree of reversal, with a major effect occurring in the first 6 to 12 months of treatment, followed by continuing reduction in urinary albumin excretion with prolonged ACE inhibitor therapy, at least for some patients.

Meta-analysis also revealed a much greater response in terms of reduction in albuminuria in those people at upper levels of the microalbuminuric range. Dr. Cooper suggested this might be because it is easier to detect change at those levels. Furthermore, monitoring needs to be improved in order to detect more sensitive treatment effects.

AII receptor antagonists have provided the opportunity to study nephropathy in type 2 diabetes and have demonstrated a very significant effect on end-stage renal disease. The IDNT (Irbesartan Diabetic Nephropathy Trial) emphasized the extra benefit of blocking the renal angiotensin system. Despite almost identical effect on blood pressure between the AII antagonist and calcium channel blocker groups, there was a significant difference between the two treatments on the doubling of serum creatinine, as well as end-stage renal disease.

Very clear effects of ACE inhibition and AII antagonism have been demonstrated, including effects on growth factors, matrix proteins, and slit pore proteins within the podocyte. One of the potential reasons that the drugs work but are not particularly effective is that the renal

angiotensin system is not adequately blocked. Dr. Cooper proposed that a combination of AII antagonists and ACE inhibitor drugs would be more beneficial, since diabetic patients seem to be particularly sensitive to a combination treatment.

ACE inhibitors may also be beneficial in diabetic retinopathy. It is interesting to note that both angiotensin receptor subtypes are also expressed in the retina, and a large body of data exists showing direct effects of angiotensin on a range of cells derived from the retina. Dr. Cooper described a study involving rats with overexpression of the renin gene that leads to major increase in components of the RAS in the retina, causing increased renin and, in particular, increased local production of AII.

When diabetes was induced in these rats, neovascularization occurred, as well as accelerated renal disease. When these rats were treated for 9 months with an ACE inhibitor this was associated with prevention of angiogenesis. . This was associated with a reduction in VEGF and VEGF receptor 2 gene expression in the retina.

A surprising result to come out of the EUCLID (EURODIAB Controlled Trial of Lisinopril in Insulin Dependent Diabetes) study was the effect of ACE inhibitors on retinal disease, another indication that this therapy might be beneficial in the treatment and prevention of diabetic retinopathy.

Currently, the DIRECT (Diabetic Retinopathy Candesartan Trial) study involving the angiotensin II receptor antagonist candesartan will address in more detail the role of blocking the RAS for proliferative retinopathy. The study includes patients with both type 1 and type 2 diabetes and those with and without evidence of retinopathy.

An issue of conjecture has been how relevant an ACE inhibitor's effect on accelerated atherosclerosis is to diabetes, but positive findings of the HOPE study have been encouraging. Dr. Cooper cited data showing a significant reduction in atherosclerosis and prevention of increased complexity of lesions, following ACE inhibitor therapy. Diabetes was associated with upregulation of various components of the RAS, and connective tissue growth factor was markedly attenuated by blocking the RAS.

Dr. Cooper concluded with the statement that knowledge from studies showing the benefit of RAS blockers will be useful in studying new compounds and approaches to the prevention of diabetic complications.

Clinical Trial Results With Growth Factor Agonists/Antagonists

Dr. Anthony P. Adamis, Cofounder and Senior Vice President for Research, Eyetech Research Center, Eyetech Pharmaceuticals, Woburn, Massachusetts

Dr. Adamis discussed growth factors, specifically VEGF and its role in diabetic retinopathy. Based on experimental evidence, the Factor X hypothesis from 1948 states that a hypothetical angiogenic factor exists that is made in the ischemic retina and is secreted, diffusible, and

responsible for the growth of abnormal vessels in the retina, optic nerve, and the iris. VEGF is one of the factors that has received an intensive amount of research in the last 10 years because its gene expression is regulated by oxygen.

Dr. Adamis described an experiment where VEGF was cultured in normal and low oxygen. Results from that study demonstrated that VEGF is the primary endothelial cell mitogen made by hypoxic retinal cells. In a laser-induced retinal hypoxial model *in vivo*, the normal oxidative state with very little VEGF production progresses to an ischemic hypoxic state with a dramatic upregulation of VEGF in the inner retina, which is where most of the pathology occurs. In addition, these animals developed very profound iris neovascularization, which leaks in the late stages of the angiogram. Following grading of neovascularization, a temporal and spatial correlation is evident between the onset of neovascularization and VEGF levels in the eye. When the neovascularization levels plateau, so do VEGF levels, and just prior to spontaneous regression of vessels, VEGF levels drop precipitously. While these results do not prove causation, they do indicate a very compelling correlation.

In humans, the same correlation exists in patients with actual proliferative diabetic retinopathy. Vitreous levels for VEGF were significantly higher than those in patients who did not have vessels growing. Dr. Adamis cited a study conducted by Dr. Lloyd Aiello that examined similar patients with diabetic retinopathy compared to patients with vessels growing on the iris and those with vessels growing secondary to severe vein occlusion, a situation very similar to the monkey model. In all three states, VEGF levels were markedly elevated.

When recombinant VEGF is injected into a normal eye, the pathology is recapitulated. Even a single injection will yield profound neovascularization and leakage from vessels. Histopathologically, this is almost a pure endothelial proliferation with widely dilated, pre-existing vessels in the iris and newer, smaller caliber vessels in the anterior third of the iris. In fact, with repeat injections, neovascular glaucoma was recapitulated, a pathology that heretofore only existed in disease. Dr. Adamis stated that this was the first time a single identified species of molecule put into the eye would result in recapitulation of the pathology.

Recombinant VEGF injected into the monkey vitreous induces a diabetic-like retinopathy, including dilated, tortuous vessels, intraretinal hemorrhages, and abnormal vasculature. Not only does VEGF induce neovascularization, it also changes, in a very profound way, the phenotype of the pre-existing vessels in the retina. VEGF inhibition results in inhibition of iris and retinal neovascularization.

From these studies, Dr. Adamis concluded that VEGF acts similarly to Factor X and justifies clinical trials of VEGF inhibition in proliferative diabetic nephropathy, studies that have yet to begin.

Before VEGF was discovered to be an angiogenic factor, it was isolated for its ability to make vessels leak. VEGF is 50,000 times more potent at causing vessel leakage than histamine at a molar basis. VEGF has been shown to be a permeability agent in the retina, but the method of its action in this area is just beginning to be understood. Recent studies suggest that VEGF is pro-inflammatory and is a fenestrae inducer. In addition, VEGF alters tight junctions, and still other

studies suggest that VEGF acts as an active transport mechanism in a transcellular fashion through vesicular vacuolar bodies. Leakage is complex and needs to be better understood.

Dr. Adamis said that retinal VEGF levels are elevated in early diabetes in both animals and humans, and VEGF is required for diabetic blood-retinal barrier breakdown.

A more recent, ongoing study is being conducted examining VEGF binding that mimics antibody action. Interesting data regarding the role of growth hormone as a pathway in diabetic retinopathy is also available. Subsequently, vitreous IGF (insulin-like growth factor) levels stimulated by growth hormone correlate with the severity of diabetic retinopathy, and studies conducted long ago established that pituitary ablation improved retinopathy in patients. Further indirect, but very suggestive circumstantial data, has shown that severe retinopathy is rare in patients who are deficient in growth hormone and IGF, and recombinant IGF–1 worsened diabetic retinopathy.

Dr. Adamis stated that preclinical data is also intriguing. Intravitreal IGF–1 injections have been shown to induce retinal neovascularization in the pig model, and IGF inhibition in the retinopathy prematurity model was able to suppress retinal neovascularization. With that rationale in hand, somatostatin, an endogenous peptide first reported in 1973 after isolation from mammalian hypothalamus, is a molecule that has been studied as a treatment for retinopathy. Of primary importance is the idea that somatostatin is an antagonist of growth hormone activity. Somatostatin analogs have been synthesized and marketed for established uses. Preliminary data indicate that these analogs may be beneficial therapy for non-proliferative and early non-high-risk proliferative retinopathy.

Given the two pathways, Dr. Adamis addressed the issue of interaction between IGF and VEGF. Citing unpublished data, he pointed out that *in vivo* studies show that IGF–1 increases retinal VEGF levels, and when IGF is blocked, VEGF induces blood-retinal barrier breakdown, possibly indicating a mechanistic link between these pathways that were previously thought to be disparate.

Discussion

Dr. Feldman asked the panel whether or not aptimers for IGF–1 were being developed, to which Dr. Adamis responded that, to his knowledge none had been as yet, but that it could be a potentially exciting target.

Dr. Kalluri asked Dr. Adamis for his opinion with regard to the administration of antagonists as chronic therapy versus imprinting of the tissue for non-induction of neovascularization.
Dr. Adamis hypothesized that administration would have to be long-term, acknowledging that diabetic retinopathy pathology does become quiescent; however, at that point, much of the neural retinal and the source of VEGF production has been lost. Potential damage could be prevented by intervention in this area, resulting in the need for chronic treatment, but any pivotal trial designed will be one in which patients are treated for a specified period of time and then re-randomized into treatment and control groups to evaluate the need for continuous treatment.

Dr. Monnier posed a question to both Drs. Aiello and Adamis regarding attempts in the PKC studies to determine its activity in the white blood cells and wondered whether a surrogate marker for VEGF production in any peripheral blood cell is known to exist. Dr. Aiello spoke to the issue of PKC activity, stating that work has been conducted in the peripheral cells, particularly in the monocyte. Currently, Dr. King is working with an assay in the monocyte that allows examination of PKC activity that correlates quite well with levels of retinopathy. Trials are being set up that will study patients on the PKC inhibitor. Dr. Aiello identified a problem with the assay in that it requires fresh monocytes, necessitating the presence of the patient at the time of the assay and rendering multi-center trials difficult.

Dr. Lorenzi stated that Dr. Adamis has invested in the definition of VEGF's possible role in retinopathy and shared concern regarding several considerations, including VEGF as a molecule that is expressed constitutively in the human retina but has not been found in that location at increased levels. Inasmuch as Dr. Aiello's group has implicated PKC as a stimulus for VEGF production, Dr. Lorenzi questioned the safety of VEGF as a target, since it may not actually increase in diabetic retinopathy and treatments that would supposedly target it may not be very effective. Dr. Adamis acknowledged that VEGF blockade is not without risk, but stated that the molecule used by his group does not block all VEGF; if complete VEGF-inhibition ultimately has a deleterious effect, clinical trials will demonstrate that.

Dr. Aiello added that PKC inhibitors ought not to be confused with VEGF inhibitors. PKC is a component in the downstream pathway of VEGF action, but its primary effect is to reverse the early biochemical changes induced by hyperlipidemia, suggesting beneficial effects in the early stages of retinopathy unlike pure VEGF inhibitors.

Dr. Whitehouse asked Dr. Vlassara to comment on the adverse events that occurred in the aminoguanidine study (ACTION I), to which she responded that very superficial flu-like symptoms and minor anemia occurred in 10 to 20 percent of patients. The more significant side effect was the development of antinuclear antibodies in a small percentage of the patients, only three of whom were reported to develop crescentic glomerulonephritis.

Dr. Aiello addressed Dr. Spiegel's question regarding the collection of renal parameters, stating that this is clearly an area where PKC has been implicated and will continue to be a focus of ongoing research. Inclusion of extensive renal parameters in the ruboxistaurin (RBX) trial would have been unwieldy and were not included, which explains the reason for limited data on renal disease in the trial. Dr. Spiegel remarked that the 4 mg and 16 mg doses used in the RBX trial did not seem to indicate a dose-response. Dr. Aiello said that 1-year study data did show a likely dose-response. However, as the trial continued, the 4 mg and16 mg doses did fluctuate somewhat. With the numbers being relatively small, Dr. Aiello stated that differences between 4 mg, 16 mg, and placebo doses could not be distinguished but that an indication exists that 32 mg doses demonstrate more response than the first three groups.

Dr. Maria Lopez-Virella, Professor, Department of Endocrinology, Diabetes, and Medical Genetics, Medical University of South Carolina, commented that people in her group examined levels of connective tissue growth factor, both in urine and plasma of type 1 diabetic patients,

and found the levels are markedly increased, correlate with albumin excretion rates, and are also involved in neuropathy, in some sense.

Dr. Monnier asked whether a VEGF surrogate marker in peripheral white blood cells has been shown to exist. Dr. Adamis responded that, to date, none that is reliable and could be used to track retinopathy has been discovered.

Dr. Szwergold wondered whether Dr. Adamis had looked at the effect of glycation on VEGF, IGF, and its potency. Dr. Adamis's group has looked at VEGF gene regulation and has identified a positive effect that will turn on the VEGF gene through free radical signaling. However, protein glycation has not been investigated.

Dr. Brownlee expressed his appreciation for the tentative results shown and hoped that at higher doses significance would be heightened. He asked whether it was more likely that blocking of PKC in the VEGF pathway occurred, or whether investigators believed VEGF production was reduced in response to hyperglycemia via PKC activation. Dr. Aiello stated that PKC has been implicated both on the hypoxia-inducing aspect of VEGF and the signaling following VEGF stimulation of receptors. The data is clear that PKC is involved downstream from VEGF binding, less so with regard to stimulation of VEGF expression. Dr. Aiello said that the PKC effect on basic endothelial cell dysfunction suggests examination of PKC inhibitors at very early stages of retinopathy, but that this would require patient numbers and trial duration similar to that of the DCCT.

Dr. Tom Hohman, Associate Director, Pharmacia Corporation, Bedminister, New Jersey, asked Dr. Aiello about data from a second study that examined the progression of severe non-proliferative to proliferative retinopathy. Dr. Aiello's group is beginning to look at this data as well, which similarly has not shown, with respect to a composite primary endpoint, the same effect. However, in that group there is a very interesting possible effect on visual loss that should prompt further research in this area.

Dr. Feldman asked Dr. Cooper whether the EUCLID study looked at neuropathy and for his comments on aspects of the renin-angiotensin system with regard to neuropathy. Dr. Cooper stated that data does suggest that ACE inhibitors might be beneficial on neuropathy and that ACE inhibitors are also very good inhibitors of AGE formation. Dr. Vlassara invited Dr. Cooper to comment on AGE breakers. Dr. Cooper responded that, although ACE inhibitors have not been determined to be AGE breakers, they might very well be, and that ALT–711 has at least two or three beneficial effects on experimental diabetic complications (reduction in cardiac diastolic dysfunction and cardiac collagen insolubility). Unpublished data on the kidney indicates ALT–711 is beneficial on matrix TGF β (transforming growth factor β) and albuminuria, which further suggests it as an alternate approach for prevention of renal AGE accumulation.

Dr. Cooper stated that primary endpoints are not reached in every study, and asked Dr. Aiello what sort of observed effect could be useful in reducing complications such as retinopathy. Dr. Aiello answered that, with regard to proliferative diseases, small treatment effects are not particularly clinically relevant. However, prevention of progression is a consideration from a very early stage, since doing so would have a significant impact. Admittedly, these sorts of

studies require more patients and a longer period of time, and these are added constraints to those that are already placed on pharmaceutical sponsors. Dr. Genuth added that the ability of the patient to maintain a lower HbA1c might also be a factor, since the patient who is able to keep HbA1c between 6 and 7 percent will face a lowered risk of retinopathy for whom additional drugs might not be necessary, as opposed to those patients who are unable to reach good HbA1c levels, who might be much better candidates for improved drugs.

Dr. Brownlee reminded the group that most diabetics die from coronary disease, and people with insulin resistance and impaired glucose tolerance suffer from the same degree of increase in mortality events as people with frank diabetes. With regard to the major killer of diabetic individuals, Dr. Brownlee urged that research continue to reach beyond glycemia. Dr. Genuth said that data from the follow-up DCCT show that macrovascular complications may be influenced by lowering HbA1c, but that event data must be analyzed before conclusions can be drawn.

Dr. Beisswenger requested clarification of AGE breaker data, since in the tail tendon data, AGEs were completely normalized, it would seem that virtually all AGEs that were formed have reactive dicarbonyl sites to react with the breaker. He asked Dr. Vlassara what percentage of accessible AGEs have a reactive dicarbonyl site observed in established AGEs, which would theoretically be necessary for the breaker to work. Dr. Vlassara said she believes that the dicarbonyl site is very common in many AGEs. Dr. Monnier stated that evidence of the presence of the structure has not been conclusively demonstrated, but potential sites have been identified.

Dr. Lorenzi suggested that, with regard to targeting drugs that can be useful for multiple complications, ACE inhibitors are very promising drugs. Her interest in retinopathy does not lead her to conclude that a major, solid effort is in place to test compounds that affect that particular complication. Dr. Cooper stated that ACE inhibitors have been available for a long time, are expensive to study, and are off-patent, but that the AII antagonist DIRECT study is addressing this issue. He did express concern that the retina has a lot of AT2 receptor subtype, as well as AT1 receptor subtype, which may prove to be an area where ACE inhibitors are more powerful than AT1 receptor blockers, although animal data seems to indicate that AT1 receptors will work in the retina. Dr. Aiello commented that retinopathy study requires exposure and duration over an extended period of time to provide reliable data.

Dr. Cooper described the DIRECT trial, which has three arms: two type 1 diabetic arms (prevention and people with early diabetes) and a type 2 arm, as well. The study has not yet completed recruitment, but it will run for 3 years, and it is placebo-controlled. Dr. Lorenzi cautioned that most clinical studies for prevention have failed to show anything within 3 years and suggested that it may be an insufficient period of time to provide answers.

Dr. Feldman asked Dr. Aiello to speculate on the PKC clinical studies on neuropathy. Dr. Aiello responded that data has shown possible benefit in phase 2 trials, particularly in those patients who still retained a reasonable amount of nerve function. These findings have prompted movement forward into phase 3 trials, which can be completed in a much shorter period of time than the ocular studies.

In conclusion, Dr. Genuth thanked the presenters for an outstanding program and the participants for their very well-directed questions and turned the meeting over to Dr. Fradkin.

Dr. Fradkin echoed Dr. Genuth's thanks for the stimulating and interactive 2-day symposium and, in particular, extended gratitude to Drs. Genuth and Nathan for their efforts in organizing the meeting, which was a tribute to the DCCT/EDIC investigators and participants. Dr. Fradkin remarked that, 20 years ago, the DCCT/EDIC researchers took on a major question and answered it definitively. However, as is the case with all good research, doing so raised new questions. This meeting has addressed some of those questions and will aid in the development of new therapies that will go beyond what the DCCT and EDIC accomplished.

Dr. Fradkin extended an invitation to all participants in the symposium to remain for the Diabetes Mellitus Interagency Coordinating Committee (DMICC) meeting immediately following the symposium. DMICC seeks to foster diabetes research and improved care for people with diabetes. She explained that the DMICC meeting would focus on a synthesis of the 20th Anniversary Symposium and attempt to distill recommendations from the symposium into initiatives to advance research in the area of diabetes.

Session 6: Diabetes Mellitus Interagency Coordinating Committee (DMICC) Meeting

Dr. Judith E. Fradkin, Director, Division of Diabetes, Endocrinology, and Metabolic Diseases, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Opening Remarks

Dr. Fradkin opened the session by thanking the speakers and attendees for their participation in the 20th anniversary symposium of the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) Study to which the DMICC meeting was appended. Dr. Fradkin then reiterated the conference goals of the DCCT/EDIC symposium, held April 10–11 and entitled "Metabolic Imprinting and the Long-Term Complications of Diabetes Mellitus: Bench to Bedside and Back":

- To celebrate and commemorate the accomplishments of the DCCT/EDIC on its 20th anniversary;
- To explore the possible mechanistic basis for what has been tentatively termed "metabolic memory" or "imprinting"; and
- To generate plans for the fostering of research in developing new therapies for the complications of type 1 diabetes.

Dr. Fradkin explained to the guests present that the DMICC is a forum for the coordination of diabetes research and healthcare aspects across multiple institutes and centers at the National Institutes of Health (NIH), other agencies within the U.S. Department of Health and Human Services (DHHS), and beyond.

Special funding for type 1 diabetes research began in 1998, with a $30 million annual budget, and has increased to five times that amount, $150 million per year, for FY 2004-2008, providing a total funding of $1.14 billion over the course of its legislative history (Balanced Budget Act of 1997, P.L. 105–33, amended by FY 2001 Consolidated Appropriations Act, P.L. 106–554). Since its inception, this funding has been the source of a number of initiatives, including the establishment of genetic consortia through the collection of patients of both type 1 and type 2 diabetes, as well as genetic collections as part of the EDIC study group. DCCT/EDIC has provided a very well-characterized group of patients in terms of metabolic control for examination of the potential genetic factors that might influence the risk of complications. Additionally, the Animal Models of Diabetic Complications Consortium (AMDCC) and the clinical research consortia, supported by type 1 funds, have been initiatives for the development of surrogate markers for diabetes complications and pilot studies for the development of new therapies.

Of particular emphasis has been the funding of studies fostering bench-to-bedside research. Dr. Fradkin stressed that development of partnerships between individuals working in type 1 diabetes with experts from outside the field, such as some of those who were very much a part of

the current DCCT/EDIC conference, is an area that will aid in the exploration and examination of new directions for diabetes research.

In May 2002, an Advisory Panel recommended expanding the areas of opportunity for type 1 diabetes research to include research in inflammation and vascular disease complications, development of improved animal models, expanded clinical research, and the application of new technologies. They further recognized that available resources and infrastructure can be enhanced by the development of consortia to examine multiple complications and the fostering of partnerships between researchers in academia, Government, and industry. Preclinical development of therapeutic applications and a central knowledge base of complications-related initiatives were also recommended. Dr. Fradkin said a Web site will be developed to identify opportunities using type 1 funds in response to these recommendations and to announce the availability of resources resulting from such initiatives.

To capitalize on what was presented during the DCCT/EDIC conference and to focus future fundamental research on potential opportunities and initiatives recommended by conference participants, Dr. Fradkin outlined several key questions concerning the pathogenesis, prevention, and therapy of complications and invited speakers to respond with specific recommendations. The following sections summarize their presentations and the attendees' comments.

What Are the Major Gaps in Our Knowledge of the Pathogenesis and Therapy of Vascular Complications?

Dr. David M. Nathan, Professor of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston

Dr. Nathan summarized the following lessons learned from DCCT/EDIC that were presented during the symposium and outlined opportunities for future research:

- Glycemia is clearly the predominant mediator of the effects of intensive versus conventional therapy, explaining more than 95 percent of the effect of intensive therapy.

- Despite the subsequent narrowing of glycemia levels, the differences in outcomes between the original intensive and conventional therapy groups persist.

- The persistent difference in diabetic complications, potentially mediated by long-term beneficial effects of lower glycemia and/or persistent adverse effects of hyperglycemia, appears to be maintained for as long as 8 years after the separation in glycemia has dissipated, a phenomenon currently termed "imprinting" or "metabolic memory."

- One of the major and most interesting observations from DCCT was the demonstration that it is the original separation in glycemia level that accounts for most of the original effect.

- Glycemic levels and the changes mediated by intensive therapy may play a role in the development of macrovascular disease, as well as microvascular disease.

- Recent data with regard to calcification in the heart appear to demonstrate a difference between intensive and conventional groups.

During the DCCT/EDIC conference, several pathophysiologic mechanisms were presented to explain the effects of glycemic control and other currently used interventions on diabetic micro- and macrovascular complications, including glycation, inflammation, glycoxidation, apoptosis, lipoxidation, cellular issues, oxidation, and genetics/epigenetics. Investigators from diverse backgrounds explored several of these potential mechanisms that might explain the imprinting effects or metabolic memory from the early intensive glycemic control on long-term complications, including glycation/receptors for advanced glycation endproducts (RAGE), genetics/epigenetics, cellular/vascular/angiogenesis issues, and immunologic factors. Topics addressed during the conference included:

- Imprinting in DCCT/EDIC.
- Pathophysiology of diabetic complications.
- Potential mechanisms for long-term effects.
- Animal models and data regarding micro- and macrovascular disease.
- New methods of detecting and tracking complications that may be useful in clinical trials.
- Results of clinical trials directed at a number of factors that may be operant in diabetic complications.

Dr. Nathan also mentioned several topics that were not discussed, but which might have been considered within the scope of the conference, such as the limitations in achieving long-term control of hyperglycemia with currently available therapy; the ways of improving glucose control in type 1 diabetes, either by biological or mechanical approaches to maintain normal glycemia; and the prevention or cure of type 1 diabetes.

A significant outcome of the symposium was the identification of several areas for additional research. First, a consensus must be reached regarding reliable, practical biomarkers or surrogates for cardiovascular disease, so that meaningful comparisons can be made in clinical trials between the effects of different interventions. Doing so will ultimately result in clarification of what sometimes appear to be contradictory results in studies and will allow for greater efficiency in the performance of interventional studies. Second, a better understanding is needed of the differences and similarities in the effects of glycemic and other interventions on different end organs, as well as the influence of genetic factors in this regard.

Dr. Nathan emphasized that the DCCT/EDIC group is the most vigorously and thoroughly studied population of type 1 diabetic patients in history, with 95 percent retention of subjects over a span of 20 years (n=1385/1441), and with an average follow-up of approximately 16 years. The population has been extensively characterized and phenotyped over time with regard to complications, diabetes therapy and chronic glycemia, and established and potential risk

factors, and it has provided researchers with an incredibly valuable resource of stored biological specimens, including DNA, which can be well utilized for the validation of biomarkers.

Dr. Nathan suggested that the DCCT/EDIC group continue to examine the relationship between the panoply of risk factors and macrovascular disease and the more severe stages of microvascular disease. As the DCCT/EDIC population evolves and develops more advanced eye, kidney, and macrovascular disease, investigators will be able to study the effects of established and putative risk factors on these clinically onerous complications. Diabetes researchers should also continue to study and to define the imprinting phenomenon described during the DCCT/EDIC symposium, including expanding epidemiologic approaches currently in use and through case-control studies.

DCCT/EDIC data can be used to identify and define clinically relevant biomarkers of complications that may be used in future studies, using phenotypic data and stored samples, which may also be used to identify biochemical steps in the pathogenesis of complications. Finally, the current DCCT/EDIC genetic initiative that is looking at the genetic contribution to susceptibility for developing complications ought to be continued.

Dr. Saul Genuth, Professor of Medicine, Division of Clinical and Molecular Endocrinology, Case Western Reserve University, added that the DCCT/EDIC cohort is not only the most vigorously and consistently studied group of type 1 diabetics, but that it is also the most accurately studied group, producing high quality data as a result of good quality control measures. Dr. Genuth stressed the importance of and opportunity for lifelong follow-up by NIH, given the high level of commitment of the patient participants in the cohort, due in part to the research mindset of the patients and to the personal bonding between patients and study investigators. He recommended that researchers capitalize on the strong research motivation of the cohort patients in their consideration of future studies and initiatives.

During the discussion following Dr. Nathan's presentation, Dr. Michael Brownlee, Anita and Jack Saltz Professor of Diabetes Research, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, pointed out a further knowledge gap with regard to the adverse effects of acute hyperglycemia or stress hyperglycemia. Data suggest that coronary disease is largely a metabolic disease; in treatment of individuals with stress hyperglycemia, the outcome in the area of infarction in the brain is proportional to the level of hyperglycemia on admission. Seventy percent of those who have myocardial infarctions are either diagnosed diabetics or people with impaired glucose tolerance. A better understanding of the mechanisms of the disease will aid in the prevention of damaging effects on outcomes, especially since the events typically measured in the diabetic population are ultimately fatal.

During the discussion following Dr. Nathan's presentation, Dr. Michael Brownlee, Anita and Jack Saltz Professor of Diabetes Research, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, pointed out a further knowledge gap with regard to the adverse effects of acute hyperglycemia or stress hyperglycemia. In addition to recent data showing that the majority of patients with coronary artery disease are either diabetic or have impaired glucose

tolerance, acute hyperglycemia has been shown to adversely affect the outcome of myocardial infarction and stroke. The area of infarction in the brain is proportional to the level of hyperglycemia on admission. Seventy percent of those who have myocardial infarctions are either diagnosed diabetics or people with impaired glucose tolerance.

Dr. Mark E. Cooper, Director, Baker Heart Research Institute, Melbourne, Australia, stressed that the development of macrovascular complications from diabetes will prove to be especially important over the next 10 years for the DCCT/EDIC population. Why some diabetic individuals are less able to withstand a given load of macrovascular disease than their non-diabetic counterparts is an area of research that may be further examined with data from the DCCT/EDIC cohort, since baseline data such as echocardiography is available for these patients. Advanced echocardiography allows diastolic dysfunction—which may be linked to the mechanisms reviewed during the DCCT/EDIC symposium—to be more easily discernible and more accurately diagnosed. Dr. Genuth added that the DCCT/EDIC patient population is exceptionally receptive to further testing or exams, especially where heart function is concerned, stating that 85 percent have already had coronary calcium scans performed.

Dr. Peter Savage, Director, Division of Epidemiology and Clinical Applications, National Heart, Lung, and Blood Institute (NHLBI), offered three points that bear closer scrutiny: (1) the subclinical cardiac dysfunction known to occur in diabetics; (2) the amount of vascular disease prior to and following the onset of renal disease and the association of renal disease with the exacerbation or progression of atherosclerosis; and (3) the importance of more efficient clinical trials to examine the means for and to document the correlate between subclinical disease measures and events, particularly in light of the new and multiple interventions available. As an example, Dr. Savage suggested that abnormalities in the system might add substantially to the subclinical disease; if not, then the subclinical disease could be used as a predictor.

Dr. David R. Matthews, Professor of Diabetic Medicine, Oxford Centre for Diabetes Endocrinology and Metabolism, England, observed that perhaps part of the "imprinting" in the DCCT/EDIC cohort is due to the education of and attention given to the patient participants.

Dr. John W. Baynes, Carolina Distinguished Professor, Department of Chemistry and Biochemistry, University of South Carolina, Columbia, cautioned that the group not become too glucocentric. While glucose might turn out to be a statistically important mediator, understanding the downstream effects is also critical. Dr. Baynes suggested that a greater emphasis be placed on insulin resistance in pre-diabetic states, which often precede the development of type 2 diabetes, during which time substantial damage can occur. Dr. Helen Vlassara, Director, Division of Experimental Diabetes and Aging, Mount Sinai School of Medicine, New York, added that researchers ought not to ignore derivatives of glucose metabolism.

The area of implementation and dissemination research, also termed translational research, was an area not covered by the symposium, but one which Dr. Denise Simons-Morton, Acting Director, Clinical Applications and Prevention, Division of Epidemiology and Clinical Applications, NHLBI, brought to the attention of the group. It was suggested by Dr. Daniel

Stryer, Acting Director, Center for Quality Improvement and Patient Safety, Agency for Healthcare Research and Quality (AHRQ), that banked clinical data studies could also be supported by R03 or hyper-accelerated grant applications, and that these data could provide information on general markers of inflammation.

Dr. John M. Lachin, Professor of Biostatistics and Epidemiology, The Biostatistics Center, George Washington University, Rockville, Maryland, offered the idea that a future challenge for researchers will be the characterization of lesions at the cellular level, which would represent the true factors that are determining the risk of further disease progression or the risk of complications.

How Can We Foster Development of Animal Models in Which Potential New Therapies Can be Explored?

Dr. Timothy S. Kern, Director, Center for Diabetes Research, Case Western Reserve University, Cleveland

Dr. Kern addressed the issues of animal models in type 1 diabetes research. Although most purely diabetic animal models do not progress to advanced stages, they nonetheless provide valuable information, including biochemical abnormalities that seem to play a role in the development of various forms of pathology.

Areas that warrant further attention and research include:

- Establishment of the validity of animal models, given that they largely tend to develop the early lesions, but fail to progress.
- Use of animal models in the development and validation of surrogate markers.
- Examination of genetic contributions to complications, since animal models offer a unique opportunity in terms of cross-breeding.
- Understanding the clonal basis or "imprinting" basis of "metabolic memory."

Considering how long complications take to develop in humans, barriers exist in the use of animal models in diabetes research on complications because the animals have relatively short lifespans. A further obstacle is the lack of macular edema models and the inability of researchers to make specialized measurements. However, the latter difficulty might be overcome through the use of core facilities to provide measurement services.

Dr. Kern encouraged the establishment of a group that would evaluate therapies and decide methods for moving therapies into the clinical setting. He also suggested expansion of the

consortium on animal models to provide an arena for discussion beyond the grant recipients and broaden the scope of researchers, a suggestion echoed by several participants at the symposium.

Dr. Cooper expressed concern that appropriate animal models be used. Since the consortium is trying to generate new animal models, they might consider starting with animals such as the db/db mice, which have fewer complications that will affect study results. Drs. Kern and Vlassara agreed with this comment, and Dr. Vlassara further challenged the definition of what constitutes a normal animal model or normal baseline. She suggested a new "hyperglycotoxemic" model be developed.

Dr. Eva L. Feldman, Professor of Neurology, University of Michigan, Ann Arbor, shared information from the AMDCC. The consortium has moved from having 2 animal models to 12 models and is now gathering interesting data on atherosclerotic and nephropathic models. The large bioinformation component of the consortium has allowed for a generous amount of shared data.

Dr. Fradkin suggested that further comments regarding expansion of the consortium be directed to Dr. Robert Star, Senior Scientific Advisor, NIDDK, at Robert_Star@nih.gov.

How Can We Foster Development of Surrogate Markers Useful for Clinical Trials of Potential New Therapies?

Dr. Ann Marie Schmidt, Associate Professor and Chief, Division of Surgical Science, College of Physicians and Surgeons, Columbia University, New York

Dr. Schmidt categorized cardiovascular disease and diabetes into three parts: (1) the innate cardiac dysfunction; (2) surrogate endpoints for long-term vascular disease, including stenting and the amount of neointimal expansion as a potential surrogate endpoint, given that diabetic individuals undergoing angioplasty and revascularization procedures do very poorly; and (3) macrovascular disease and atherosclerosis itself. (Dr. Schmidt served on the May 16, 2002, Advisory Panel.)

Dr. Schmidt proposed that intravascular ultrasonography (IVUS) might also be used as an endpoint. Since the increase in IVUS quantification of macrovascular disease has been demonstrated, it appears that the Food and Drug Administration (FDA) might be softening with respect to endpoints other than death and clinical events.

With regard to plaque and instability, examination of the inflammatory mediators and inflammatory markers produced by peripheral monocytes in humans following intervention may provide a useful surrogate marker. MMP9, antigen activity, and procoagulant response are also being investigated, as well as impaired endothelial independent relaxation, although the last is not an FDA-approved endpoint. The response to acetylcholine is very abnormal in diabetic individuals and can in and of itself be a surrogate marker.

Clearly, a very important surrogate marker is C-reactive protein (CRP), which might prove useful not only with regard to defining response to therapy, but also when examining quartiles of elevated CRP levels at baseline and their application to relative risk.

Dr. Schmidt identified the following methods for development of surrogate markers:

- Functional MRI is a promising study method, particularly because of its wide availability, but one which may require incentives to encourage study participation.
- Urine protocytes may be a potential marker of early injury, although albuminuria is not an FDA-approved endpoint.

- Degree of alveolar bone loss and periodontal disease are potential surrogate markers for inflammatory baseline and response, since epidemiological data suggest that periodontal disease, regardless of the presence or absence of diabetes, is a risk factor for the development of atherosclerosis.

- Erectile dysfunction, because it involves not only neurology but also vasculature, is a possible surrogate marker.

- Skin biopsies could be surrogate markers for levels of collagen abnormalities.

- Live oxidation products are possible surrogate markers for measurement.

Information presented during the DCCT/EDIC symposium suggested proteomics and genomics as possible surrogate markers, an idea Dr. Schmidt found attractive not only because of the availability of DCCT/EDIC samples, but also because research in these areas encourages basic researchers and clinical trialists to partner with biotech companies, thereby increasing the sample pool and fostering further multidisciplinary action.

Following Dr. Schmidt's presentation, Dr. Bruce Berkowitz, Professor, Department of Cell Biology and Opthalmology, Wayne State University School of Medicine, Detroit, cautioned researchers to use the most finely honed tools available, and as the MRI community possesses an extremely powerful set of tools for diabetic research, they ought to be enticed to form partnerships.

Dr. Matthews commented that better data would become available if researchers could get repeat measures where some specific change or threshold could be predefined. Surrogate markers for the process as an endpoint would reduce regulators' dependency on hard endpoints such as myocardial infarction and death.

Dr. Josephine Briggs, Director, Division of Kidney, Urologic, and Hematologic Diseases, NIDDK, offered a follow-up to Dr. Matthew's remarks on working with regulators, saying that she and Dr. Thomas Hostetter, Director, National Kidney Disease Education Program, NIDDK,

have been in contact with FDA regarding the development of a research agenda that would lead to clarity in proteinuria as a process marker.

How Can We Foster Identification of New Therapeutic Targets and Agents?

Dr. Lloyd Paul Aiello, Assistant Director, Beetham Eye Institute and Associate Professor of Ophthalmology, Harvard Medical School, Joslin Diabetes Center

Dr. Aiello suggested that increased consortium or network approaches would prove useful in moving research findings into clinical trials more rapidly. Excellent characterization and uniformly standardized evaluation of consortia resources would speed evaluation, provide larger sample numbers, and improve comparability between studies. These repositories could also provide some fundamental analyses that are helpful or commonly utilized for this transition, either within the collected samples or perhaps within the repositories. Benefits would include improved comparability between studies, more efficient and consistent evaluation, and services for investigators who are in possession of samples but are unfamiliar with a particular evaluation technique.

Dr. Aiello pointed out the need to rapidly identify, evaluate, characterize, and implement new technologies that may become increasingly important both in the identification of new targets and the evaluation of potential surrogate markers. Such approaches, in addition to providing novel targets may provide cross-fertilization among different complication disciplines and characterize new mechanisms by which researchers could evaluate markers in clinical trials in an efficient and rigorous manner.

During subsequent discussion, Dr. Aiello emphasized that a functional genomics/proteomics approach, conducted with homogenous patients or animal models and identifying different targets, would aid in fostering identification of new therapeutic targets.

Dr. Feldman proposed that some type 1 funds might be directed toward discovery studies, which could lead to new mechanisms, particularly in the proteomics field. Dr. Brownlee commented that discussion seemed to center on two general topics: (1) a focus on optimizing what is currently available, and (2) the concept of discovery. Dr. Vlassara remarked that the DCCT was basically an era of intervention, focusing on the control and modification of blood sugar; perhaps now it was time to add another dimension to the DCCT.

How Can We Move Promising Therapeutic Agents From Bench to Bedside?

Dr. Nigel Calcutt, Associate Professor, Department of Pathology, University of California San Diego

Dr. Calcutt used his experience with moving a molecule (prosaptide) from discovery to phase 2 clinical trials over a relatively short time as an analysis of the bench-to-bedside procedure. According to Dr. Calcutt, doing so involves correctly targeted funding. He identified several factors which contributed to the successful process:

- Personal drive and focus of the Principal Investigators, which included discovery of the molecule and raising money through private funds and venture capital.

- Availability of the STAR program, a fast-moving funding mechanism, where funding was provided in part by the State of California, part by the company of interest. A most important aspect of this funding program is the recognition of the academic as Principal Investigator. The funding is therapy-oriented and results-driven, protects company intellectual property, and provides for initial proof-of-concept studies, allowing investigators to produce the preliminary data necessary to qualify for NIH funding.
- Availability of an NIH Request for Application (RFA), an important aspect because it targeted money at therapy-driven research, rather than purely mechanistic-driven studies.

- Luck and opportunity for collaboration between researchers.

Dr. Calcutt noted that, while these conditions are admittedly unlikely to reoccur in the near future, there are steps NIH can take to create a similarly helpful environment. For example, NIH could fund exploratory research programs that provide money for 1-year rolling, results-driven projects, such as those provided by the Juvenile Diabetes Research Foundation (JDRF) International. An incentive for academics to participate could be initiated through the creation of modified STAR/SBIR (Small Business Innovation Research) funding to include both industry and academia, where both parties would receive recognition for participation. RFAs for R01s to support therapy-driven research should be made available, not to the exclusion of mechanistic-driven research, but to allow for quicker progression. Support systems, both informational banks and funding sources, to connect Principal Investigators having potential therapeutics with those skilled in phase 1 and 2 trials, might be made available through the use of paired grants. Further, NIH could provide assistance through both funding and information to small biotechnology companies to aid them in moving potential therapeutic agents through phase 1 and 2 trials.

During discussion, the point was made that using surrogate markers and non-regulatory approved endpoints may speed up the process. Dr. Calcutt suggested the formation of a body to negotiate a compromise between NIH’s scientific position and FDA's required position from a safety point of view.

Dr. Spiegel, Director, NIDDK, recommended Rapid Access to Interventional Development (RAID), a program used at the National Cancer Institute that provides, on a contract basis, some functions such as producing a sufficient quantity or quality of a product, by means that ordinarily would not be available to an investigator who has a patented therapeutic agent. Production issues might also be expanded through this program.

Dr. Fradkin pointed out the availability of the innovative partnerships RFA that pairs researchers working in diabetes with scientists who have expertise relative to diabetes but who are working in other fields, and proposed the notion that rather than a single grant, two paired grants might be a more attractive option for investigators, a suggestion that was met with general agreement.

Dr. Spiegel concluded with the observation that future research teams ought to embody the concept of a multidisciplinary approach, acknowledging that equal credit for more than a single Principal Investigator is a crucial aspect of team research.

What Are the Most Promising Opportunities To Advance Research To Develop New Therapies for Complications?

Dr. Michael Brownlee, Anita and Jack Saltz Professor of Diabetes Research, Albert Einstein College of Medicine

Dr. Brownlee posed several possible research questions for consideration by those in attendance:

- What are the mechanisms responsible for microvascular complications?
- What are the mechanisms responsible for macrovascular complications?
- What genetic issues determine the development and progression of diabetic complications?

He expressed the opinion that further investigation and definition of the issue of metabolic memory is certainly necessary, including expansion of the concept to include other areas such as insulin-resistance and fatty acid memory.

Dr. Brownlee recognized the importance of drugs with regard to the prevention of diabetic complications, but remarked that perhaps a greater focus ought to be placed on secondary prevention, since the mechanisms responsible for initiation may not be the same mechanisms responsible for progression of complications. Surrogate markers and a new clinical study paradigm are also areas that he believed warrant additional study, because current paradigms are too costly and require too many years to effectively screen treatments that show promise in animal models.

It is generally accepted that, when considering genetic susceptibility to complications, animal models such as those provided by the AMDCC provide investigators with the advantage of using animals with known genetic backgrounds. These models ought to be further utilized.

As researchers focus design attempts on drugs aimed at specific targets, Dr. Brownlee identified high throughput screening for new therapeutic targets and agents as the new wave of the future.

Dr. Brownlee noted that the DCCT/EDIC symposium's emphasis on multidisciplinary research and collaboration between areas of expertise strongly suggests that dual Principal Investigator grants and exploratory research programs that promote discovery and innovative research should be a priority. In conclusion, he listed the following areas as the most important and most promising research opportunities:

- Development of a mechanism for real discovery and innovation.
- Multidisciplinary efforts fostered through dual investigator grants between researchers in complementary fields to produce innovative work.
- Funding for non-patented therapeutic agent trials.

Dr. Fradkin closed the session with the comment that the meeting produced not only intriguing ideas in the area of diabetes research, but identified available resources for carrying them to fruition.