Regulation of Inflammatory Responses: Influence of Sex and Gender

Bethesda, Maryland
September 19–20, 2006

WORKSHOP SUMMARY

Office of Research on Women's Health, Office of the Director
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Department of Health and Human Services

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EXECUTIVE SUMMARY

Several studies have shown that inflammation, which involves a constellation of symptoms and comprises a series of activities, underlies chronic diseases such as cardiovascular disease, as well as autoimmune diseases such as rheumatoid arthritis and lupus. Because of this increasing evidence, inflammation has become a hot topic in the public arena, as shown by articles in *Time*, *Newsweek*, and *Wall Street Journal*. The molecular and cellular details of inflammatory processes are well understood and are similar among several diseases, but initiating events for inflammation are still unknown. Oxidative stress, tissue damage, and age have all been implicated in the process, but no common specific stimuli have been identified.

Sex-related differences have been observed in several diseases or injuries, including lupus, rheumatoid arthritis, heart disease, asthma, periodontal disease, and response to major injury, shock, or trauma. These studies further hint that sex hormones such as estrogen modulate both inflammation and immune response, and that these effects could be classified based on systemic hormone levels, the role of hormone receptor isoforms and variants, and the stage of disease. However, more work is needed to increase understanding of hormonal effects on inflammation, immune response, and disease. For example, much work has focused on how estrogens modulate cellular responses, but little information is available about other hormones, such as testosterone, progesterone, glucocorticoids, and insulin, and the effects of interactions among hormones on inflammatory processes. Moreover, existing treatments for inflammatory and autoimmune disease offer only limited efficacy and induce severe side effects. New treatment strategies will not only have to restore immune tolerance, prevent sensitization or neutralizing antibody production, and limit unintended biological effects, but also address sex differences and hormonal effects in the context of these diseases.

On September 19–20, 2006, the National Institutes of Health Office of Research on Women's Health and the National Institute of Allergy and Infectious Diseases convened a workshop on the influence of sex and gender on the regulation of inflammatory responses. Lisa Begg, Dr.P.H., R.N., and Christopher Taylor, Sc.D., served as workshop chairs. During this workshop, researchers from across the United States and the world presented their research to provide participants with a state of the science. Representatives of Institutes across the NIH attended the workshop, including representatives of the National Cancer Institute; the National Heart, Lung, and Blood Institute; National Institute on Aging; the National Institute on Alcohol Abuse and Alcoholism; the National Institute of Arthritis and Musculoskeletal and Skin Diseases; the National Institute of Child Health and Human Development; the National Institute of

Dental and Craniofacial Research; the National Institute on Drug Abuse; the National Institute of General Medical Sciences; the National Institute of Mental Health; and the National Institute of Neurological Disorders and Stroke. The first session provided a basic foundation of what inflammation is, and subsequent sessions explored sex differences in the burden of disease and response to insult, cellular and molecular mechanisms in inflammation, and emerging strategies to treat inflammatory diseases. These presentations were followed by discussions in which workshop participants identified gaps in knowledge, research needs, and opportunities for future research. Recommendations for research opportunities will be incorporated into a trans-NIH initiative on inflammation.

Future Research

Session moderators and workshop participants identified the following gaps and research questions:

- What is the exact stimulus or trigger for inflammatory processes?
- How is lipid metabolism regulated by hormones?
- How do regional differences in adiposity or energetics contribute to the differing burden of the metabolic syndrome?
- What are the anatomical differences in vascular lesion formation?
- What is the role of sex hormones in regulating HDL production, metabolism, and modification?
- What is the role of hormone receptor activation in inflammatory responses?
- Are there epigenetic effects of gender that influence sex bias?
- What are the effects of hormones on innate immunity in healthy subjects?
- How do sex and/or gonadal steroid hormones alter inflammatory and immune responses after injury?
- How do aging, menopause, and andropause affect these responses?
- What are the mechanisms by which hormones influence cellular responses to insult?
- What is the temporal response of sex hormones to shock and trauma?
- Can hormonal effects on immune or other cells account for sex biases in autoimmunity, infection, and recovery from trauma?
- What is the mechanism of action of estrogen or the ER in particular cell types?
- Which ER isoforms or variants are specific to which cell types?
- Which hormones mediate sex-specific effects?
- How do sex hormones interact with pathogen responses?
- How do symptoms and responses differ by sex in humans?
- How can new strategies employ the concept of “outflammation,” using chemorepellants to remove cells from places where they normally should not appear?
- How do resolvins and the lipoxin pathway, which enhance the resolution of inflammation, be used?
- Can non-steroidal anti-inflammatory drugs provide a benefit?

Research Considerations

- Development of new models to further explore sex differences.

- Maintaining a focus on physiology, even while conducting experiments *in vitro* in cell systems and on particular systems of interest.
- Sorting out direct causality of chronic disease, when the initiating event has long passed and tissue response to that event is well established.
- Activation and function of pro- and anti-inflammatory proteins.
- Effects of hormone receptor resistance and hormone metabolism.
- Influence of tissue specificity in inflammatory response.

LIST OF ACRONYMS AND ABBREVIATIONS

ACR	American College of Rheumatology
AR	androgen receptor
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
CVD	cardiovascular disease
CNS	central nervous system
C. rectus	*Campylobacter rectus*
CRP	C-reactive protein
CVB3	coxsackievirus B3
DAS	Disease Activity Score
DC	dendritic cell
DHEA	dehydroepiandrosterone
DHT	dehydrotestosterone
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
E. coli	*Escherichia coli*
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ER-α	estrogen receptor alpha
ER-β	estrogen receptor beta
EULAR	European League Against Rheumatism
GR	glucocorticoid receptor
HDL	high-density lipoprotein (cholesterol)
HPA	hypothalamic-pituitary-adrenal
HPT	hypothalamic-pituitary-thyroid
HRT	hormone replacement therapy
HUVEC	human umbilical vein endothelial cells
ICAM	intracellular adhesion molecule
ICU	intensive care unit
IFN	interferon
IIT	intensive insulin therapy
IκB	inhibitor of nuclear factor kappa B
IL	interleukin
iNOS	inducible nitric oxide synthase

LDL	low-density lipoprotein (cholesterol)
LeTx	anthrax lethal toxin
15-LO	15-lipoxygenase
LPS	lipopolysaccharide
MIC-1	macrophage inhibitory cytokine
MODS	multiple organ dysfunction syndrome
MCP-1	monocyte chemoattractant protein 1
M-CSF	monocyte colony stimulating factor
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MS	multiple sclerosis
NFκB	nuclear factor kappa B
NHLBI	National Heart, Lung, and Blood Institute
NIA	National Institute on Aging
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NK	natural killer
NKT	natural killer T cell
NO	nitric oxide
ORWH	Office of Research on Women's Diseases
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E2
PGI	prostacyclin
PI3K	phosphatidylinositol 3-kinase
PPT	propylpyrazole triol
PR	progesterone receptor
PR-A	progesterone receptor A
PR-B	progesterone receptor B
RA	rheumatoid arthritis
RRMS	relapse-remitting course of MS
SAA	serum amyloid A
SERM	selective estrogen receptor modulator
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythromatosus
T/HS	trauma and hemorrhagic shock
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha

VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WBC	white blood cells
WHI	Women's Health Initiative

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WORKSHOP SUMMARY

INTRODUCTION

The classic definition of inflammation was first proposed in the first-century description by Celsus of redness, swelling, heat, and pain. More recently, the study of inflammation has focused more and more on the cellular and molecular players involved, and recent work has revealed the roles of these players in several diseases and disorders, such as cardiovascular disease (CVD), diabetes, arthritis, and cancer. For example, atherosclerosis was once viewed as a bland, non-inflammatory process resulting in a benign tumor of the smooth muscle cells. With the identification of various cytokines as modulators of cell proliferation in fibrotic diseases,[1] this view of the disease process has now evolved to describe atherosclerosis as an inflammatory process.[2,3] The idea that inflammation plays a role in CVD and pulmonary diseases represented a "frontier" in research, even as Pasceri and Yeh published an editorial noting the similarities in pathology between atherosclerosis and rheumatoid arthritis (RA) and between atherosclerosis and tuberculosis.[4] Yet now, following a tremendous shift in thinking, the role of inflammation in atherosclerosis, along with other diseases, is a matter of public interest, having appeared in *Time*, *Newsweek,* and the *New York Times*.

Although the understanding of inflammatory processes and the role they play in disease has increased, this field is still evolving, particularly in the area of sex and gender differences in the regulation of inflammatory responses. For example, recent work has shown that varying sex hormone levels can influence immune function and the type of immune response, but the effects of hormone receptor resistance are poorly understood. On September 19–20, 2006, the National Institutes of Health (NIH) Office of Research on Women's Health (ORWH) and the National Institute of Allergy and Infectious Diseases (NIAID) co-sponsored a workshop on the influence of sex and gender in the regulation of inflammatory responses. The goals of this workshop were to assess and advance the state of the science in inflammation research and to generate ideas for research opportunities. Research presentations focused on the biology of inflammation, sex-related inflammatory disorders, cellular and molecular mechanisms in inflammation research, and emerging strategies in treating inflammatory diseases. Following these presentations, moderators presented themes from each session, and workshop participants discussed research needs and opportunities. It is hoped that these ideas will be incorporated into a NIH Roadmap initiative focused on inflammation.

SESSION I: INFLAMMATION: WHAT IS IT?

Moderator: Virginia Miller, Ph.D., Mayo Clinic

The presentations during this session provided a foundation for the rest of the workshop by discussing what inflammation is, whether biomarkers are the cause or the effects of disease, and how hormones affect host-pathogen interactions and inflammatory/immune responses. CVD was discussed as an example of the involvement of inflammatory processes in chronic disease.

An Overview of Inflammatory Mechanisms in Disease

Peter Libby, M.D., Harvard University

The role of inflammation in disease pathogenesis involves an interplay between innate and adaptive responses and a balance between pro- and anti-inflammatory stimuli.[5] Because these factors appear in several diseases and disorders, it could be said that regardless of specialty, researchers most likely all study the same generic disease, in which monocyte attachment facilitates leukocyte adhesion to epithelial or mesenchymal cells, which in turn promote migration and penetration of the monocytes and their differentiation into tissue macrophages. The types of epithelial and mesenchymal cells, as well as the type of tissue macrophage, are specific to the disease of interest.

Atherosclerosis begins when risk factors, such as high levels of low-density lipoprotein (LDL) cholesterol, induce monocytes to attach to the artery wall and secrete adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), that facilitate the adhesion of leukocytes to the endothelium. VCAM-1 specifically binds monocytes and lymphocytes found in atherosclerotic plaques and can be induced by cytokines. Within 1 week after rabbits are fed a diet rich in cholesterol and saturated fat, patches of aortic endothelial cells express VCAM-1, then bind leukocytes.[6] Endothelial cells can modulate the type of adhesion molecules expressed and thus the recruitment of innate immune, adaptive immune, or acute inflammatory effector cells.

Following leukocyte adhesion, endothelial and smooth muscle cells express chemoattractants or chemokines, such as monocyte chemoattractant protein 1 (MCP-1), that induce monocyte migration and penetration into the intima. Inactivation of MCP-1 in mutant mice rendered susceptible to atherosclerosis show fewer atherosclerotic plaques and less atherosclerosis overall.[7] Following migration and penetration, monocytes in the atherosclerotic plaque differentiate into tissue macrophages, or foam cells, which then secrete factors promoting oxidative stress, coagulation, and apoptosis, resulting in the formation of a necrotic core in the plaque. Among the signals that stimulate the metamorphosis from monocyte to foam cell is monocyte colony stimulating factor (M-CSF). Inactivation of M-CSF decreases atherosclerosis, but it also causes problems in the bone.[8]

Following the initiation of atherosclerosis, lesions and plaques remain clinically stable for decades. Inflammation also plays a role in progression. Libby and colleagues have shown that cells other than leukocytes can secrete interleukin 1-beta (IL-1β). Vascular endothelial cells produce other cytokines, including tumor necrosis factor alpha (TNF-α), TNF-β, IL-1α, IL-1β, IL-6, M-CSF, MCP-1, and IL-18. Smooth muscle cells also express CD40 ligand, which is important for B-cell activation. In addition, CD40 ligand is expressed on vascular endothelial cells, smooth muscle cells, and macrophages in atherosclerotic plaques, and expression of COX-2, which is induced by CD40 ligation, is augmented. Experiments also have shown that mutant mice susceptible to atherosclerosis and fed a high-fat diet show arrest of atherosclerosis progression when CD40 signaling is disrupted.[9] Thus, vascular cells actively participate in inflammation, rather than simply respond to it.

Inflammation also contributes to the thrombotic complications that bring the patient most dramatically to the clinic. An atherosclerotic plaque consists of a lipid core surrounded by a fibrous cap that is formed by extracellular matrix made by smooth muscle cells. The integrity of

the fibrous cap depends on type I and type III interstitial collagen. Matrix metalloproteinases (MMP) cleave collagen between glycine 775 and leucine or isoleucine 776, breaking the triple helix into fragments. The persistence of risk factors result in the accumulation of inflammatory cells and instructions to the macrophages to overexpress the collagenases MMP1, MMP8, and MMP13, which degrade the fibrous cap and render it susceptible to rupture at the same time production of interferon gamma (IFN-γ) halts new collagen synthesis by the smooth muscle cells.[10] Other inflammatory mediators, including CD40 ligand, IL-1, TNF-α, MCP-1, and M-CSF, also contribute to acute thrombotic complications,[11] and inflammation induces the expression of the pro-coagulant tissue factor. Reducing risk factors such as lipid levels can limit inflammation, stabilize the fibrous cap, and facilitate the reversal or resorption of the atherosclerotic plaque.

Several pro-inflammatory molecules have been proposed as independent predictors of myocardial infarction and stroke, two complications of atherosclerosis. Among these are the primary pro-inflammatory cytokines such as TNF-α; the messenger cytokine IL-6; endothelial adhesion molecules; and acute-phase reactants such as fibrinogen, plasminogen inhibitor, C-reactive protein (CRP), and serum amyloid A (SAA). Among these molecules, CRP is most attractive because it is chemically stable; can be measured by a reliable, standardized, convenient, and inexpensive assay; has a long half-life; and shows no diurnal variation. Serum concentrations of CRP add prognostic value beyond the Framingham risk score. Activities that decrease the risk for CVD, such as exercise, weight loss, and smoking cessation, also decrease serum CRP concentrations. Oral estrogen and estrogen/progestin combinations, which have been associated with increased thrombotic liability in the populations studied, increase CRP.

Discussion

Although both sexes are at risk for CVD, the face of this disease has changed. In the 1950s, the typical CVD patient was white, middle-aged, male, hypercholesterolemic, and hypertensive. Now, the risk profile is shifting from smoking and hypertension to nutritional factors such as diabetes and the metabolic syndrome. Coronary disease has become more prevalent in women and in disadvantaged, minority populations, partly because of the shift in risk profiles toward metabolic disorders, including low high-density lipoprotein (HDL) levels, high triglyceride levels, and glucose intolerance or insulin resistance. In addition, although death rates associated with CVD have declined, these rates are age adjusted, and obesity is fast becoming a pediatric disease. More data is needed about diet, behavior, and exercise, and ways to encourage people to maintain a healthy body weight and choose a diet to forestall obesity should be explored. In addition, in some studies, hormone replacement therapy (HRT) has been demonstrated to be somewhat protective in premenopausal women, in contrast to large clinical trials that suggest that HRT increases the risk for heart disease in postmenopausal women. More work is needed to sort out the protective and detrimental effects of estrogen on the cardiovascular system, as well as the increased association of CVD with diabetes and the metabolic syndrome. Clinicians are only starting to realize that women with CVD may present with symptoms different from those traditionally associated with the disease. In addition, women often serve as caregivers and are less likely to seek care for themselves, especially for symptoms not traditionally associated with CVD. Because of these factors, CVD may be underdiagnosed in women.

Overall, CRP levels are higher in women than in men, but only slightly, and they vary by race and ethnicity, although how much of this difference is associated with dietary differences is not known. Estrogen affects CRP levels, although the effects of oral estrogens are more pronounced than those of endogenous estrogen. Despite these differences, reference values for both men and women are the same.

The specific effects of foam cell production on major vessels may depend on several factors. One is pressure; pulmonary hypertension promotes atherosclerosis of the pulmonary arteries. In addition, inflammation and high cholesterol results in an accumulation of foam cells in other tissues, such as tendinous xanthomata. Orange tonsils are found when cholesterol cannot be transported out of macrophages, as in Tangiers disease.

Inflammatory Modulators of Cardiovascular Risk: Cause or Effect

Godfrey Getz, M.D., Ph.D., University of Chicago

As discussed by Dr. Libby, atherosclerosis takes several decades to develop, and for many of those decades, patients are not aware they have it. Most risk factors have been identified through their relationship with clinical endpoints, even though most CVD involves underlying atherosclerotic processes. In a component of the PDAY Study,[12] topographic distributions of atherosclerotic lesions were examined in autopsied persons aged 15 to 34 years. Within this population, males had more fatty streaks and raised lesions in the right coronary artery, compared with females; thus the development of atherosclerosis was more prevalent among men. In the future, imaging may be used to identify the presence of atherosclerosis long before a patient complains of symptoms. Although several modalities are under development, however, they are at various stages of maturity and offer differing degrees of invasiveness, and none are at the point of precisely determining the nature of a patient's atherosclerotic lesion.

Elevated LDL most likely is a cause of CVD, based on the positive correlation between total plasma cholesterol and LDL cholesterol with the incidence of CVD, the evidence of genetic hypercholesterolemia in cases of premature atherosclerosis, the reduced progression of atherosclerosis when total cholesterol and LDL are lowered, and the requirement for dyslipidemia for atherosclerosis to develop in experimental animals. In particular, oxidized LDL most likely plays a causal role in atherogenesis. LDL is a globular particle with a core of cholesterol ester and a surface consisting of cholesterol, phospholipid, and proteins, primarily apoB-100. Most circulating LDL is native, but as it transits through endothelial cells, the surface components of several LDL molecules become oxidized through the action of 15-lipoxygenase (15-LO) and inducible nitric oxide synthase (iNOS).[13] The oxidation of phospholipids in LDL is highly pro-inflammatory.[13] The appearance of modified LDL molecules in the subendothelial space activates signaling pathways, including those involving VCAM-1 and several chemokines, which promote the recruitment of circulating monocytes in areas where oxidized LDL has accumulated. Under the influence of M-CSF, these monocytes differentiate into macrophages and express scavenger receptors that facilitate the uptake of modified LDL and the formation of foam cells. Once activated, these macrophages secrete factors that promote further monocyte and T-cell recruitment and differentiation. Oxidized LDL inhibits the production of nitric oxide (NO) by endothelial NOS (eNOS), resulting in a less-pliant blood vessel. Results from several studies have correlated the increase in the levels of antibodies against oxidized phospholipids with several aspects of CVD.

High levels of HDL, on the other hand are atherprotective, as shown by an inverse relationship between CVD and HDL levels and studies, mostly in animals, of the influence of apoA-1, the major HDL protein, on atherosclerosis. Like LDL, HDL is a globular particle, but it is more heterogeneous. It is smaller, with many more proteins and fewer steryl esters associated with it.[14] Thus, HDL is more readily remodeled in the circulation. HDL participates in reverse cholesterol transport, where the cell surface molecules ABCA1 and ABCG1 promote the transfer of cholesterol from foam cells to apoA-1. Ultimately, as remodeled HDL, apoA-1 is transported through the plasma to the liver for secretion as bile acid.[15] Persons lacking the ABCA1 protein are unable to carry out reverse cholesterol transport and exhibit orange or yellow tonsils. The presence of apoA-1 is the primary protective property of HDL. Mice deficient in both the LDL receptor and apoA-1 show increased development of atherosclerotic lesions.[16] The administration of an apoA-1 mimetic to apoE-deficient mice, even in low concentrations, minimizes the number of atherosclerotic lesions.[17] These studies point to apoA-1 as a potential therapeutic target, but studies assessing the effect of apoA-1 on human atherosclerosis are still in preliminary stages.

HDL is an anti-inflammatory agent.[18] A culture system consisting of a synthetic artery composed of human endothelial cells overlying cultivated smooth muscle cells has been used to assess effects on monocyte recruitment.[19] Oxidized LDL promotes monocyte recruitment, while the addition of HDL attenuates the effects of oxidized LDL. The anti-oxidant paraoxonase plays a role in this inhibition. Navab and colleagues[19] plotted an inflammatory index based on their cell culture system and found that HDL reduces inflammation by about 60%. However, when an index was plotted for the same amount of HDL taken from patients who had experienced a myocardial infarction, the anti-inflammatory properties of the HDL molecules had been lost. Thus the quality and composition of HDL is probably just as important as the quantity, if not more so.

Elevated levels of the acute inflammatory markers CRP and SAA have also been cited as risk factors for CVD. The relative risk for future CVD events is higher for these markers than it is for elevated LDL.[20,21] CRP has been used as a biomarker in several CVD studies, although at the low limit of the acute phase reaction. SAA is transported in the bloodstream in association with HDL. However, it is not clear whether CRP and SAA are simply biomarkers or whether they have any pathogenetic influence on atherosclerotic progression. Mice do not produce CRP, and work with SAA has not advanced to the point of decisive genetic manipulation.

Several inflammatory cell types are present in an atherosclerotic lesion. Genetic manipulation of mouse models and *in vitro* cell systems has shown that macrophages are central to inflammatory processes underlying atherosclerosis. Yet natural killer (NK) cells, $CD4^+$ T cells, NK T (NKT) cells, and $CD8^+$ T cells all serve pro-inflammatory functions and increase atherogenesis in mouse models, whereas $CD4^+$ regulatory T cells and B cells perform anti-inflammatory functions and decrease atherogenesis. Although few B cells are observed in atherosclerotic lesions, work with mouse models suggests they are protective. The pro-inflammatory cytokines IFN-γ, IL-12, and IL-18 and the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β) are also present in these lesions. Work exploring the role of mature T and B cells in atherosclerosis is under way. However, it is clear that robust atherosclerosis may develop in the

absence of both T and B cells, and these responses may be gender biased in some vascular beds. Results from the human PDAY study showed that unlike males, young females have relatively more abdominal aortic atherosclerosis than coronary artery atherosclerosis. Thus vascular beds differ in relation to gender influences in humans, as well.

Discussion

Studies examining expression of the estrogen receptor (ER) in the vasculature have focused primarily on the alpha (ER-α) isoform. However, ER-α expression in various regions of the vasculature has not been examined. ER studies using vascular models are discussed further in Dr. Bender's section in Session III.

Recent data has been published showing an increased fraction of pro-inflammatory HDL in autoimmune diseases such as systemic lupus erythromatosus (SLE) and RA. These studies also examined the effects of oxidation. It could be that in these diseases, the level of oxidative stress is high enough to overwhelm anti-oxidant enzymes. Administration of the apoA-1 mimetic results in the transport of paraoxonase from HDL to the mimetic. Thus, oxidative stress could substantially modify the composition of HDL so that it no longer functions as an anti-inflammatory agent.

It is not clear whether patients with a family history of elevated cholesterol based on high HDL levels are protected from atherosclerosis. Some genetic abnormalities of HDL homeostasis have been identified that influence their atheroprotective properties. In Japan, families with high HDL resulting from partial deficiency of cholesterol ester transfer protein (CETP) seem to be protected from atherosclerosis. CETP transfers cholesterol esters between HDL and other lipoproteins. Trials are in process to determine the therapeutic benefit of CETP inhibition. In Italy families with the apoA-1 mutation apoA-1 Milano also seem to be protected from atherosclerosis, though their HDL level is not increased. In addition premenopausal women show different distributions of HDL particle size. HDL2, the larger HDL component, is higher in females than in males, which may afford some premenopausal protection. More study is needed about the relationship between the composition of HDL and protection against atherosclerosis. Future research also should focus on the role of sex hormones in regulating HDL production, metabolism, and modification.

Hormones and Inflammation: Host-Pathogen-Hormone Interactions

Esther Sternberg, M.D., National Institute of Mental Health, NIH

Steroid hormones affect host inflammatory/immune responses in a variety of ways, through the central nervous system (CNS), the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, and the hypothalamic-pituitary-gonadal (HPG) axis.[22,23] The effects of estrogen, glucocorticoids, or progesterone on host inflammatory responses depend on its concentration. Low levels of estrogen tend to shift responses toward the Th1 or cellular phenotype, whereas high levels shift them toward the Th2 or humoral phenotype.[24,25] Glucocorticoids and progesterone generally drive anti-inflammatory responses and tend toward immune suppressive states. Thus, immune function changes during the menstrual cycle, where the levels of estrogen and progesterone vary, and during periods of stress. During the luteal phase, when estrogen levels are low and progesterone levels high, the expression of T-lymphocyte receptors decrease, antibody production increases, and susceptibility to infection increases. Likewise, immune

function also varies during pregnancy, with strong immunosuppression occurring in the third trimester as hormone levels (estrogen, progesterone, and glucocorticoids) increase dramatically: the number of suppressor T cells increases, the number of cytotoxic T cells decrease, and susceptibility to infections increase. Work is under way in Dr. Sternberg's laboratory to assess the effects of sex hormones on dendritic cells, which are important in pathogen recognition and serve as a bridge between adaptive and innate immunity.

Recent work has shown that bacterial toxins can interact with steroid hormone receptors and the associated host hormone responses. Anthrax lethal toxin (LeTx) comprises two proteins: lethal factor (LF), which must be transported into the cell to exert its effects, and protective antigen (PA), which facilitates entry of LF into the cell. Using a transient transfection system, Dr. Sternberg's group showed that at extremely low concentrations, LeTx is able to repress activation of the glucocorticoid receptor (GR), primarily by interfering with its ability to bind DNA and promote downstream histone acetylation, as shown by chromatin immunoprecipitation.[26] LeTx does not, however, affect GR ligand binding, the number of GR expressed by cells, GR translocation to the nucleus, or GR transrepression. The toxin also represses specific forms of the ER (ER-α) and progesterone receptor (PR-B) in a manner dependent on both the receptor and the promoter.[27] Work is under way to determine whether repression of sex steroid receptor activity is a general phenomenon among bacterial toxins.

Resistance to glucocorticoids, which can result from mutations or polymorphisms in the GR or in associated proteins, elevated levels of the antagonistic GR-β, decreased numbers of GR, or bacterial toxins, which results in the interruptions of the HPA axis at the tissue level that have been implicated in autoimmune or inflammatory diseases such as RA, SLE, Crohn's disease, asthma, and multiple sclerosis (MS).[22,28-36] Inhibition of the HPA axis predisposes a host to increased inflammation. For example, anthrax-resistant mice show increased mortality in response to LeTx when the adrenal glands of these animals were removed or when the glucocorticoid response was blocked with the GR antagonist mifepristone (RU-486).[37] This increased mortality cannot be rescued by treatment with dexamethasone. The effects of mifepristone treatment on LeTx-associated mortality suggest that adrenal factors other than glucocorticoid activity also may be involved. Thus, treating autoimmune or inflammatory diseases with high concentrations of glucocorticoid might not be effective.

Steroid hormones may play a critical role in the first line of host defense, and more attention should be paid to the effects of hormone receptor resistance. In addition to studies of on the effects of GR, other studies have shown that estrogen and ER activity influence response to lipopolysaccharide (LPS).[38] In addition to focusing on how much hormone is present, future research should consider the role of steroid hormone receptor activity, including the sex hormone receptors, in susceptibility to inflammation.

Discussion

More work should be done to assess whether receptors are uniformly downregulated across all cell types, as receptor isoforms vary across tissue. It should be noted that the studies of LeTx effects on GR activation also assessed the induction of GR-induced enzymes and found this induction blocked.

SESSION II: SEX-RELATED INFLAMMATORY DISORDERS

Moderator: Elizabeth Kovacs, Ph.D., Loyola University

Presentations in this session focused on the use of epidemiological studies and animal models to explore sex differences in MS, shock and trauma, environmental insult, and periodontal disease.

Estrogen Receptors and Autoimmune Disease

Rhonda Voskuhl, M.D., University of California, Los Angeles

The relapse-remitting course of MS (RRMS), which involves a cycle of symptomatic relapses followed by return to baseline, gradually progresses to a point where patients are unable to recover. As shown by magnetic resonance imaging, active, relapses involve gadolinium-positive inflammatory lesions in the white matter, but permanent disability goes beyond these lesions, involving atrophy in the gray matter. Existing treatment options, which are anti-inflammatory and only indirectly neuroprotective, are expensive and only partially effective. They reduce relapses by a third, but disability continues to progress. Women with RRMS are somewhat protected from relapse during late pregnancy, when progesterone and estrogen levels increase, but they become prone to relapse following delivery.[39] Further understanding of the effects of sex hormones on RRMS may provide insight into new, more effective treatment options.

Experimental autoimmune encephalomyelitis (EAE), a classic Th1-mediated disease with a cell infiltrate at the core, is a mouse model corresponding to MS. Several groups have observed that estriol and estradiol are protective against EAE in several genetic backgrounds. Expression of Th1 cytokines decreases, and in some cases, expression of Th2 cytokines such as IL-10 increases. Estrogen treatment also reduces the function of dendritic cells and macrophages and expression of inflammatory chemokines, and it increases the number of $CD4^{+}CD25^{+}$ regulatory T cells. These mechanisms, as well as others that have yet to be identified, decrease the number of inflammatory cells in the CNS. Yet estrogens also appear to be neuroprotective in this model. Microglial activation, neuronal cell death, and oligodendrocyte cell death are all reduced, neurotrophic factors are induced, and the number of dendritic spines and synapses are increased in response to estrogen. Expression of TNF, IFN, and IL-2 decreases, and expression of IL-5 increases. Work by two groups using knockout mice has shown that the overall protective effect of estrogen is mediated by ER-α.[40,41] However, this does not rule out the influence of other ER isoforms.

Dr. Voskuhl's group has further examined the anti-inflammatory and neuroprotective effects of the ER using the ER-α agonist propyl pyrazole triol (PPT).[42] PPT works as well as estradiol in protecting against EAE in wild-type mice and mice deficient in ER-β, but not in mice deficient in ER-α, indicating a high degree of specificity for this agonist in a disease setting. Treatment with PPT, like that with estradiol, reduces the number of white-matter lesions and the amount of axonal transaction, consistent with an anti-inflammatory effect mediated through ER-α. Treatment with PPT also decreases the amount of permanent disability-associated neuronal abnormalities in the gray matter. Thus, both estradiol and the ER-α agonist PPT exert anti-inflammatory and neuroprotective effects. However, whether these effects are direct and whether gray matter atrophy can be prevented is not yet clear. Work is under way to assess possible effects mediated by ER-β.

Discussion

The effects of oral contraceptives or non-pregnancy concentrations of endogenous estrogen on MS or EAE are not known. No differences have been observed in ovariectomized mice, patients who have undergone hysterectomies, or postmenopausal women. However, these concentrations of estrogen are much lower than those associated with pregnancy. Clinical trials are under way.

Sex Differences in Inflammatory Responses to Shock/Trauma

Edwin Deitch, M.D., New Jersey Medical School

Systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), and multiple organ dysfunction (MODS) are common occurrences following major shock or trauma. Gut barrier function plays a critical role in these syndromes. Several studies have shown that gut ischemia, which occurs through a shunting of blood from the intestinal circulation to the central circulation, is a better predictor of ARDS and MODS than are systemic hemodynamics. This is consistent with prospective clinical trials in which increased gut permeability predicts the development of ARDS and MODS and early enteral feeding, which improves the gut barrier, decreases the likelihood for infections and the duration of hospital stays for trauma patients. In a review of nine registry-based clinical studies and one prospective cohort study, Ananthakrishnan and colleagues found that seven of the ten studies showed improved outcomes in premenopausal women,[43] which suggests that sex might influence outcomes following major shock or trauma.

There are more bacteria in one person's gut than there are people on earth, and the amount of endotoxin and other bacterial products in the gut is sufficient to kill the host a thousand times over. In the early 1980s, Dr. Deitch's group proposed a paradigm in which loss of gut barrier function allows bacteria to translocate and spread to the lymphatics, resulting in bacteremia and systemic infection. However, this paradigm proved difficult to apply. In recent work, Dr. Deitch's group subjected male Sprague-Dawley rats to trauma and hemorrhagic shock (T/HS) or trauma and sham shock, then collected mesenteric lymph and tested it *in vitro* on endothelial cells and neutrophils or *in vivo* by injecting it into normal rats.[44-47] T/HS and lymph and plasma from T/HS rats induced lung and red-blood-cell injury, neutrophil activation, and bone marrow growth suppression. This was consistent with what was observed in human trauma patients, and the fraction of plasma with biologic activity was the same between rats and humans. These experiments have been repeated in a pig model, where the lymph collected from T/HS males increased the permeability of a monolayer of human umbilical vein endothelial cells and activated rat and human neutrophils.[48] These results support a model of gut-origin SIRS, ARDS, and MODS, in which mesenteric lymph serves as the link between the gut and distant organ injury or inflammation. In this model, gut ischemia results in the production and release of biologically active factors into the lymph vein, and gut-derived factors in lymph lead to distant organ injuries through interactions between neutrophils and endothelial cells. The lymph factors responsible for biologic activity are not bacterial or cytokines, but rather large proteins and lipids that have yet to be identified.

The extent of T/HS-induced organ injury and cellular dysfunction is influenced by sex.[49-52] In the rat experiments, females appeared relatively resistant to T/HS, and unlike T/HS lymph from male rats, T/HS lymph from proestrus female rats did not have a biologic effect, except for mild neutrophil activation. Lymph from castrated males appeared somewhat protective against injury associated with T/HS, whereas lymph from ovariectomized females had the same biologic

activity as that from normal T/HS males. Moreover, the inflammatory response to hypoxia and reoxygenation was reduced in the gut in female rats, compared with that in male rats,[53] and intestinal iNOS activity and plasma NO concentrations were increased in males subjected to T/HS, but not in females.[50] Similar sex differences were observed in pig[48] and baboon models.[54] Prospective clinical trials are under way.

There are some caveats associated with the gut lymph model of gut-origin SIRS, ARDS, and MODS. Sex hormone effects might only be relevant during the early, acute post-trauma period after T/HS, and sex hormone levels change rapidly, both in males and females, following T/HS. Moreover, the effect of sex on clinical outcome following burn injury appears to differ from that following shock and trauma (see Dr. Kovacs' presentation, below), and females become more susceptible to injury as hormone levels change. Future work should examine the temporal response of sex hormones to T/HS, the mechanisms by which these hormones influence cellular response to insult, and the potential role for sex hormone modulation in acute clinical conditions.

Discussion

The biologic factors in mesenteric lymph from male rats subjected to T/HS have yet to be identified. They are not traditional cytokines, polysaccharides, or endotoxins. Because work on the gut lymph model is still evolving, it is not clear whether inflammatory bowel disease or other chronic disease affects the ability of gut lymph to induce injury.

Treatment of Burn Injury: Sex Effects

Elizabeth J. Kovacs, Ph.D., Loyola University

Of the more than 100,000 patients admitted to hospitals for burns, 80% of them are male and 50% have ethanol in their blood at the time of admission. In most cases, ethanol exposure is acute rather than chronic. Patients with ethanol in their systems face increased morbidity and mortality associated with these injuries. They are twice as likely to experience serious complications and 60% more likely to undergo surgical procedures, require more rigorous antibiotic therapy, stay in the hospital longer, and succumb to their injuries. Yet regardless of alcohol exposure and sex, a similar cascade of inflammatory and immune events occurs in response to a burn. This cascade is initiated by an increase in the expression of pro-inflammatory mediators, including IL-6, followed by a decrease in T-lymphocyte activation and IL-2 expression and a shift in the phenotype of cytokines produced from Th1 to Th2, as assessed by decreased expression of IFN-γ and increased expression of IL-4. Because of this, burn patients become more susceptible to infection. Evidence suggests that high levels of the pro-inflammatory mediator IL-6 for a prolonged period is associated with poor prognosis.

Mice given ethanol before a burn injury experience immunosuppression to a greater degree than do those experiencing ethanol exposure or burn injury alone. These mice show decreased survival following bacterial challenge, decreased leukocyte chemotaxis to the skin, increased leukocyte chemotaxis to the lung, and diminished type hypersensitivity (DTH) response. Lymphocyte proliferation and IL-2 production are decreased, and the levels of several pro-inflammatory mediators, including IL-1, TNF-α, IL-6, and prostaglandin E_2 (PGE_2), are increased. This mouse model is therefore a useful one in teasing out sex differences in the response to burn injury. Dr. Kovacs' group has investigated the use of hormonal approaches to reduce the amount of aberrant pro-inflammatory cytokine production following burn injury and

to restore immunity in a mouse model. One of the hormones under investigation is estrogen, which controls the expression of many cytokines, up- or downregulating them at varying concentrations. Cycling females exhibit a more vigorous cellular and humoral immune response than males do, depending on the stage in the cycle. Proestrus levels of estrogen are protective, whereas testosterone and pregnancy levels of estrogen are immunosuppressive.

In Dr. Kovacs' experiments, male mice were given ethanol and subjected to a scald injury, then resuscitated and treated with 17β-estradiol. Immune responses were measured at various time points after injury. Male mice treated with proestrus levels of 17β-estradiol exhibited a marked recovery in DTH response and splenocyte proliferation. Additionally, in splenocyte proliferation assays, if macrophages were removed or antibodies against CD3 were used to stimulate proliferation, the administration of 17β-estradiol had no effect. When splenic macrophages were removed from mice given ethanol and burn injury, given estrogen *in vivo*, and cultured *in vitro* with LPS, estrogen treatment reduced the increase in IL-6 production by 50%. The observed changes did not result from differences in ER expression; ER levels were the same across all treatment groups. Although treatment with 17β-estradiol did not affect expression of nuclear factor kappa B (NFκB) or inhibitor of NFκB (IκB) or phosphorylation of IκB overall, it did decrease the level of NFκB activation in macrophages from mice exposed to ethanol and burn injury. In addition, mice given the combined insult of ethanol exposure and burn injury that were treated with 17β-estradiol experienced improved survival following bacterial challenge, to the level observed in mice subjected to burn injury alone. Thus, treatment with 17β-estradiol improved immunity in male mice exposed to ethanol and burn injury. Hormonal approaches that failed to improve immunity in these mice included the testosterone derivative dehydrotestosterone (DHT), the androgen receptor (AR) antagonist flutamide, gonadectomy, the testosterone and estrogen precursor dehydroepiandrosterone (DHEA), and the ER antagonist ICI 182,780.

On the basis of these results, one would expect that female mice exposed to burn injury would have a better prognosis than males. However, the 10-day mortality following burn injury is similar between males and females, and the mortality following a combination of ethanol exposure and burn injury is more marked among females. Moreover, clinical evidence indicates that women do worse after burn injury than men do (Loyola Burn Registry).[55-58] Although these studies were limited to moderate-sized burn injury—mortality following large burn injuries was similar between men and women and for postmenopausal women—the increased mortality among female burn victims suggests decreased immune response. Dr. Kovacs' group explored potential differences in immune response using the same injury model described above. At 48 hours following exposure to ethanol and burn injury, both males and females were immunosuppressed, as indicated by a decrease in the DTH response. At 7 days, however, female mice exposed to ethanol and burned remained immunosuppressed, whereas males did not. The DTH response was attenuated, splenocyte proliferation was suppressed, and IL-6 was elevated more dramatically among these females. Ovariectomy improved immunity, as shown by restored DTH response, greater mitogen-induced splenocyte proliferation, and reduced aberrant expression of IL-6.

On the basis of these results, treatment with low, proestrus levels of estrogen can improve cellular immunity in male mice following ethanol exposure and burn injury, via control of IL-6

production. Although the IL-6 promoter has no estrogen responsive elements, estrogen modulates its production through interaction of the ER with transcription factors such as NF-κB and Ap-1. Thus, sex-specific treatment regimens should be considered for burn patients.

Alterations in estrogen levels are somewhat confusing. Ethanol exposure decreases endogenous estrogen levels, whereas burn injury increases it. Both males and females experience an increase in estrogen following burns. In males this increase occurs at a moderate level and is transient, but in females the level of estrogen reaches pregnancy levels and is maintained for a much longer period of time: weeks to months, depending on the extent of the injuries. It is not clear what the combined effects of ethanol exposure and burn injury would be. More work is needed to determine the effects of hormones on innate immunity in healthy participants; how sex and/or gonadal steroid hormones alter inflammatory and immune responses after injury; and how aging, menopause or andropause, and conditions associated with aging, such as diabetes, affect these responses.

Discussion

The relationship between IL-6 and estrogen is somewhat complex. *In vitro,* small but highly concentrated doses of estrogen can affect IL-6 activation and coactivator binding to NFκB, yet *in vivo,* as shown by Dr. Kovacs' group, IL-6 increases in response to estrogen treatment. This bimodal activation of IL-6 may result from differences in mechanisms and related receptors, or it may represent indirect effects.

The data indicating early protection from injury in females is consistent with other work showing that females are protected from injury to the gut and lung during the first 24 hours following burn injury. The mechanism for these effects is not clear. These sex differences might reflect differing cell populations activated early and late, differences in bone marrow response, or a combination of hormonal effects and differing cell populations.

Sex Differences in Lung Function and Response to Environmental Agents

Darryl C. Zeldin, M.D., National Institute of Environmental Health Sciences, NIH

Asthma is a chronic lung disease characterized by intermittent bouts of wheezing, coughing, reversible airflow obstruction, airway inflammation, and bronchial hyperresponsiveness. It is of enormous public health importance because of its prevalence of about 11%, its mortality rate of more than 5,000 deaths per year, and its associations with hospital admissions, emergency-room visits, poorer quality of life, and high costs. On the basis of data from the Third National Health and Nutrition Examination Survey, clear sex differences in asthma prevalence exist. Among children, prevalence is higher among males than females, but around the age of 20 years and in most of adult life, prevalence is higher among females. At older ages, the trends change again, and prevalence is higher among males.

Several studies have suggested that sex hormones influence asthma. Severity and bronchial hyperresponsiveness vary during the menstrual cycle;[59] and half of all female patients admitted to the hospital for asthma are admitted during the premenstrual phase.[60] Use of oral contraceptives can attenuate the effects of the menstrual cycle on lung function,[61] and postmenopausal women receiving HRT experience less airway obstruction than those not taking HRT.[62] In addition, females with Turner's syndrome, which is characterized by low circulating

levels of estrogen, experience increased airway obstruction that can be alleviated with estrogen treatment.[63]

Dr. Zeldin's group has used mouse models to further explore these differences. Using an invasive measure of lung function, they found that response to methacholine, an airway-restrictive agent, is more enhanced in males than in females, as shown by decreased Newtonian and tissue resistance and decreased elastance.[64] This group also found that bacterial LPS, which triggers an inflammatory response in the lung, induced an exaggerated hypothermic response in males, compared with that seen in females. Inflammatory response in males was also exaggerated, as shown by the presence of neutrophils in bronchoalveolar lavage (BAL) fluid, increased histopathological scores, and increased cytokine response. Ovariectomy had no effect on hypothermic response in females, but castration abolished the inflammatory response in males, as demonstrated by attenuated cytokine response. Females treated with the androgen DHT exhibited exaggerated response to LPS, as shown by increased production of TNF-α, increased numbers of cells and neutrophils in BAL fluid, and enhanced hypothermic response. Thus, airway responsiveness is influenced by sex, and the exaggerated response seen in males is mediated in part by androgens.

Despite the apparent sex differences in this mouse model, the physiologic roles of sex hormone receptors in the adult lung are not known, although knockout mouse models for both ER-α and ER-β are available.[65,66] Both ER-α and ER-β are expressed in the lung,[67] and although ER-β is a critical regulator of alveoli formation and surfactant homeostasis,[68] both receptors are required for alveoli formation in mice.[69] How the expression and function of these receptors correlate with the exaggerated airway response observed in male mice exposed to methacholine remains to be determined.

Discussion

No work has been done to compare estrus cycles and differences in responsiveness in females.

Periodontal Disease Inflammation and Obstetric Complications

Steven Offenbacher, D.D.S., Ph.D., University of North Carolina, Chapel Hill

Periodontal problems are common in pregnancy and are more prevalent than all other obstetric and sexually transmitted infections combined. Several studies have shown that maternal periodontal disease significantly increases the risk for preterm births and preeclampsia,[70] and active periodontal progression increases the risk independently of other factors such as socioeconomic status, smoking, or drug abuse. Organisms living in the oral biofilm, a slime layer that forms on the teeth and includes clusters of several different organisms,[71] create an ulceration of the epithelium, gain access to the system circulation, and are disseminated through the liver and to the fetus, resulting in placental and fetal exposure to microbes, poor placental perfusion, and inflammation. These events ultimately give rise to fetal growth restriction, preterm delivery, and neonatal morbidity. Exposure to oral organisms is highly associated with admission to and lengthy stays in neonatal intensive care units, and because low birth weight has been associated with several adverse outcomes, including neurological deficits, intrauterine exposure to oral organisms can contribute to lifetime disabilities and impairments.

Most common among these organisms is *Campylobacter rectus* (*C. rectus*), a commensal inhabitant of the oral cavity in humans. *Campylobacter* species have been associated with abortion, premature labor, and severe perinatal infection in humans and induce abortions in horses, cattle, and sheep. In mice, exposure to *C. fetus* or *C. jejuni* during pregnancy results in adverse pregnancy outcomes and fetal complications.[72] Fetal exposure *C. rectus* is found in approximately 28% of all pregnant women and in more than half of infants at 32 weeks of gestation.[73] Although exposure to *C. rectus* increases the risk of premature delivery, high levels of CRP, the oxidative stress marker 8-iso, TNF-α, and PGE_2 also increase this risk exponentially.

Dr. Offenbacher's group has studied the effects of infection by *C. rectus* in a mouse model.[74,75] In this model, a sterile chamber was implanted in the dorsolumbar subcutaneous tissue and inoculated with *C. rectus*, then mice were bred and subjected to a timed challenge. Exposure to *C. rectus* increased the frequency of resorption and fetal growth restriction and decreased neonatal survival. The weight of growth-restricted fetuses was reduced by 27%, compared with controls. Mild inflammation was observed in the decidual zone of the placenta, and development of the labyrinth zone decreased in response to challenge with *C. rectus*. Expression of Th1 cytokines increased, and that of Th2 cytokines and TGF-β decreased. To further explore the relationship between inflammation and growth restriction, Dr. Offenbacher's group conducted microarray analyses on placenta from challenged mice. These analyses revealed that 202 genes were differentially expressed. The majority of these genes were downregulated, including genes encoding placental growth factors, suggesting that placental development is suppressed in response to *C. rectus* exposure. Among the genes significantly downregulated was *Igf2*, an imprinted gene that is critical for somatic growth of the fetus.

Imprinting is a process in which DNA methylation at CpG sites converts biallelic expression to monoallelic expression. CpG doublets, which are often present in high-density regions of selected genes, are highly prevalent among master control genes that regulate transcription, cell growth and differentiation, morphogenesis, and neoplasm development. DNA methylation is often associated with gene silencing through alterations in chromatin structure, and it is conserved following DNA replication, resulting in a stable, somatic, heritable DNA modification that does not involve a sequence change. This process generally increases throughout the life span and controls the differentiation of $CD4^+$ T cells.

A comparison of murine imprinted genes and array signals showed that among 21 shared genes, 9 were significantly downregulated, indicating that in addition to suppressing growth and differentiation factors in the placenta, exposure to oral organisms also enhances imprinting. Bisulfite sequencing of DNA extracted from the placenta of organism-challenged mice revealed some sequences in *Igf2* that were hypermethylated as a result of *C. rectus* challenge (in press).

These results are consistent with work by McMinn and colleagues,[76] who reported suppressed placental expression of *Igf2* mRNA in human intrauterine growth restriction. The functional significance of *Igf2* hypermethylation is not known, but it might block an Sp-1 binding element. Moreover, because methylation patterns are heritable in somatic cells, infection-mediated epigenetic alterations could affect the well-being of the offspring.

Discussion

Other placental growth factor genes that were downregulated in response to *C. rectus* challenge have CpG doublets available for DNA methylation, and other bacterial infections, such as *Helicobacter pylori*, have also been linked with increased DNA methylation. Many intrauterine methylations are thought to persist throughout a person's lifetime.

Methylation analyses using human placenta have not yet been done.

SESSION III: CELLULAR AND MOLECULAR MECHANISMS IN INFLAMMATION RESEARCH

Moderator: Susan Kovats, Ph.D., Beckman Research Institute

As shown by the work presented in Session II, sex differences exist in the host response to injury, environmental insult, and chronic disease. The presentations in Session III focused on the cellular and molecular mechanisms by which sex hormones influence inflammatory processes and immune response.

Vascular Cell Signaling by Membrane Estrogen Receptors

Jeff Bender, M.D., Yale University

Although the endothelium has historically been considered a static barrier, recent work has shown it to be highly dynamic. In addition to its barrier function, other roles for the endothelium include regulation of vasomotor tone, anticoagulant and prothrombotic functions, and a role in inflammatory events. Endothelial dysfunction is prothrombic, present in atherosclerosis and hypercholesterolemic states, involved in impairment of vasomotor tone, permissive of leukocyte adhesion and transmigration, and involved in the alteration of barrier properties. Under normal conditions, the endothelium produces large quantities of NO, which inhibits platelet aggregation, monocyte adhesion, and smooth muscle cell proliferation and migration and promotes the relaxation of smooth muscle cells. These functions are also carried out by prostacyclin (PGI2), which is also released by the endothelium. Much less NO and PGI2 are produced by a dysfunctional endothelium, and macrophages and foam cells produce large amounts of superoxides, which quench NO and form peroxynitrite, a toxic agent that damages the vascular wall.

Estrogen can have a protective effect against CVD, as suggested by low rates of coronary heart disease (CHD) in premenopausal women, the narrowing gender gap of CHD after menopause, and the increased risk for CHD in oophorectomized young women not taking estrogen. Estrogen might diminish the degree of monocyte or T-cell adhesion to the arterial intima, an early component of atherogenesis. It also might enhance the release of NO, thus impairing monocyte adhesion, neointima formation, and platelet aggregation. Dr. Bender's group has shown that estrogen treatment of the endothelium prevents cytokine-mediated induction of adhesion molecules and that this protective effect is mediated by ER-α.[77]

Yet there is a conundrum associated with estrogen. Data from *in vitro* and *in vivo* animal studies show a favorable effect in terms of CVD, but clinical trials, such as the Women's Health Initiative (WHI), demonstrate an unfavorable effect. A review by Grodstein and colleagues found a somewhat protective effect in postmenopausal users of estrogen and HRT,[78] which

contradicted earlier studies showing no protective effect as well as the increased incidence of cardiovascular events observed in the WHI. Part of the explanation for this conundrum may lie in the conduct of these studies. Mikkola and colleagues found a large burden of atherosclerosis in ovariectomized primates fed an atherogenic diet.[79] If these primates had been treated with conjugate equine estrogen at the time of ovariectomy, the burden of atherosclerosis was reduced by 70%. However, if the initiation of estrogen treatment was delayed by 2 years, there was no protective effect. Thus, timing may be critical, and the results from the WHI should be considered in this light. In the WHI, the average age at enrollment was 63 years, participants had been postmenopausal an average of 12 years, and many had established, significant vascular disease.

Dr. Bender's group has focused on estrogen effects triggered by the activation of membrane-bound ER. Unlike the traditional nuclear receptor pathway, which is usually delayed, the membrane ER pathway is rapid and induces eNOS through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The resulting release of NO induces the production of cyclic GMP in smooth muscle cells, thereby interfering with proliferation and migration and promoting relaxation. Membrane-bound ER is distributed in a punctate pattern in a subset of endothelial cells and is found in two forms: the full-length 66 kDa ER-α, and a shorter, 46 kDa form that arises from a translation initiation site found in the second exon of the ER-α gene.[80] No full-length ER-α is observed in estrogen-deprived HUVEC. Treatment with estrogen induces some expression of the full-length ER-α in these cells, but the shorter form is predominant. Cardiovascular studies in ER-knockout mice have been carried out by several groups, using the wire loop carotid injury remodeling model, where smooth muscle cell proliferation and matrix deposition is inhibited by 17β-estradiol.[81] Elimination of ER-α or ER-β has no effect, and elimination of both has only a modest effect. The shorter form of membrane ER is present in knockout mice in which exon 1 h as been targeted, but in knockout mice in which exon 2 has been targeted, there is no ER-α protein and vascular response to estrogen is eliminated. Thus, the shorter form of membrane ER plays an important role in vascular tissue.

Work is under way in Dr. Bender's laboratory to further characterize the shorter, membrane-bound form and its role in mediating the vascular effects of estrogen. In addition, new clinical trials are in progress to further clarify the effects of estrogen on CVD. Future work should determine how selective estrogen receptor modulators (SERMs) affect the estrogen responses mediated by membrane ER versus nuclear ER, how the membrane-bound ER is structured, and what the possible roles are for associated proteins in vascular remodeling responses.

Discussion

Work discussed by Dr. Bender has focused only on 17β-estradiol. The effects of pregnancy levels of estrogen are not known. The short, 46 kDa form of the ER most likely is not unique to endothelial cells, although there may be some tissue specificity with regard to splicing and any complexes the ER forms with coregulators. The shorter form of the ER is more efficient at transducing rapid responses and less so at mediating traditional nuclear receptor responses, although it does have that capability. It is not known whether the shorter ER is active in the absence of ligand. The ectodomain of this receptor is also unknown, although its sequence predicts a C-terminal ectodomain. Studies of the PR and GR have also shown receptors of varying sizes specific to the tissue in which they are expressed.

It is not clear whether work has been done to examine the ability of ER-α splice variants to dimerize with ER-β.

Sex Differences in Host Defense Against Pneumonia

Lester Kobzik, M.D., Harvard University

Pneumococcal pneumonia is the most common form of community-acquired pneumonia and is associated with morbidity and mortality in hospital patients.[82,83] This is partly caused by frequent, low-dose infectious challenges to the lungs through nocturnal aspiration.[84,85] Alveolar macrophages play a critical role in lung defense. They are avidly phagocytic and use scavenger receptors to interact with unopsonized inhaled particles and pathogens.[86] Depletion or dysfunction of alveolar macrophages has been linked to the decreased clearance of bacteria and particles.

Using murine pneumococcal pneumonia as a model, Dr. Kobzik's group has found that resistance to pneumonia is greater among females than among males. The percentage of neutrophils and bacteria found in the lung is lower in females, and survival rates are higher. Although these sex differences are only observed at low doses of pathogen, these doses might be relevant to early defense events. Sex differences in susceptibility to *Pneumococcus* have been demonstrated by several groups for many decades, beginning with Weiss and colleagues[87] in the 1970s. Yet further exploration of host defense has yielded confusing results.[88]

In experiments conducted by Dr. Kobzik's group, the number of bacterial cells ingested by alveolar macrophages was identical between male and female mice, but the kill rate was higher among females. This was true also for human alveolar macrophages. The difference in kill rates could not be explained by differences in NADPH oxidase activity, but it was abolished by non-selective NOS inhibition, indicating involvement of the NO pathway in sex differences in host defense. The female advantage could be explained by a role for NOS2, which is constitutively expressed in both murine and human alveolar macrophages. Further examination revealed divergent pathways of host defense, with males relying more on iNOS/NOS2 and females relying more on eNOS/NOS3. NOS3 is activated by the PI3K pathway, which is triggered by macrophage phagocytosis and by estrogen.[89] *In vitro* studies by Dr. Kobzik's group using estrogen treatment of macrophages reproduced the female advantage observed *in vivo*, as well as the abolition of that advantage by treatment with a NOS inhibitor. Consistent with other studies of the endothelium, estrogen-mediated phosphorylation of Akt induced phosphorylation of NOS3, and inhibition of Akt blocked the estrogen-mediated advantage.

Although *in vitro* studies, studies in animal models, and some epidemiological studies indicate sex differences in susceptibility to community-acquired pneumoccoccal pneumonia, no clear data has been generated to date in humans. In addition, it is not clear whether the estrogen-mediated resistance to pneumoccoccal pneumonia will have therapeutic relevance. Models of other problems with innate immunity are needed, as well as studies exploring neonatal host defense susceptibility. More epidemiological studies are needed to further clarify the relevance of the murine pneumococcal pneumonia model.

Discussion

The differences in activity between alveolar macrophages from females and those from males is likely entirely hormone driven. *In vitro*, an advantage is slowly conferred by estrogen treatment on macrophages from male humans or mice. If adoptive transfer experiments were conducted, alveolar macrophages most likely would assume the characteristics of their environment.

Although eNOS is expressed in murine alveolar macrophages, the use of eNOS knockout models does not necessarily prove that eNOS is responsible for the observed sex differences in susceptibility. The use of knockout models in a macrophage-specific fashion could further determine whether eNOS function is important in the lungs, as opposed to the sex differences being related to a vascular issue. However, in the lung, macrophages encounter bacteria first, and it is unlikely that other cells are necessary in the time window studied. Moreover, macrophage-depletion studies support a role for eNOS in the sex differences in alveolar macrophage function.

eNOS levels are identical between males and females within 2 hours of bacterial challenge, and no iNOS is observed. By 24 hours, however, iNOS has been induced. The enzymatic activities of eNOS and iNOS have not been compared between males and females. However, differences in activity may not correlate with differences in macrophage activity. In a different setting, Dr. Deitch's group has demonstrated increased activity for iNOS, but this did not explain the ability of macrophages to kill pathogens.

Estrogen Modulation of Dendritic Cells

Susan Kovats, Ph.D., Beckman Research Institute

Most lymphoid and myeloid cells express ER and AR, pointing to a role for sex hormone regulation in autoimmunity and response to infection. Systemic manipulation of sex hormones or the response to them is an attractive therapeutic approach, but it requires further understanding of the regulation of immune function by sex hormones, both in normal biology and in disease states. Dendritic cells (DC) serve as a bridge between innate and adaptive immunity. They facilitate contact with the antigen and pathogen products; activate antigen-presenting cells through antigen processing and presentation, costimulation, and cytokine production; and activate naïve T cells and $CD4^+$ T helper cell polarization. Although several studies have provided evidence of estrogen action at these different steps, little is known about the effects of estrogen on the DC themselves.

In an *ex vivo* model for DC differentiation, Dr. Kovats' group added granulocyte M-CSF (GM-CSF) to total bone marrow, then monitored cellular development using flow cytometry. In some experiments they removed estrogen from the culture medium and added back lower physiologic doses, and in others they added ER antagonists to standard culture medium. The addition of estrogen promoted the differentiation of DC from bone marrow cells, and at the end of the time period studied, these cells expressed ER but not AR.[90] The addition of testosterone or DHT had no effect. Estrogen-dependent DC expressed CD11c and intermediate levels of CD11b but were negative for Ly6C. These cells also expressed langerin, suggesting that estrogen stimulates the development of Langerhans cells, which are more capable of responding to LPS than the estrogen-dependent DC in these cultures, have a reduced endocytic capacity, and express lower levels of Toll-like receptor 4 (TLR4) but higher levels of CD86 and major histocompatibility complex (MHC) I and II.[91] Efforts are under way to determine the mechanism of estrogen action

on differentiating DC, the role of the ER in DC differentiation and antigen-presenting function *in vivo*, and the role of ER-mediated events in DC function during autoimmunity.

Dr. Kovats' group has also explored the effects of the SERMs tamoxifen and raloxifene on DC differentiation. Both drugs have been compared in breast cancer prevention trials. Tamoxifen exerts antagonist activity in breast tissue but agonist activity in endometrial tissue and bone. Raloxifene, which was developed as a drug targeted toward osteoporosis, exerts agonist activity in bone but antagonist activity in breast and endometrial tissue. Using their *ex vivo* culture system, Dr. Kovats' group found that exposure to either drug inhibits estrogen-mediated DC differentiation.[92] The DC that differentiated were hyporesponsive to TLR4 and TLR9 ligands and exhibited decreased production of MHC-II, CD86, and IL-12. However, tamoxifen exposure did not affect the expression of TLR4 or TLR9. These cells were therefore more likely to exist in a resting state than in an activated one. Future work will explore how SERMs modulate tissue DC differentiation *in vivo*, during infection, and during autoimmunity and how they modulate DC function in innate and adaptive immunity.

In general, more optimal models are needed to further our understanding of sex hormone regulation of immune function. Development of these models should address sex and age, as well as systemic exogenous manipulation of hormones versus endogenous variations, ways to measure those variations, ER deficiency versus systemic or cell-type–specific blockade, disease models with sex-based differences in immune function, other hormones such as progesterone, and the HPA axis.

Discussion

The role of estrogen in the regulation of DC differentiation or function *in vivo* during autoimmunity is still unknown. In young mice prone to SLE, DC are already increased, and in older mice that already have SLE, more DC are observed in females than in males. More work is needed in a gender-divergent lupus model to determine whether this increase is a cause or effect of SLE and whether early stages of pathogenesis involve changes in the number or function of DC, or both.

It is not clear whether the number of Langerhans cells differs between males and females.

Estrogen, Inflammation, and Thrombotic Risk

Virginia Miller, Ph.D., Mayo Clinic

For thrombosis to occur, a mechanical or biochemical lesion must be present in the vascular wall and blood components must interact with that lesion. Components in the blood include soluble factors such as coagulation proteins and cellular factors such as platelets and leukocytes. Platelets represent a dynamic cell system, as their population turns over in about 12 days, and they therefore represent a target for phenotypic modulation. When activated, platelets express membrane adhesion proteins and secrete vasoactive, inflammatory and mitogenic cytokines from dense and alpha granules. Thus, when activated, they can interact with leukocytes and the vascular wall and facilitate the generation of thrombin. Platelet aggregation therefore plays a role in thrombotic risk.

Platelets are produced in the bone marrow from megakaryocytes, which express ER and AR, and they turn over every few days. Dr. Miller's group has examined platelet aggregation in platelet-rich plasma in pigs. As these pigs reached sexual maturity, aggregation decreased in females and increased in males. Ovariectomy in females caused an increase in platelet aggregation, which was reversed by treatment with estrogen or a SERM. Estrogen also modulated the secretion of TGF-β and platelet-derived growth factor (PDGF) by activated platelets.[93,94] Thus, hormones can modulate platelet phenotypes and regulate their aggregation. However, to date, no studies have examined platelet function across the lifespan, and no longitiudinal studies have been done to relate platelet phenotype with vascular lesions in humans.

In another set of experiments, mice were injected with *Escherichia coli* LPS, and platelets were examined before injection and at 7 days afterward, when platelets usually turn over. Platelet aggregation and secretion of alpha and dense granules increased following LPS injection, but fibrinogen binding did not.[95] Thus, a single inflammatory exposure, LPS, can affect systemic inflammatory processes days after the initial event. In preliminary experiments, expression of fibrinogen receptors was examined in early menopausal women in whom platelets had been stimulated with ADP or sTRAP. Although the collective response, illustrated as the percentage of fibrinogen-positive platelets, showed large variability, individual responses to both agonists were consistent, suggesting that a platelet phenotype might be characteristic for an individual.

These results, along with those in mice, point to difficulties in using any one assay to define changes in procoagulative phenotypes. However, individuals could be monitored to determine whether their responses to interventions increase or decrease, and using a panel of assays can provide insight into factors that contribute to procoagulative activity. In addition, the effects of cytokines, hormones, and pathogens can be added to a model examining changes in platelet phenotype and function. Dr. Miller's group has employed this approach by conducting array analysis on some of their samples, and they have found increased expression of PDGF and RANTES expression in females, compared with males. More assays are available to determine what constitutes risk, but more work is needed to clarify the relationships among serology, hormones, cytokines, and platelet characteristics.

A review by Herrington and colleagues found no predictive differences in allele or genotype frequencies to establish thrombotic risk in an analysis of proteins involved in the coagulation cascade.[96] Thus, efforts to characterize thrombotic risk must move beyond these proteins. Dr. Miller's group has examined relationships between the ER and platelet activity. In mice deficient for ER-β, no differences were observed in the phenotype of platelets or reticulated platelets in young mice. However, the percentage of reticulated platelets was increased in aged mice deficient in ER-β. Under these conditions, the platelets observed were positive for P-selectin, fibrinogen, and annexin V expression[97]. In addition the number of mice who died within 24 hours of LPS injection increased slightly for aged mice deficient in ER-β, suggesting that hormonal status influences phenotypic expression in animals with genotypic variation in ERs.

These results provide some insight into thrombotic risk, and they point to a paradigm shift in the way risk is defined. New models should further assess coagulability of the blood, the role of genetics and environment in platelet phenotypes, and new assays to determine individual risk. A randomized trial is under way to translate findings from animal models to humans.

Discussion

At present, the location of ER-β in platelet membranes (alpha, dense granules) is not known, and much remains to be learned about platelet energetics with changes in hormonal status. Phosphorylated antibodies and assays to assess coagulative risk can be added to a model defining thrombotic risk.

Use of Insulin Therapy in Treatment of Inflammatory Diseases

Greet van den Berghe, M.D., Ph.D., University of Leuven, Belgium

Activation of the insulin receptor results in the transduction of metabolic signals, through the action of PI3K, as well as transduction of a mitogenic signal.[98] Type II diabetes occurs when excess insulin bypasses the receptor and directly influences mitogenic events. Under these conditions, the metabolic signal is abolished, and the number of mitogenic complications increases. Critical illnesses such as sepsis, burn injury, and renal failure involve a high degree of inflammation, but they also involve hyperglycemia arising from insulin resistance in the liver and muscle. High levels of insulin-like growth factor binding protein 1 can predict survival during critical illness.[99,100]

Dr. van den Berghe's group has studied the effects of intensive insulin therapy (IIT) on critical illness. They found that IIT controlled blood glucose and improved survival among patients in a surgical intensive care unit (ICU).[101,102] They also found that improvement was maintained long term among patients in a cardiac ICU.[103] In addition, IIT alleviated morbidity associated with brain injury and improved recovery among neurosurgery and neurology survivors.[104] Further analyses revealed that IIT was highly cost effective, saving a median of 2,683 euro per patient.[105]

Illness is more severe among patients admitted to the medical ICU, and more comorbidities, such as HIV/AIDS, cancer, and end-stage diseases, are present. Thus the hospital mortality rate is higher among patients who stay in the medical ICU for a few days. In a study similar to the surgical ICU study, Dr. van den Berghe and colleagues explored whether IIT would prove beneficial to patients in the medical ICU for at least 3 days.[106] IIT significantly reduced mortality among patients in this group, to a degree comparable to the reduction in mortality observed among surgical ICU patients.[101,106] Patients were weaned from the ventilator, moved from ICU, and discharged from the hospital at an earlier time than patients who received conventional treatment. The risk for kidney injury, hyperbilirubinemia, and critical illness polyneuropathy was also reduced. However, IIT did not affect bacteremia or infection.

The IIT-associated cumulative reduction in risk for hospital mortality was 4% among intent-to-treat patients and 8% among patients staying at least 3 days (van den Berghe et al., *Diabetes* 2006, in press). Multivariate logistic regression analysis revealed no sex differences in the efficacy of IIT. Although a recent editorial suggested that IIT only works to offset deleterious effects of feeding, analysis of efficacy among three tertiles of intravenous glucose showed a benefit even among patients who received little glucose.

Although IIT is effective among surgical and medical ICU patients, potential harms exist, such as hypoglycemia and dangers associated with treatment for fewer than 3 days. Brief hypoglycemia increased among patients receiving IIT in the surgical and medical ICUs,[102,106] but

this hypoglycemia did not appear to affect mortality in these studies or in a nested case control study.[107] Nor was mortality increased when IIT was given to surgical ICU patients for fewer than 3 days. Mortality did increase among medical ICU patients, but multivariate logistic regression revealed history of cancer, diabetes, or kidney infection as the reasons for increased risk.[106] Elevated levels of CRP, creatinine, and alanine aminotransferase also were shown as reasons to restrict IIT. Potential harms associated with the high insulin dose used in IIT are under investigation, but thus far, the clinical benefit of IIT in the ICU outweighs the potential harm.

IIT reduces the levels of CRP and mannose-binding lectin.[102] In a rabbit model of acute and prolonged critical illness,[108] neuropathy, kidney function, and mortality increased when glucose levels were high, regardless of insulin.[109] IIT counteracted the effects of glucose toxicity, thereby protecting against mitochondrial damage; increasing activity of the respiratory chain; and suppressing adhesion molecule expression, iNOS expression and circulating NO levels.[98,110,111] Thus, IIT improves outcome by preventing glucose toxicity and its associated effects. This does not rule out direct effects of insulin, but these effects most likely are not independent of glucose control.

Discussion

Further work is under way to determine the role, if any, of glycated proteins in the outcomes associated with critical illness. Although the liver is important in estrogen metabolism, no studies have examined the relationship between this and illness-associated hyperglycemia.

SESSION IV: EMERGING STRATEGIES IN TREATMENT OF INFLAMMATORY DISEASES

Moderator: DeLisa Fairweather, Ph.D., Johns Hopkins University

Presentations in this session explored flaws in current treatments, agents designed to interfere with the activity of inflammatory mediators, and new players in or markers of inflammation.

TNF-Blocking Agents: Raising the Bar for Effective Treatment

Daniel Furst, M.D., University of California, Los Angeles

The autoimmune disease RA is more prevalent among women than men. TNF-blocking agents and other biologics have the potential to radically change the symptoms, quality of life, and outcomes associated with RA, but the mechanisms underlying sex differences in predisposition to RA are not known, and sex differences in response to treatment have not been tested formally. Raising the bar for effective treatment means improving methods to measure response and improving medications for RA, and expectations for successfully treating this disease are high.

Historically, response to RA treatments was measured by counting the number of tender or painful joints or conducting a patient global assessment. In 1993, a panel determined that the number of swollen joints were most important in determining response, followed by physical disability and pain.[112] This made it harder to compare drugs and determine efficacy, because two drugs, for example leflunomide and methotrexate, might look similar in terms of tender joint count, but not in other efficacy end points. In addition, sex differences in response were not tested. van der Heijde and colleagues proposed a system that examined changes in the underlying pathology, with an aim toward preventing structural damage.[113] This scheme involved the

systematic measurement of joints and scoring them based on the amount of erosion observed. On the basis of these scores, an inverse longitudinal relationship between radiographic damage and physical function could be observed.[114,115]

Comparison among RA treatments was also hampered by multiple outcome measures across clinical trials. To address this problem, a group of committed rheumatologists sought to develop a specific set of outcome measures and provide uniformity and standardization. In a consensus statement,[116] the ACR issued criteria that include a decrease in both tender and swollen joints and an improvement in at least three of five measures: patient global assessment, pain, physician's global assessment, validated functional measures, and acute phase reactants. These criteria could be applied to a 20%, 50%, or 70% response, where a 20% response equated to a patient feeling better and a 70% response equated to a patient being close to remission. This approach has allowed comparisons among studies and moved the development of anti-rheumatic drugs forward. On the basis of these criteria, for example, results from a trial of the TNF-blocker adalimumab showed a dose response at 26 weeks.[117-119] In this trial, 50% of patients showed a 20% response and only 10 to 20% showed a 70% response. ACR response criteria were also applied to the ASPIRE study,[120,121] which compared infliximab and methotrexate, and to studies comparing etanercept, infliximab, methotrexate, and leflunomide. Yet although the criteria facilitated comparisons, they were dichotomous and did not relay information about disease activity at baseline.

Investigators in the Netherlands devised the Disease Activity Score (DAS) to assess improvement or response.[122,123] Although this was not an intuitive index,[124] the DAS provided a continuous measure and was incorporated into the European League Against Rheumatism's (EULAR) definition of response.[125,126] This measurement corroborated the ACR Response criteria and moved the field forward as well as allowing better, quantitative comparisons. For example, in a trial comparing methotrexate, sulfasalzine, and a combination of the two, no differences were observed among these regimens, based on the DAS.[127] In addition, on the basis of the area under the curve of improvement in DAS, researchers were able to determine that TNF blockers worked more quickly than methotrexate. DAS could also be correlated with ACR response criteria, as shown in trials of TNF blockers, methotrexate, and other therapeutic agents.[128,129] On the basis of these indices, these drugs inhibited radiographic damage, even though patients did not report feeling better. For the first time, investigators could see that these regimens or combinations caused radiographic healing. Although none of these response definitions incorporated testing for sex differences, multivariate regression analyses showed no obvious differences.

Thus, the development of improved outcome measures improved the ability to develop effective therapeutic agents and, with the development of the biologic anti-rheumatic agents, have "raised the bar," in terms of both expectations and results. The DAS most likely improves the ability to separate drugs, but the ACR criteria form a more intuitive measure of response. Trials are in progress, using these indices to assess sequential monotherapy and varying combinations. Early results show that, on the basis of X-ray progression, DAS, and Health Assessment Questionnaire indices, treatment with a TNF blocker or an aggressive combination with prednisone induces a rapid response at an early time, and patients go into remission more quickly.[130-132] Future work should formally test sex differences in disease activity and response to treatment in the studies

already done and assess the causes of these differences, including genetic, hormonal, and environmental effects.

Discussion

According to some anecdotal reports, some women who have taken TNF blockers for RA and become pregnant reported improvement after delivery. Existing data indicate that the effects of pregnancy on RA vary. TNF blockers are used increasingly in pregnant patients.

Biomarkers that can predict responsiveness to RA treatments are not yet known.

MIC-1, a Poorly Understood TGF-β Superfamily Cytokine with Roles in Inflammation and Cancer

Samuel Breit, M.D., Ph.D., University of New South Wales, Australia

Macrophage inhibitory cytokine 1 (MIC-1), a distantly related member of the TGF-β superfamily of cytokines, is induced in most cells by injury, inflammation, and malignancy, primarily through the action of p53 and EGR-1. It is variably processed, secreted cytokine; unprocessed MIC-1 remains tissue bound through interactions with the extracellular matrix. The H6D polymorphism, which changes a histidine residue to aspartic acid at position 6 in the mature protein, has been associated with altered risk for cancer development and outcome.

Although MIC-1 is not generally expressed in normal tissue, serum levels increase dramatically and rapidly during pregnancy. The MIC-1 protein is highly expressed in the placenta and involved in interactions between the placenta and maternal tissue, and it inhibits trophoblast outgrowth and proliferation. Serum concentrations of MIC-1 have not been associated with preeclampsia or Down syndrome pregnancies, but they are much lower in women who miscarry than those who carry to term.[133] In a large cohort of patients monitored through blood samples and imaging, MIC-1 levels decrease as early as 3 weeks before miscarriage occurs. In a smaller cohort of patients whose pregnancies were proven viable by ultrasound at the time of sample collection, MIC-1 levels were lower in women who later miscarried. No diagnostic test is available to predict the likelihood of miscarriage. Although more prospective studies are needed, serum MIC-1 levels might form the basis for such a test, predicting miscarriage early enough to apply therapeutic interventions. Further study should address the possible involvement of MIC-1 in the immunoregulatory processes associated with placental implantation, growth, and development and roles for MIC-1 in placental health. The high expression of MIC-1 in the placenta, as well as its overexpression by many sex-specific cancers, suggests that this cytokine is influenced by sex and gender and may be regulated hormonally.

Studies have associated MIC-1 serum levels with chronic inflammatory diseases. In a prospective case-control study of women aged 45 years or older with no signs of CVD or cancer, MIC-1 serum levels predicted cardiovascular risk, independently of CRP and other inflammatory factors.[134] Other studies found that MIC-1 expression was upregulated in the hypertrophic myocardium and that MIC-1 protected against cardiac failure in animal models. MIC-1 also has been implicated in RA. In a pilot cross-sectional study in 60 patients, MIC-1 levels were elevated in unselected RA but were much more so in patients whose disease was so severe as to require therapy using autologous bone marrow transplant (Brown et al., *Arthritis Rheum* 2006, in press). At 3 months following transplantation, however, MIC-1 levels had returned to normal. In

addition, the H6D allele of MIC-1 was associated with early-onset erosive disease and severe, treatment-resistant RA, and an algorithm incorporating serum MIC-1 levels and the H6D allele could predict response to hematopoietic stem cell transplantation and the presence of severe disease and erosions.

Discussion

The protective effect of overexpressed MIC-1 against cardiac failure appears to contradict study results showing MIC-1 as a risk factor for CVD events. It could be that MIC-1 is produced to mitigate injury but offers only partial protection. Higher concentrations of MIC-1 could result in improved outcomes, but removal of MIC-1 could be deleterious. Such effects have been found with IL-10 in other chronic inflammatory processes.

Current Lupus Treatments Neither Control Disease Activity or Prevent Organ Damage: The Case for New Approaches

Michelle Petri, M.D., M.P.H., Johns Hopkins University

SLE is a prototypical autoimmune disease characterized by rashes, arthritis, and hair loss. SLE autoantibodies, which appear years before clinical lupus presents,[135] mediate an inflammatory process leading to complement activation and ultimately, to fibrosis. The disease affects about 1.5 million Americans, more commonly among women, African Americans, and Asians. The manifestation of SLE is similar to that for MS, with a relapse-remission pattern that progresses in spite of modern therapy. The most common cause of death among patients with SLE is CVD. Although SLE is more common among women, it is more severe among men. Production of antibodies against double-stranded DNA is similar between men and women, as are low levels of C3 and C4, but men are more likely to experience proteinuria, renal insufficience, renal failure, and myocardial infarction.

The incidence of SLE has tripled since the 1950s to 1970s.[136]

Although the prognosis for patients with SLE has improved since the 1950s, it has remained about the same since the 1980s. In addition, 50% of patients with SLE have permanent organ damage, and for 75% of these patients, organ damage results from treatment with corticosteroids. In a study 498 women with SLE and 2,208 women in the Framingham Offspring Study, myocardial infarction was 50 times more likely among female SLE patients aged 35 to 44 years.[137] Other risk factors included older age at the time of SLE diagnosis, longer SLE disease duration, longer duration of corticosteroid use, and hypercholesterolemia. In Canadian studies, once data were adjusted for traditional CVD risk factors, hypertension was the only risk factor associated with increased atherosclerosis in SLE patients.[138] Elevated LDL was also a factor, but less so. Thus existing therapies do not appear to interrupt the processes leading to accelerated atherosclerosis.

The immunopathogenesis of SLE represents an interlocking circle between innate and adaptive immunity.[139] Plasmacytoid DC produce IFN-α, which activates myeloid DC to stimulate T cells and B cells. Activated B cells produce autoantibodies, which form immune complexes that stimulate plasmacytoid DC and present antigen to T cells. Several biological approaches have been devised to interrupt this circle, but interruption increases SLE patients' susceptibility to bacterial and fungal infections. Thus, how successful these therapies will be is not clear.

Estrogen most likely has a role in SLE. It enhances B-cell differentiation,[140] autoantibody production,[141] and cytokine production. Peripheral blood mononuclear cells are more responsive to estrogen, as shown by increased production of IL-10 and antibodies against double-stranded DNA and decreased apoptosis.[142] In addition, treatment with 17β-estradiol increases production of T-cell activation markers,[143] including calcineurin and CD154. Levels of DHEA are low in women with SLE and further reduced by prednisone treatment. In double-blind, randomized clinical trials, Dr. Petri's group found that treatment with DHEA resulted in sustained prednisone reduction in patients with SLE, although effects were better at higher doses of DHEA.[144] This effect was generalizable, as shown by studies conducted in Taiwan.[145] Results from these studies are consistent with DHEA functioning as an immunomodulator. Other studies have shown that DHEA treatment reduces IL-6 production from bone marrow stromal cells through downregulation of NFκB,[146] increases expression of peroxisome proliferator activated receptor beta,[146] and decreases IL-10 synthesis in patients with SLE.[147]

Dr. Petri's group has carried out clinical trials to determine whether exogenous estrogen is harmful to patients with SLE. In one randomized trial, they found that women receiving HRT experienced an increase in the probability of mild to severe flares, indicating that HRT increases the relapse-remission pattern.[148] Previous studies in both animal models and humans had yielded contradictory data with regard to oral contraceptives,[149-152] although one study found that 43% of women taking oral contraceptive experienced renal flares, suggesting that exogenous estrogen exacerbated SLE. However, most premenopausal women with SLE want to preserve ovarian function, and oral contraceptives alleviate their increased risk for benign gynecologic diseases. In a clinical trial carried out by Dr. Petri's group, no differences in severe, moderate, or mild flares were observed.[153] Thus oral contraceptives do not appear to exacerbate SLE, as long as the women receiving them are not hypercoagulative.

Discussion

Estrogen metabolism in patients with SLE is more likely to use the hydroxylation pathway, although polymorphisms predisposing to this pathway also can be found in relatives who do not have lupus. This predisposition might be exploited with targeted therapies, but so far no beneficial approaches have been identified. For example, certain foods such as broccoli can reverse the effects of the hydroxylation pathway, but they do not provide a clinical benefit. It may be that hydroxylation is important in early pathogenesis but not in clinical disease.

Existing mouse models are not useful in studying SLE, because the disease progresses so quickly to death in mice. Relapse is detected in these mice based on proteinuria and end-stage renal disease. Other murine models are needed in which lupus can be detected based on rash or arthritis.

The literature on the ER appears to conflict with what has been observed in these lupus studies. It might be that genetic predisposition plays a key role in ER effects. Studies of GR in lupus have been done, though they are not reviewed here.

IL-12 and Tim-3 Protect Against Coxsackievirus B3-induced Myocarditis

DeLisa Fairweather, Ph.D., Johns Hopkins University

Chronic disease involves both genetic susceptibility and environmental factors, and once a patient presents clinically, he or she is well past the initiating event. Dr. Fairweather's group studies each stage of chronic disease pathogenesis, using an animal model of coxsackievirus B3 (CVB3)-induced myocarditis.[154] Mice are inoculated with virus, which has been passaged through the heart and is cleared within 16 days. Death is not induced in this model. Both BALB/c and BL/6 mice develop acute inflammation of the heart, but only BALB/c mice progress to a chronic stage of autoimmunity. Necrosis is not observed during the acute stage, but it is seen at the chronic stage.

In this model at acute stages, IFN-γ deficiency results in increased viral replication and decreased production of neutrophils and macrophages, but it does not increase inflammation overall in the heart.[155] Thus, IL-12–induced IFN-γ is needed to reduce viral replication. However, Th1 responses are responsible for increasing acute inflammation of the heart in CVB3-induced myocarditis. Thus treatments to reduce inflammation might increase susceptibility to infection by CVB3 or other organisms. At chronic stages, IFN-γ deficiency results in increased myocarditis and fibrosis, as well as increased production of the profibrotic cytokines TGF-β_1, IL-1β, and IL-4.[156] Dilated cardiomyopathy is observed, in addition to increased calcified fibrotic pericarditis and increased immune complex deposition, particularly along the pericardium. Increased pericarditis is associated with increased degranulating mast cells. Thus, the Th2-mediated response observed in IFN-γ–deficient mice increases the likelihood of chronic heart disease, and treatments to reduce acute inflammation might increase susceptibility to Th2-mediated heart disease.

Males experience both acute and chronic myocarditis, but their response is more inflammatory. The number of macrophages, neutrophils, mast cells, NK cells, and $CD8^+$ T cells is larger in males, and the Th1 response is enhanced. Production of IL-1 and IL-18 increases in the heart, and macrophages and mast cells, which behave as antigen-presenting cells following CVB3 infection,[157] express TLR4, as shown by flow cytometry.[158] In mice deficient in TLR4 and infected with CVB3, the immune cell types in the heart resembled those observed in female mice infected with the virus. In TLR4-deficient mice expression of the inhibitory receptor Tim-3, which prevents apoptosis of Th1 cells during adaptive immune response, was increased on mast cells and on B and T lymphocytes, and apoptosis of $CD11b^+$ cells was observed. On the other hand, if Tim-3 function was inhibited in male mice infected with CVB3 during the innate immune response, inflammation and TLR4 expression increased, and the number of T regulatory cells decreased.[159] The female response to CVB3 infection appears to be more Th2 mediated and fibrotic during chronic myocarditis, although the amount of viral replication is similar to that seen in males during the innate and acute response. In females the number of T regulatory cells increases, and mast cells and B and T lymphocytes express Tim-3. Thus cross-talk by TLR4 and Tim-3 signaling regulate inflammation in the heart: Tim-3 increases CD80, CTLA-4 expression, and regulatory T-cell populations, whereas TLR4 increases production of IL-1, IL-18, and inflammation.

Many chronic diseases involve progression from an acute disease state to a chronic one, but as the above results suggest, differences between acute and chronic disease must be dissected out

for treatments to be effective. The presence of infection also will affect efficacy. Studies focused on estrogens, IL-4, anti-inflammatory therapies, and therapies that block TNF, IL-1, or IFN-γ are under way.

Discussion

Early studies showed that TNF-blocking agents increased mortality in humans with congestive heart failure. However, this was in the case of advanced heart failure; studies have not examined the use of these agents during primary inflammatory myocarditis. More recent data show that TNF-blocking agents might not be as detrimental as once feared.

SESSION V: PANEL DISCUSSIONS AND RECOMMENDATIONS

Moderator: Christopher E. Taylor, National Institute of Allergy and Infectious Diseases, NIH

Session I Discussion and Recommendations

Session Moderator's Summary

The classic definition of inflammation describes a constellation of symptoms. Inflammation itself has been described in a large amount of cellular and molecular detail, but it could be defined differently by different people, depending on the diseases they study. Dr. Libby's presentation highlighted cellular events that may be common among various diseases or disorders, including changes in lipids and cytokines and genetic influences. One must be careful when assessing a potential biomarker, however, as clinically useful biomarkers should be both specific and sensitive. Measuring hs-CRP as a biomarker of CVD relies on a highly sensitive assay, but the cytokine itself is not specifically regulated by a single stimulus and thus lacks specificity.

Knowledge Gaps and Research Needs

- **What is the exact stimulus or trigger for inflammatory processes?**
  Tissue damage, oxidative stress, and aging have been discussed, but no clear-cut trigger has been identified.
- **How is lipid metabolism regulated by hormones?**
- **How do regional differences in adiposity or energetics contribute to the differing burden of the metabolic syndrome?**
- **What are the anatomical differences in vascular lesion formation?**
- **What is the role of sex hormones in regulating HDL production, metabolism, and modification?**
- **What is the role of hormone receptor activation in inflammatory responses?**
- **Are there epigenetic effects of gender that influence sex biases?**

Session II Discussion and Recommendations

Session Moderator's Summary

Presentations during Session II discussed models of sex-related differences in autoimmune disease and responses to injury, environmental insult, and chronic periodontal infection. They also hinted toward the role of varying levels of estrogens in inflammatory processes. The estrogen story is particularly confusing. Results from some studies suggest that estrogen exerts anti-inflammatory effects, but several diseases affect women more than men, and some studies suggest that this increased susceptibility might arise from divergent inflammatory responses. In

addition, varying levels of estrogen, for example levels associated with pregnancy or menopause, can also modulate effects on inflammatory processes.

Knowledge Gaps and Research Needs

- **What are the effects of hormones on innate immunity in healthy subjects?**
  Future studies should address baseline sex differences in the function of innate immune cells and how sex hormones modulate activation of these cells. In addition, the interactions among inflammatory cells and between these and non-inflammatory cells should be explored further. When considering the effects of sex hormones, it should be acknowledged that both estrogen and testosterone circulate in both sexes. How these hormones are balanced throughout the lifespan also should be considered. In addition, differences in the way sex hormones are used by animal models, compared with humans, should be kept in mind.
- **How do sex and/or gonadal steroid hormones alter inflammatory and immune responses after injury?**
  More attention should be paid to the organ systems involved in inflammatory and immune responses, and systemic effects versus organ-specific effects should be teased out. In addition, to estrogen, other hormones such as progesterone, glucocorticoids, and insulin should be studied in this context.

  Efforts to understand sex differences among these diseases might focus too much on sex hormones and not enough on processes that may be encoded by sex chromosomes. It could be that the hormonal differences discussed in these presentations are indirect effects. Future studies should clarify that these effects are directly caused by sex hormones and do not arise from the sex chromosomes themselves. However, it should be noted that the gene encoding the AR lies on the X chromosome, and efforts to rule out sex chromosome effects might be difficult because of imprinting and X-chromosome silencing in women.

  Most studies so far have focused only on specific pathways. Future studies should employ a systems biology approach. For example, expression of the ER is widespread and most likely encompasses a complex network *in vivo*. New tools should be developed, including tissue-specific mouse knockouts in which individual receptors are under temporal control. Some models with inducible ER have already been devised.
- **How do aging, menopause, and andropause affect these responses?**
  Differences among children, adults of reproductive age, and older adults, should be studied, as well as the effects of comorbidities associated with aging, such as diabetes, CVD, and cancer.
- **What are the mechanisms by which hormones influence cellular responses to insult?**
- **What is the temporal response of sex hormones to shock and trauma?**

Session III Discussion and Recommendations

Session Moderator's Summary

Key issues discussed during this session included the roles of ER isoforms and rapid versus nuclear ER signaling; the effects of variable systemic estrogen levels, for example male versus female, non-pregnancy versus pregnancy, and hormone replacement therapy; the effects of ER levels or function and hormone resistance; hormone effects on cellular differentiation, activation, and function; and consideration of all sex hormones and their intersection with the HPA axis.

Several ligands exist for the ER, including estrogens, phytoestrogens, and SERMs. Thus work with animal models must take into account other sources of estrogen or ER ligands. In addition, several ER heterodimers exist, and the differential outcome of ER-α and ER-β ligation depends on ligand concentration, ligand and receptor conformation, and relative expression of the ER and its coregulators.

The development of new treatment strategies must overcome several roadblocks, including hormone interactions, effects during the acute stage of disease versus those during the chronic stage, and ubiquitous expression of ER-α. Clinical observations in genetically diverse populations must be reconciled with the results of laboratory experiments in model systems. Moreover, appropriate doses of estrogen must be determined for effects on a particular tissue, and specificity and potential molecular targets within ER-mediated signaling pathways should be addressed. Future work should also employ global approaches such as microarray and serial analysis of gene expression to generate a more complete picture of the effects of environmental modulation.

Knowledge Gaps and Research Needs

- **Can hormonal effects on immune or other cells account for sex biases in autoimmunity, infection, and recovery from trauma?**
- **What is the mechanism of action of estrogen or the ER in particular cell types?**
- **Which ER isoforms or variants are specific to which cell types?**
- **Which hormones mediate sex-specific effects?**
- **How do sex hormones interact with pathogen responses?**
- **How do symptoms and responses differ by sex in humans?**

Session IV Discussion and Recommendations

Session Moderator's Summary

Current strategies in the treatment of inflammatory diseases include palliative or substitutive approaches such as insulin or thyroid hormone therapy, anti-inflammatory approaches such as the use of corticosteroids, and immunosuppressive approaches such as the use of cyclophosphamide. However, these strategies offer limited efficacy and severe side effects. New approaches should restore immune tolerance and medical benefit, prevent sensitization or neutralizing antibody production, and limit unintended biological effects.

Emerging strategies include:

- Monoclonal antibodies. To overcome the problems of sensitization and cytokine release, one could use humanized monoclonal antibodies and immunosuppressive agents. Engineered Fc regions could be used to avoid side effects and prolong half-life, and engineered variable regions could be used to increase affinity.
- Approaches targeting proteins involved in inflammation and immune response, such as adhesion molecules, cytokines, or cell differentiation antigens. However, at present, these approaches induce severe side effects.
- Soluble autoantigens, delivered nasally or orally to induce tolerance. These treatments are effective if given early, but lag times might increase the risk for disease acceleration or anaphylaxis.

- Soluble receptors and protein fusion conjugates, such as TNF, CTLA-4–immunoglobulin, or IL-1R.
- Cell therapy, such as the use of tolerogenic DC or cultured T regulatory cells.
- Gene therapy, such as the administration of immunoregulatory cytokines such as IL-10.

Knowledge Gaps and Research Needs

- **How can new strategies employ the concept of "outflammation," using chemorepellants to remove cells from places where they normally should not appear?**
- **How do resolvins and the lipoxin pathway, which enhance the resolution of inflammation, be used?**
  Several derivatives have been shown to be enhanced in through aspirin therapy, and some industry-supported work is exploring this further.
- **Can nonsteroidal anti-inflammatory drugs provide a benefit?**
  These drugs might prove problematic because they inhibit side pathways, but recent papers suggest that these drugs block atherosclerosis.

Research Models and Other Research Considerations

Future research exploring sex differences in the regulation of inflammatory processes will require the development of new models and more completely exploit existing ones. For example, periodontal disease in pregnancy, in which a natural biofilm is immediately adjacent to tissue, is a model that offers easy access to tissue and a wealth of information, including microarray profiles, effects of hormonal changes throughout pregnancy, effects of constant infectious challenge, and *ex vivo* responses. The use of LPS as a stimulant in several model systems is advantageous because the pathways involved have been well defined, but it also might be problematic.

The development of new models should account for different sources of estrogens as well as factors involved in the differential outcomes of ER isoforms. Several Institutes within the NIH, including the National Institute on Aging (NIA), are involved in creating mouse models. A series of discussions are under way across several agencies, including the NIH and the U.S. Department of Agriculture, to explore the issue of soy, which contains phytoestrogens.

Differences among animal models and humans should also be considered. For example, studying the effects of hormone resistance might prove difficult, as animal models might not fully recapitulate it, and simply measuring hormone levels might not be useful. Protocols to measure GR resistance functionally in human whole blood *ex vivo*, rather than molecularly, could be adapted for use in studying other hormone receptors. However, it should be kept in mind that hormone receptor levels modulate when cells are kept in culture and that some phenomena might be observed that are not relevant to biologic processes.

In addition, a "Rosetta stone" of effects should be established. For example, what happens in the whole animal following ovariectomy, including effects on various systems, is not known. This problem points to the need for a systems biology approach to more carefully define models and model systems. In addition, investigators can share with other investigators the tissues they do not use, facilitating the beginning of a more complete picture of what happens in a particular model.

Other research considerations include:

- Maintaining a focus on physiology, even while conducting experiments *in vitro* in cell systems and on particular systems of interest.
- Sorting out direct causality of chronic disease, when the initiating event has long passed and tissue response to that event is well established.
- Activation and function of pro- and anti-inflammatory proteins.
- Effects of hormone receptor resistance and hormone metabolism.
- Influence of tissue specificity in inflammatory response.

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