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Radiat Res. Author manuscript; available in PMC 2016 October 05.

Published in final edited form as:

Radiat Res. 2016 August ; 186(2): 99–111. doi:10.1667/RR14436.1.

Understanding the Pathophysiology and Challenges of Development of Medical Countermeasures for Radiation-Induced Vascular/Endothelial Cell Injuries: Report of a NIAID Workshop, August 20, 2015

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Abstract

After the events of September 11, 2001, a decade of research on the development of medical countermeasures (MCMs) to treat victims of a radiological incident has yielded two FDA-approved agents to mitigate acute radiation syndrome. These licensed agents specifically target the mitigation of radiation-induced neutropenia and infection potential, while the ramifications of the exposure event in a public health emergency incident could include the entire body, causing additional acute and/or delayed organ/tissue injuries. Anecdotal data as well as recent findings from both radiation accident survivors and animal experiments implicate radiation-induced injury or dysfunction of the vascular endothelium leading to tissue and organ injuries. There are significant gaps in our understanding of the disease processes and progression, as well as the optimum approaches to develop medical countermeasures to mitigate radiation vascular injury. To address this issue, the Radiation and Nuclear Countermeasures Program of the National Institute of Allergy and Infectious Diseases (NIAID) organized a one-day workshop to examine the current state of the science in radiation-induced vascular injuries and organ dysfunction, the natural history of the pathophysiology and the product development maturity of potential medical countermeasures to treat these injuries. Meeting presentations were followed by a NIAID-led open discussion among academic investigators, industry researchers and government agency representatives. This article provides a summary of these presentations and subsequent discussion from the workshop.

INTRODUCTION

The current geopolitical environment has heightened fears of an imminent mass casualty event from the detonation of an improvised nuclear device (IND) or weaponized radiological material. Further, radiological accidents at Chernobyl (1986), Goiania (1987), Tokaimura (1997) and Fukushima-Daiichi (2011) underscore the pressing need for medical countermeasures (MCMs) to mitigate the complex injuries arising from unanticipated radiation exposure. To this end, the National Institute of Allergy and Infectious Diseases

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(NIAID) has been directed by the U.S. Department of Health and Human Services (HHS) to identify, characterize and develop appropriate MCMs to treat injured victims of a large-scale radiological/nuclear incident. NIAID implemented the Radiation and Nuclear Countermeasure Program (RNCP) in 2004 to accelerate research and product development of radiation MCMs, with the end goal of MCM licensure and purchase for the Strategic National Stockpile.

Licensure by the Food and Drug Administration (FDA) is feasible under the FDA's Animal Rule, and relies on section 21 CFR, part 314, subpart I ("Approval of New Drugs when Human Efficacy Studies are not Ethical or Feasible") and part 601, subpart H ("Approval of Biological Products when Human Efficacy Studies are not Ethical or Feasible") (1). After a decade of intense effort, the FDA approved two MCMs for hematopoietic acute radiation syndrome (H-ARS); Neupogen® (granulocyte colony stimulating factor or G-CSF) was approved in March 2015 (2) followed by the approval of Neulasta® in November 2015 (3). However, several additional radiation-induced injury sequelae in individuals who survive H-ARS can result in multitissue/multiorgan dysfunction and failure, and late effects (4), for which there continues to be no specific treatment modalities. Delayed effects of acute radiation exposure (DEARE) are collectively characterized as a chronic condition manifested in multiple major organ systems of H-ARS survivors, including the gastrointestinal (GI) tract, bone marrow, lung, kidney, heart and brain. The vascular endothelium is an organ central to all tissues and may be important in acute radiation injuries and in DEAREs. Therefore, research focused on the mechanisms of these injury types and the development of MCMs to mitigate them is essential.

The RNCP conducted a one-day workshop on August 20, 2015 to address the current state of the research, MCM development and animal models used to assess and mitigate radiation injury to the vascular endothelium. Speakers included academicians and industry partners (Table 1). The objectives of this meeting were to: 1. To capture the current status of research in radiation-induced vascular injury; 2. Obtain scientific updates from researchers regarding MCM development; 3. Identify research gaps in this specific area; and 4. Provide a platform for an open, informal dialogue among the scientists with expertise in radiation-induced endothelial cell and vascular injuries, and representatives from U.S. government funding and regulatory agencies tasked with facilitating the development of MCMs for FDA licensure. Participating U.S. government panelists at the NIAID-led discussion included: RNCP program officers, the NIAID Office of Regulatory Affairs, the Biomedical Advanced Research and Development Authority (BARDA), the National Cancer Institute (NCI), the Department of Defense, and from the FDA, members of the Counterterrorism and Emergency Coordination Staff (CTECS), the Center for Drugs Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER). Discussion topics centered on: 1. Identifying research gaps in the development of MCMs to treat vascular-endothelial injuries resulting from radiation exposure; 2. Understanding the complexity, progression, biomarkers and fate of the irradiated vasculature; and 3. Providing general guidance on NIAID requirements for investigators addressing radiation injury to the vascular endothelium, in the context of a radiation incident. This report summarizes the primary approaches discussed during the meeting.

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Background on Vasculature Biology

Radiation-induced damage to various organs/tissues, either in the context of a partial- or total-body irradiation (TBI), is accompanied by perturbations of the vascular endothelium. Four years after the discovery of X rays, Gassmann (5) published his findings on injury to the endothelium in irradiated skin. Historically, after irradiation, different tissues present with histological evidence of vascular/endothelial damage, lending credence to the pivotal role of vascular injury in tissue toxicity (6). Endothelial damage and subsequent progressive changes in the vasculature can contribute to chronic lesions in lung, liver, kidney (7, 8), heart and brain (9). Exposed populations that survive initial irradiation, whether during radiotherapy or a nuclear incident, are at risk of developing multiple organ dysfunction syndrome (MODS) associated with progression of vascular dysregulation. Therefore, the radiobiological response of the vascular network, which is still not completely understood, is of major importance for the medical management of radiation injury.

The endothelium is a monolayer of endothelial cells (ECs) lining the lumen of all blood vessels and is therefore present in every organ, with the exception of the lens and the cartilage. The endothelial surface area in an adult human is composed of approximately 1–6 $\times 10^{13}$ cells, weighs approximately 1 kg and covers a surface area of approximately 4,000–7,000 m^2 (10). Initially regarded as an inert “tube” lining of the circulatory system, providing a conduit for transport of various substances within the bloodstream, the dynamic and tissue-specific nature of the vasculature endothelium continues to be of great interest, specifically in relationship to its role in the pathologies of radiation-induced injuries.

Juxtapositioned between the flowing blood and the vessel wall, the endothelium plays a key role in vasoregulation, homeostasis and in selectively controlling the traffic of choice hematopoietic cells and nutrients. Descriptions of heterogeneity of the EC phenotype, differences in function, interactions and cell communication are detailed in recent reviews (11, 12). As an active paracrine, endocrine and autocrine organ, the endothelium regulates vascular tone and blood flow, and maintains vascular homeostasis by releasing vasodilators such as nitric oxide (NO) and prostacyclin, as well as vasoconstrictors, including endothelin, thromboxane and platelet-activating factor (PAF). In addition to promoting vasodilatation, a healthy endothelium has antioxidant, anti-inflammatory, antiatherogenic, anticoagulant and fibrinolytic effects (13). Further, the EC surface consists of a layer of surface glycoprotein (glycocalyx) that provides not only a local charged barrier to the trans-endothelial migration of blood cells and plasma proteins under normal physiological conditions, but is also very metabolically active (14).

The Role of the Vasculature in Past Radiological Incidents

The vascular endothelium is intimately linked to both early and late radiation pathologies affecting several major organs. In the aftermath of the Hiroshima and Nagasaki atomic bombings, it is estimated that 60–100% of the casualties had evidence of hemorrhage at death (15). In survivors who developed late injury, the vascular tissue was involved in tissue damage, as is evident from studies on cerebrovascular disease (16), cardiovascular diseases (17, 18), chronic kidney disease (19) and gastrointestinal diseases (4). Of the 28 people who died within 98 days of the Chernobyl criticality incident, deaths were attributed to skin,

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gastrointestinal and lung reactions, but most deaths were characterized by circulatory problems with high incidence of edema and focal hemorrhages (20). After the Tokaimura incident, Akashi (21) discussed the possible role of inflammation and hemorrhage in multiple organ failure (MOF). In an elegant review of 110 case histories of radiation accidents spanning from 1945 through 2000, the authors analyzed MOF after TBI and stated, “It also became clear that the symptomatology of organ system involvement could be traced not only to the pathophysiology of the rapidly turning over cell renewal systems but—of equal or more importance—to the vascular system and specifically, to the endothelial components” (22). Although vascular injury is recognized as a key component in addressing radiation-mitigation strategies, the cellular, molecular and systemic mechanisms involved in the pathologies of MOF are still obscure, and therefore require tremendous efforts to elucidate the pathways and to develop MCM strategies for the vascular endothelium.

Pathophysiology of Radiation Injury to the Vascular Endothelium

The microvasculature response to radiation can be classified according to acute, delayed and late effects, which contribute to the initiation, progression and maintenance of damage, both to the vasculature as well as the organs and tissues associated with it. The primary target of radiation injury to the vasculature is the endothelial cell. The acute phase of damage occurs within hours to weeks postirradiation, and is characterized by endothelial swelling, vascular permeability and edema, lymphocyte adhesion and infiltration and apoptosis (23). The denudation of ECs is followed by loss of barrier integrity and changes in permeability of the vessel. This phase is often accompanied by inflammation and migration of leukocytes and platelets as well as cellular debris with fibrin deposition, micro-thrombi formation and edema (Fig. 1). Later vascular effects occur weeks to months postirradiation and include capillary collapse, thickening of basement membranes, scarring and fibrosis, telangiectasia and a loss of clonogenic capacity (24).

Pharmaceutical strategies to treat vascular injury often assume intervention in one or more of the pathways from onset of insult to fibrosis/major morbidities. Amid this complex multitude of cross-talk and interactions, key processes and pathways emerge as potential targets. Endothelial apoptosis is the heralding event in injury to the vasculature, accompanied by production of short and long-lived reactive oxygen species (ROS) and inflammation. The smaller vessels occlude due to swelling of the ECs, and activation of infiltrating granulocytes and platelets result in formation of microthrombi and fibrin deposition, all of which are accompanied by unique signatures of biochemical and/or signaling molecules (6). Although early endothelial and accompanying events precede late tissue damage such as fibrosis, the critical roles of key players and pathways involved in the late effects are as yet unclear.

Meeting Presentations Overview

The NIAID-sponsored, one-day workshop consisted of three sessions:

1. Understanding Radiation Injury to Endothelial Cells;
2. Identifying Pathways to Target Medical Countermeasures Development;
3. Identification of Medical Countermeasures Targeting Endothelial Injury.

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This report summarizes the presented data, hypotheses and key discussion topics from the workshop. However, it is not intended to constitute a comprehensive review of radiation-induced vascular injury and all the possible mitigation strategies. Where prepublication data are mentioned below, the presenter's name is provided in parentheses. Additional articles on the science presented at the meeting are included in this issue (Radiat Res, 186.2, 2016).

Session 1—In Session 1 of the workshop, speakers focused on describing: 1. Major pathways in radiation-induced endothelial injuries; 2. The role of vascular injury in delayed organ toxicities; and 3. Expression of cell surface markers as targets for MCMs.

Dr. Martin Hauer-Jensen described the role of radiation-induced injury to the ECs in gastrointestinal acute radiation syndrome (GI-ARS). GI-ARS, a sub-syndrome of radiation lethality, is attributed to the radiation sensitivity of the microvascular EC (23). The sensitivity of the enterocytic endothelium microvasculature is likely related to a stress-related surge of acid sphingomyelinase (ASMase) in ECs. Endothelial dysfunction, marked by a loss in thromboresistance and increased inflammatory markers, inhibits the recovery of the villus epithelium and leads to the breakdown of the epithelial barrier (25). These pathologies are mediated by thrombomodulin (TM), a transmembrane glycoprotein regulator of thrombin function, located on endothelial microvasculature cells. TM reduction appears to result from direct oxidative damage and a genetic downregulation of TM by inflammatory stimulus including, IL-1β, TNF-α and TGF-β (25–28). Reduction in TM levels causes over-activation of cellular thrombin receptors, and decreased activation of protein C, a plasma protein with anticoagulant, anti-inflammatory and cytoprotective functions. The presence of inflammatory proteases promotes ectodomain shedding and the ensuing breakdown of the vasculature (25, 29) (M. Hauer-Jensen).

Under normal conditions, TM removes thrombin from circulation by forming a binding complex; consequently, with thrombin unregulated, thrombin-dependent cellular receptors are hyperactivated. In addition, thrombin loses its ability to activate protein C (25, 30). Together these underlying mechanisms lead to a fibro-proliferative response and contribute to delayed intestinal dysfunction as well as the subsequent inactivation of endothelial cell membranes (25).

Altogether, TM and its related players in radiation enteropathy present a very attractive target for intervention strategies, such as administration of exogenous TM or activated protein C (APC), restoration of TM-protein C system or inhibition of downstream effectors of TM dysregulation. For instance, $TM^{-/-}$ mice demonstrated increased mortality after TBI. However, survival was significantly improved by administering gamma-tocotrienol (GT3), an unsaturated analogue of tocopherol, prior to irradiation. Tocols have antioxidant properties that may inhibit the enzyme hydroxyl-methyl-glutaryl-coenzyme A reductase (HMGCR). The inhibition of HMGCR leads to an upregulation of TM *in vitro*, protecting cells against radiation (31) (M. Hauer-Jensen). Furthermore, the GI tract can also be protected from radiation injury by endothelium survival factors such as vascular endothelial growth factor (VEGF), acidic and basic FGFs and interleukin 11(IL-11) (32–34).

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Thrombomodulin and its modulation by irradiation are also involved in delayed response of intestinal radiation toxicity. Late vascular/endothelial tissue damage can occur several weeks to months postirradiation and fibrosis is considered an irreversible damage. These late effects are characterized by microvessel collapse, thickening of the basement membrane, and persistence of an activated, procoagulant endothelial phenotype, which is ultimately senescent and can result in fibrosis. Telangiectasia and sclerosis result from capillary dysfunction due to loss of endothelial and smooth muscle cells, and are accompanied by vessel wall thickening, and enlarged ECs. Fibrosis, a common feature in late tissue damage, has its origins in the primary damage to ECs. Endothelial cell death and denudation is followed by perivascular edema, changes in the vessel wall and fibrin deposition. A “Model of Mechanism of Self Perpetuation” for fibrosis in radiation-induced enteropathy has been proposed in which radiation injury to the ECs leads to increased levels of thrombin and inflammation mediated by TGF-β and TNF-α signaling (M. Hauer-Jensen).

Researchers at Tufts University School of Medicine have attempted to elucidate the differences in the mechanisms of damage to normal tissue, including the endothelium, in response to different qualities of radiation, protons and gamma rays. Radiation qualities elicit varying EC injury, vascular and remodeling responses. In fact, proton and gamma irradiation have produced contrasting effects with regards to angiogenesis (35). These studies present a very insightful overview of response, and a means to modulate vascular injury. Because there are considerable similarities between radiation-induced systemic response and the biological processes found in aging, such as angiogenesis and immunogenicity (L. Hlatky), the researchers identified a hub signaling molecule, TGF-β1, which is modulated with age and a prominent player in VEGF-mediated angiogenesis response.

Apart from the initial DNA damaging effect on the endothelium, radiation also promotes ROS, tissue and systemic senescence (36, 37). The accumulation of this damage leads to the dysfunction of stem cells and the onset of vascular endothelial cell senescence. The damage continues as senescent cells secrete inflammatory cytokines, proteases and other molecules that disrupt the normal tissue microenvironment (38, 39). Cell senescence has been associated with various delayed pathologies including atherosclerosis, osteoarthritis and chronic lung disease (40). In contrast, senescent cells have also been shown to secrete factors such as senescence-associated secretory phenotype (SASP) that mediate wound healing and tissue repair (40). While the roles of senescent cells are complex and conflicting, *in vivo* clearance of senescent cells has demonstrated a delay in aging-associated disorders (41). In fact, senolytic drugs were shown to significantly improve vascular endothelial function and improve cardiovascular function in mice (42). It is therefore possible that senolytic drugs (e.g., ABT263) represent a “fountain of youth” and can be effective MCMs in treating radiation injury (D. Zhou).

ABT263, an inhibitor of anti-apoptotic proteins BCL-2 and BCL-XL, is cytotoxic to senescent human fibroblasts, human renal epithelial cells and mouse embryo fibroblasts (D. Zhou). In addition, *in vivo* TBI mouse studies revealed that lung tissue expressed the greatest amount of senescent cells, followed by skeletal muscle and brain tissue, whereas liver and heart had a minimal increase in senescent cells. Senescent cells are the “new

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targets" for anti-aging therapy. Since radiation exposure may cause premature aging of the hematopoietic system, novel MCMs to prevent delayed effects via clearance of senescent cells are of significant interest.

In summary, this session noted differences in radiation injury due to radiation quality, identified signal-transduction specific, and highlighted systemic changes in the vasculature that involved inflammation, ROS and accelerated aging. These processes presented unique opportunities to disrupt the injury pathway using a variety of strategies.

Session 2—In Session 2 of the workshop, presenters discussed: 1. EC dysfunction and inflammation pathways after exposure with an emphasis on potential MCM targets against delayed cardiovascular disease; 2. The biology of high, single-dose radiation exposure on the microvasculature; and 3. Radiation responses of ECs that contribute to radiation-induced coagulopathies.

The primary lesion resulting from exposure to ionizing radiation is damage to the ECs, leading to cell death, apoptosis, faulty repair and/or dysfunction. Apoptosis or programmed cell death in ECs is mediated by the sphingomyelinase pathway (43, 44). This ubiquitous pathway links specific cell surface receptors to external stressors, such as radiation, to the nucleus. Sphingomyelin (SM) hydrolysis occurs rapidly after the initial insult via the action of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases, to generate ceramide. Ceramide then serves as a second messenger, leading to apoptotic DNA degradation (A. Haimovitz-Friedman). Bovine aortic endothelial cells are sensitive to radiation doses as low as 1– 2 Gy. The transfer of apoptotic signals from the irradiated cell membrane apparently employs the stress-activated protein kinase/Jun kinase cascade (44), which presents an attractive target for modulation of radiation injury. For instance, basic fibroblast growth factor (bFGF) reduced radiation-induced ASMase translocation and ceramide production, thus decreasing apoptosis. bFGF administered to mice, before and after whole-thorax irradiation, reduced endothelial apoptosis and lethality from radiation pneumonitis (45). Further, the investigators explored the role of VEGF in inhibiting ASMase activity, as well as an anti-ceramide antibody to prevent apoptosis (A. Haimovitz-Friedman). However, selection of appropriate model systems, radiation quality and schedule, dose, as well as timing of administration of anti-angiogenic therapies are crucial considerations to successful intervention strategies.

Work centering on the ASMase pathway has identified a causal link between apoptosis of ECs and radiation-induced GI death (46). The microvascular endothelium is the primary target for radiation injury to the GI tract with the secondary messenger, ceramide, central to injury (R. Kolesnick). The biology of this injury is ascribed to ceramide-driven, vascular dysfunction mediated by oxidative damage and alterations to the fidelity of the repair apparatus. Ceramide activity is facilitated by ceramide-rich platforms (CRPs), which are transient structures that form as lipid vesicles on the plasma membrane of cells in response to increase in ceramide (47). These CRPs afford a scaffold for protein polymerization. Targeting the ceramide pathway using sphingosine-1-phosphate (SIP), a metabolite of the ceramide pathway as well as a known inhibitor of ceramide-mediated apoptosis, has been shown to reduce cell death (43). A variety of other ceramide antagonists that are currently

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being investigated include anti-ceramide antibodies that inhibit apoptosis in cell culture, as well as murine gastrointestinal crypts, while mitigating GI-ARS lethality. Other modulators of ceramide, however, do not protect ASMase$^{-/-}$ mice, underscoring the participation of the ASMase pathway in radiation-induced vascular injury to the GI tract (R. Kolesnick).

Investigators from the University of Texas Health Science Center described the processes of vascular inflammation and potential target for radiation-mediated cardiovascular disease (M. Natarajan). Oxidative stress upregulates numerous pathways pertinent to vascular disease, including matrix metalloproteinases, adhesion molecules, pro-inflammatory cytokines and smooth muscle cell proliferation and apoptosis, while inactivating vasculo-protective nitric oxide (NO). Irradiated vascular cells suffer from persistent oxidative stress and inflammation subsequent to irradiation. Radiation negates EC migration and tube network formation (48), while simultaneously reducing the number of endothelial progenitor cells. Additionally, a fast interaction between NO and superoxide anions results in the formation of peroxynitrite (49). The accelerated degradation of NO and formation of peroxynitrite then reduces the availability of endothelium-derived NO by activation of the IKK/NF-κB pathway after irradiation, resulting in depression of several key aortic vessel functions. Radioprotectants that scavenge free radicals such as the aminothiols, and sulfhydryl containing ACE inhibitors such as captopril, attenuate radiation damage (50), while organic nitrates and sodium nitroprusside that can release NO in the body are severely limited due to their poor distribution and toxicity, and these prophylactics are impractical for use in the event of an unanticipated exposure. Enhancement of the endothelial nitric oxide synthase (eNOS)/NO pathway combined with sepiapterin and simultaneous inhibition of IKK-β represents a potential countermeasure approach for radiation-induced cardiovascular diseases (M. Natarajan).

Radiation-induced coagulopathy (RIC) manifests as a veno-occlusive disease in which the lumens of veins are obstructed, leading to hemorrhage, microthrombi formation and lethality due to changes in homeostasis. These events are particularly evident in irradiated lung (51), liver (52) and heart (53). Recently, it was hypothesized that RIC results in mortality from disseminated intravascular coagulopathy (DIC), especially in ferrets and pigs (54). The hallmark of DIC is an activated coagulation pathway, accompanied by simultaneous clotting and bleeding, with the presence of soluble fibrin in blood. Some researchers hypothesize that depletion of circulating end-cells of the myeloid, lymphoid and megakaryocytic lineages is not the primary cause of H-ARS mortality, which is instead due to the activation of the coagulation pathway and DIC (A. Kennedy). DIC is accompanied by coagulopathies, high levels of circulating nucleosomes/histones (cNH), which are highly toxic and associated with death, and the prevalence of hemorrhages. Treatment of radiation-induced coagulopathy requires a multipronged approach. Since ferrets exhibit highly significant reduction in clotting factors (55), treatment of ferrets with BeneFIX® (recombinant factor IX) can abolish the radiation-induced alteration in blood clotting times. This field is still in its infancy, and preliminary results suggest that clotting factors may serve as effective MCMs in radiation-induced DIC (A. Kennedy). Other interventions include the anticoagulants heparin, dicumarol and warfarin, which spare injuries to the lung, liver and heart, and steroids are also reported to decrease these injuries in the liver (56).

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An increase in plasminogen activator inbibitor-1 (PAI-1) represses the activity of tissue plasminogen activator (tPA) and hence increases the deposition of fibrin in the injured organ (A. Kennedy). Further, ECs release von Willebrand factor (VWF), an acute phase protein that increases in systemic inflammation. VWF is produced constitutively in ECs, and is secreted as an ultra-large form of the molecule (ULVWF), which is rapidly cleaved into smaller pieces. Increased circulating levels of ULVWF increase pro-inflammatory states and are correlated with the degree of organ failure (A. Kennedy). There is evidence that treatment with dextran sulfate (a fibrinolytic stimulator) as well as actinomycin D and ACE inhibitors benefits the management of late tissue injury (56).

Session 3—In Session 3 of the meeting, presenters discussed approaches that show promise for the mitigation of radiation-induced injury to endothelial cells. These approaches included: 1. Targeting of pathways that are known to be activated by radiation exposure; 2. Cellular therapies to reconstitute the damaged bone marrow niche; and 3. Physical approaches to minimize leakiness of radiation-injured blood vessels.

As discussed previously (M. Hauer-Jensen), the TM molecule has an important role in mediating radiation injury to the vasculature. The thrombin molecule itself is a key initiator of tissue repair, activating both anti- and pro-inflammatory signaling. Radiation exposure leads to loss of EC-derived TM, which then limits repair. Accordingly, TP508, a single 23-amino acid natural thrombin breakdown product released after blood clot formation to initiate tissue repair, is being proposed as a novel therapeutic. TP508 has been shown to restore endothelial function through activation of eNOS (57), and to be safe and effective in human clinical trials (D. Carney). Licensure of this drug is being pursued and considered for other indications such cardiovascular repair (58–61), fracture healing (62) and diabetic foot ulcers (63). By increasing eNOS, TP508 minimizes inflammation and interferes with radiation-induced endothelial dysfunction, which leads to an overall significant increase in survival and mean survival time after lethal irradiation in mice (D. Carney). The drug has also has been shown to minimize radiation injury in the GI tract, brain and lungs, all of which are heavily vascularized.

Several laboratories are studying the role of the vascular niche and endothelial cells in radiation-induced injury and repair (S. Rafii). Because hematopoiesis occurs in the bone marrow niche (64), damage to this compartment can have a dramatic impact on the circulating immune cells. The niche involves a complex interplay of bone, immune cells, adipocytes and ECs, the latter of which are known to have a role in both angiogenesis as well as organ regeneration and repair. The vasculature that feeds different areas of the body is composed of ECs that have distinct geographical properties unique to the tissue that they perfuse, such as liver, bone marrow, lung and brain. Within specialized vascular niches such as the bone marrow, the vascular ECs interact with other cells via cell-to-cell signaling and provide additional signaling to other niche components. As a result, the blood vessels in each organ system can have differential radiation sensitivities (65). The vasculature can secrete specific factors at the sites of tissue injury that can influence tissue regeneration, as seen in the lung (66) and liver (67). This effect has also been noted in various tissues, including: pancreatic islet cells (68), adipose stem cells (69), bone (70, 71) and cardiac endothelium (72). Beginning in 2000, Rafii and other groups showed that it was possible to

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produce organ-specific endothelial cells (73–76). These endothelial cells expand hematopoietic stem and progenitor cells by deploying angiocrine growth factors (77) and restore hematopoietic recovery in myelosuppressed mice. Endothelial cells can also be generated from embryonic or induced pluripotent stem cells (iPS) (78). The problem with cells derived from these sources, however, is that they can still retain some of their fetal characteristics and can be unstable. To address this, mature amniotic cells have been reprogrammed into endothelial cells with success (79).

Radiation exposure can cause significant injury to the vasculature in the bone marrow (80). In previous studies, infusion of ECs into irradiated mice was reported to improve survival and lead to renewal of hematopoietic stem cells (81, 82). Mouse brain and fetal-derived ECs were both able to rescue the animals, however, mesenchymal cells were not (J. Chute). In addition, compounds that influence and support endothelial cells represent potential countermeasures to increase tissue survival after radiation injury. For example, pleiotrophin (PTN) administration yields a >25-fold increase in survival of hematopoietic stem cell (HSC)-supportive human ECs (83–85). Pleiotrophin is a 15 kD-secreted, heparin-binding growth factor that is produced by ECs as well as neurons and other cell types. The PTN molecule has been shown to have a role in angiogenesis and organ repair; additionally as an MCM it has demonstrated an increase in survival after radiation exposure in mice (86). Epidermal growth factor, a receptor expressed by hematopoietic stem cells, has also been shown to increase survival after radiation exposure and promote reconstitution of the bone marrow (87).

Other radiation mitigation approaches include targeting repair of the vascular leakiness that results from radiation injury (A. Sung). Breakdown of the EC wall can lead to bleeding in the tissues and edema. In an unirradiated, and otherwise uninjured vasculature, platelets roll along the vessels and interact with the ECs, leading to the release of soluble signaling molecules by both cell types. In turn, these molecules can modify the behavior of the cells. Radiation-induced platelet loss from the circulation leads to thrombocytopenia, and can play a major role in radiation-induced lethality (88). Fibrinogen-coated albumin nanospheres (FCN) have been shown to prevent bleeding from radiation-induced thrombocytopenia and vascular injury (A. Sung). These nanospheres interact with the endothelium to effectively seal up leaks in the vasculature. Even low levels of circulating platelets are able to bind to the FCN at the site of endothelial damage and create a plug. Mechanism-of-action studies, conducted *in vitro* with human umbilical vein endothelial cells (HUVECs), suggest that the FCNs bind only to activated ECs. In an irradiated mouse model, FCNs were shown to increase survival and reduce bleeding time, while having no effect on the level of circulating platelets (A. Sung). Studies were also undertaken using an anti-platelet antibody, (anti-CD42b) to achieve a more severe thrombocytopenia in the mouse model. In these experiments, FCNs also yielded a survival benefit when injected intravenously after irradiation (A. Sung).

Other MCMs (not discussed at the meeting), which have shown efficacy in mitigating injury to ECs and the vasculature include atorvastatin’s protection of HUVECs (89). This statin appears to exert its effect via upregulation of TM and APC levels. Another MCM that appears to have efficacy in reversing radiation-induced vascular changes is gamma-

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tocotrienol (GT3). While providing for hematopoietic progenitor cell mobilization as one of its mechanisms of action, the compound also induces VEGF, which is important for new vessel growth and endothelial progenitor mobilization (90). In summary, in addition to the approaches discussed during Session 3 of the meeting, anti-ceramide antibodies (R. Kolesnick), HMG-CoA reductase inhibitors, growth factors (bFGF, VEGF), exogenous APC or recombinant TM (rTM) administration, pharmacological upregulation of TM, the senolytic drug ABT263 (D. Zhou) and antioxidants represent interesting and novel approaches to the mitigation of vasculature radiation injury.

Biomarkers of Radiation Injury to the Vasculature

Radiation-induced damage can potentially be monitored *in vivo* by harnessing the signals produced by circulating ECs. Mature ECs are released into the circulation due to the normal turnover and renewal of the EC lining of the vasculature. These cells are heterogeneous in size (15–50 μm), carry the markers of ECs (e.g., VWF, CD144 and CD146), but not leukocyte markers (CD45), and serve as noninvasive markers of EC damage and dysfunction. Al-Massarani and colleagues demonstrated a reduction in circulating ECs after acute- and fractionated-dose gamma irradiation in rat peripheral blood, with an incomplete recovery 2 months postirradiaton (91), and in patients undergoing chemotherapy (92, 93). The presence of systemic signalers of processes, such as the oxidative stress pathway and inflammation, is attractive since there is vast potential for a noninvasive or minimally invasive source of biomarkers for early and delayed progression of injury.

Studies indicate that specific panels of growth factors, matrix metalloproteins and DNA damage markers are not only predictive of damage to a specific major organ, but in some cases are predictive of injury progression (lethality versus survival). For instance, IL-1, IL-6 and CXCL1 are markers of pulmonary damage (94), while TM downregulation after irradiation (M. Hauer-Jensen, D. Carney) in addition to inflammatory cytokines such as IL-1, TNF-α and TGF-β are noted in radiation enteropathy (25). Biomarkers may also function as predictors of radiation injury as well as the efficacy of the MCM in mitigating damage, ameliorating major morbidities and rescuing the organism from lethality. For instance, the presence of ULVWF (discussed earlier) is indicative of the degree of organ failure, while the presence of high levels of cNH is often associated with death (95). The presence of fibrin in blood is an indicator of delayed vascular injury. If fibrin decreases in the organ after antifibrin therapy, this indicates the treatment is efficacious. However, the kinetics of the biomarkers, as well as their multiphasic manifestation and intimate cross-talk with other signal transducers, pose a challenge.

Preclinical Models to Study Radiation Responses of the Vascular Endothelium

In vivo models—NIAID supports the development of appropriate animal models in several species, e.g., rodents (mice and rats), canines and non-human primates (NHPs) since the FDA Animal Rule licensure pathway requires that the MCM demonstrate proof of efficacy in two animal species that accurately model the expected human response (1). It is necessary that the mechanism of action of mitigation/therapy closely resembles the action of the MCM in humans. Similarly, selection of the primary end points must have relevance to the end user, and are usually mortality and major morbidities inherent to the vasculature.

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The selection of appropriate models must be dictated by the primary end point and the mechanism of action of the MCM. These models, as well as the pathophysiology of injury, should be clearly linked to the human experience.

The most studied model for evaluating radiation injury to the vasculature is the use of mouse strains such as C57BL/6, CD-1, CD2F1, C3H and BALB/c. These were used to evaluate both direct insult to the vasculature and late effect in major organs where the vascular/endothelial dysfunction mediates the delayed injury. NO-mediated EC dysfunction was studied in C57BL/6 wild-type and knockout strains (M. Natarajan). Others have studied the role of vascular dysfunction in different tissues after irradiation; C57BL/6 and CD-1 in gastrointestinal crypt protection and CNS injuries (M. Hauer-Jensen, R. Kolesnick, D. Carney). To study radiation-induced delayed cardiovascular diseases such as atherosclerosis in wild-type animals, Dr. Natarajan's laboratory repurposed an *in vivo* model with interrupted blood flow in wild-type C57BL/6 mice for radiation response.

Knockout mice were another preferred model among the presenters and were mainly developed to understand the complexity of radiation response. The use of $ASMase^{+/+}$ or $ASMase^{-/-}$ mice demonstrated the key role of sphingomyelin pathway and ceramide signaling in radiation-induced dysregulation of the vascular EC (R. Kolesnick). $TM^{+/+}$ mice demonstrated higher survival than the $TM^{Pro/-}$ (protein C deficient) strain, and B6.129P2-Nos (eNOS-deficient mice) were significantly more sensitive to radiation lethality than wild-type C57BL/6J mice (M. Hauer-Jensen). In addition to eNOS knockout mice, Dr. Natarajan's group cross-bred mice to obtain vessel-specific eNOS-deficient mice (Tie_2-$eNOS^{-/-}$) and also constructed targeted deletion of IKK-β in the vascular endothelium using $IKK\text{-}\beta^{flox/flox}$ mice and Tie_2-Cre transgene to elucidate the contribution of the IKK/NFκB pathway in progression of eNOS and radiation dysregulation in cardiovascular diseases. Since wild-type mice are rarely atherosclerotic, most rodent models of atherosclerosis are transgenic mice (96).

Knockout mice were also used to explain the mechanism of actions of PTN, which expands bone marrow hematopoietic stem cells (BM-HSC). While $PTN^{+/+}$ mice survive lethal irradiation, $PTN^{-/-}$ mice with defective HSPC regeneration succumb to the insult (J. Chute). Mitigation of aortic endothelial function and maintenance of endothelial sprouting have also been demonstrated in irradiated CD-1 outbred mice after MCM administration (D. Carney).

Another group suggested that the mouse, with its LD_{50} far removed from the human LD_{50} and its distinctly different pathophysiology, is not the optimum model to study hemorrhage and coagulopathies that are hallmarks of radiation vascular injury (A. Kennedy). They noted that at the LD_{50}, mouse mortality was predominantly due to infection, and this species did not exhibit hemorrhage, while fatalities of the atom-bomb attacks presented with widespread hemorrhage (97), as did patients from radiation accidents in Norway (98) and Brazil (99). Given these limitations in the rodent model, the investigators cited their work in ferrets and minipigs, and referenced publications by other researchers in dogs and cows, in which radiation response is more closely aligned with the human radiation experience.

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To study radiation-induced cardiovascular disease, Dr. Natarajan's laboratory developed an *in vivo* model with interrupted blood flow, since shear stress under normal flow conditions is considered atheroprotective. Since laminar flow shear that occurs in the long arteries is more atheroprotective, and the disturbed flow in the arterial bifurcation sites and curved arteries are more atherogenic, the group developed a technique to manipulate the arterial flow condition at a specific site and estimate the stimulatory effect of radiation on the development of vascular occlusions in a much shorter time in normal animals. In the GI-ARS model, others have utilized a two-tiered approach to spare hematopoietic deaths, which was comprised of TBI followed by a bone marrow cell transplant, or abdominal irradiation with a partial bone marrow shield (R. Kolesnick).

Few large mammals are currently being studied as models for radiation-induced vascular/endothelial injury. Researchers at the University of Pennsylvania have developed a ferret model to compare solar particle event ionizing radiation effects to gamma irradiation, with emesis as the primary consideration (100). This may represent a potential model for vascular injury, since the irradiated ferrets demonstrated hemorrhage and coagulation abnormalities, very similar to the human response at the LD_{50} dose. A study with the Yucatan minipig (101) indicated that minipigs respond very similarly to irradiated ferrets, while other large animal models, like irradiated canines and guinea pigs (54), provide some background information on hemorrhage for these species and underscore the need for robust research to develop appropriate models for MCM testing for vascular damage.

In vitro models—It is recognized that *in vitro* systems are highly attractive models for elucidating the radiation injury mechanism at the cellular level, to understand the contributions of the cell response to the tissue, as well as the signaling pathways. The investigators provided data generated from studies in HUVEC, bovine aortic ECs (BAEC), WI38 human diploid fibroblasts, renal epithelial cells and mouse embryonic cells. There were considerable differences in the culture conditions depending on the end points under study. For example, while most investigators used standard static culture conditions, one group described an *in vitro* system that simulated *in vivo* flow shear stress (M. Natarajan), while other laboratories used a serum- and growth factor-free culture condition (S. Rafii). At this preliminary stage, allowing the end point (apoptosis, signal pathways, transplantation assays) to drive culture conditions is recommended. Required guidance from regulatory bodies at this juncture can reduce the scope of research and limit the versatile approaches that can potentially uncover novel MCMs.

Irradiation Protocols to Enable Accurate Exposure and Interpretation of Results

As part of the current NIH-wide strategic plan objective to enhance scientific stewardship by ensuring rigor and reproducibility of scientific experiments, there is a renewed need for diligence in the development of irradiation protocols for the study of radiation effects in all organs and tissues (not just the vasculature). This effort requires greater details from the scientific community concerning radiation source, instruments, radiation field (size of the field, volume and uniformity), dose rate, source-to-surface distance, geometry of irradiation and animal orientation, details of most recent calibration and in-run dosimetry details. Animal details such as species, strain, sex, age, vendor source, health report and strategies

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for individual identification are also critical (102) This information helps to ensure that experiments performed in one laboratory can be repeated elsewhere, and that the data generated can be judged as accurate.

Apart from the volume of irradiated tissue/organ/organism, another consideration is the radiation quality used. Radiations of different quality are not merely the “insult”, but differ in modulating vascular response and can inform about the vastly different responses of radiation-induced vascular injury and ensuing unique response. The DNA damage that occurs at the molecular level in ECs is dependent not only on the radiation dose, but also on the radiation source (e.g. proton, gamma, etc.) (103, 104). Proton and gamma irradiation each result in unique changes in transcriptome regulation (105), gene methylation (106) and miRNA biogenesis (107, 108). Using protons and gamma sources, investigators at the Tufts University School of Medicine showed opposite angiogenic responses (L. Hlatky).

DISCUSSION

Panel Discussion Comments

During the meeting, oral presentations were followed by an open discussion among the speakers and a panel of other academicians, researchers and representatives from U.S. government funding and regulatory agencies. The discussions covered the global role of the vascular endothelium in radiation injury, potential targets for MCM intervention, biomarkers of radiation injury in the vasculature and the challenges of developing MCMs for these types of injuries.

Role of the ECs/Vasculature in Radiation Injury

The consensus among the panel was that radiation injury to the vascular endothelium is a complex, highly diverse and convoluted series of events. The panel emphasized that not all ECs are equal; there is significant heterogeneity found among ECs derived from different organs and tissue. Although ECs are present within all vessels, they display different structures and functions depending on the vessel types (arteries, veins, capillary and lymphatics). For example, ECs on the arteries and veins are continuous and robust compared to thinner capillary ECs. The response of ECs to radiation exposure also differs based on geographical location, proximity to organ–organ systems, and association with the vascular bed as well as the size of the vessel. The heterogeneity of ECs on arterioles, venules, microvasculature and capillaries is hypothesized to be due to intercellular differences, transcription factors, signals from major cells within the organs of immediacy and to interactions with the vessel bed. It is assumed that radiosensitive tissues have radiosensitive vasculature while the radioresistant organs (lung, kidney, brain) have radioresistant vasculature. Because the radiation sensitivity of ECs in specific organs is relatively unknown, a systematic approach is recommended to study this scientific gap. Since the normal, unstressed function of ECs in different organs is known to be different, it is not surprising that their radiation responses would also differ.

Differences in response are also dependent on the vascular bed associated with the endothelium, as well as the size, structure and function of the vessel. For example, the APC

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receptor presenting to TM-thrombin complex is found predominantly on the macrovasculature, but not in the microvasculature, a finding which could be attributed to different flow rates in vessels. The structure of the endothelium presents another challenge: while the micro-vasculature, with its single layer of endothelial cells and a few associated pericytes, is more sensitive to radiation damage, the microvasculature, with support structures such as smooth muscle, vascular bed and drainage, is more resistant. The ratio of pericytes to ECs may be another factor in radiation sensitivity within a specific organ. For example, the brain microvascular bed is known to have more pericytes, which could contribute to its radiosensitivity. Other aspects to consider are the inherent radiation sensitivity of cells surrounding/supporting the ECs, cycling rates of different ECs, oxygenation status of organs where ECs are located and how those ECs are grown in culture (e.g., use of serum in cell growth media) and the need for pure cellular populations for study. Therefore, the meeting participants supported the idea that very basic, mechanistic studies were still warranted in this area.

Early and Delayed Expression of Biomarkers of Vascular/Endothelial Injury

Although the pathobiology of vascular injury is not completely understood, an abundance of data from *in vitro*, *in situ* and *in vivo* studies suggest biomarkers that describe specific aspects of the damage, which can lead to a better understanding of the late effects of radiation. The biomarkers can be signal transducers (e.g., cytokines, chemokines, exosomes), cells and cellular products as well as signaling pathways (e.g., apoptosis, oxidative stress, inflammation, TM and senolytic). In addition, given that ECs appear to retain a memory of previous exposure, it is likely that there are also epigenetic signals involved. The pathophysiology of aging is thought to be extremely similar to radiation-induced late effects, and monitoring aging-related markers is one potential approach to quantify radiation-induced vascular injury. The panel also expressed concern about sectioning the injury into early and late effects; in particular, distinctions are needed between the early appearance of biomarkers and biokinetics in relationship to the time of exposure. Tissue damage to the vasculature is an evolving process that can take from seconds to years to manifest in humans. Thus, some of the processes can take more time than others to reach a level of detection.

Pathway to FDA Licensure

Ultimately, NIAID's mission is to facilitate development and licensure of MCMs to ameliorate radiation mortality and reduce the consequences of vascular injury after irradiation. Although, there is an immediate need for very basic research in this field, it is still critical for investigators and organizations to meet with the FDA to consult on all aspects of MCM development. The licensing pathways under section 21 CFR Parts 314 and 601 of the FDA's Animal Rule provide excellent guidance for forecasting appropriate animal models (1). In addition to the Animal Rule and selection of an appropriate animal model, considerable attention must be paid to the exposure protocols, the pathobiology of the MCM and its mechanism of action with bridging studies linking animal data to the human response (109), for successful pathways to licensure. The most optimal regulatory strategy will require continued, repeated and consistent discussions with the funding agency

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and the FDA to obtain feedback, affect midcourse corrections and achieve approval of MCMs for radiation-induced vascular dysfunction.

Identified Research Gaps and Meeting Conclusions

Radiation-induced vascular injury has been a recognized complication of radiation exposure since the discovery of radiation. However, research on the role of the vasculature in radiation injury and the development of MCMs specific to vascular/endothelial damage is inadequate. The topics discussed during this meeting demonstrate the need for early-research-stage investigations of radiation-induced injury to the vasculature. It is clear that research in this area is too premature to consider standardization of *in vitro* and *in vivo* models for studying the phenomenon. In addition, the role of TM in the thrombohemorrhagic imbalance after radiation injury requires additional study, as does distinguishing between responses in lymphatic ECs versus other vessel ECs. Tools continue to become available, including transgenic mouse models, which will simplify the kinds of studies that need to be performed. In addition, a better understanding of the cross-talk between ECs and other niche cells is required. More studies are necessary to understand the pathology of vascular damage, modulation of signaling pathways and the development of MCMs to treat the injury.

Acknowledgments

The authors are grateful to the participants for contributions to the workshop, especially to the speakers for their active engagement on topics of major interest in this research area and those who assisted with the preparation of this manuscript. The authors also thank NIAID colleagues David Cassatt, Carmen Rios and Bert Maidment for their careful review of the manuscript.

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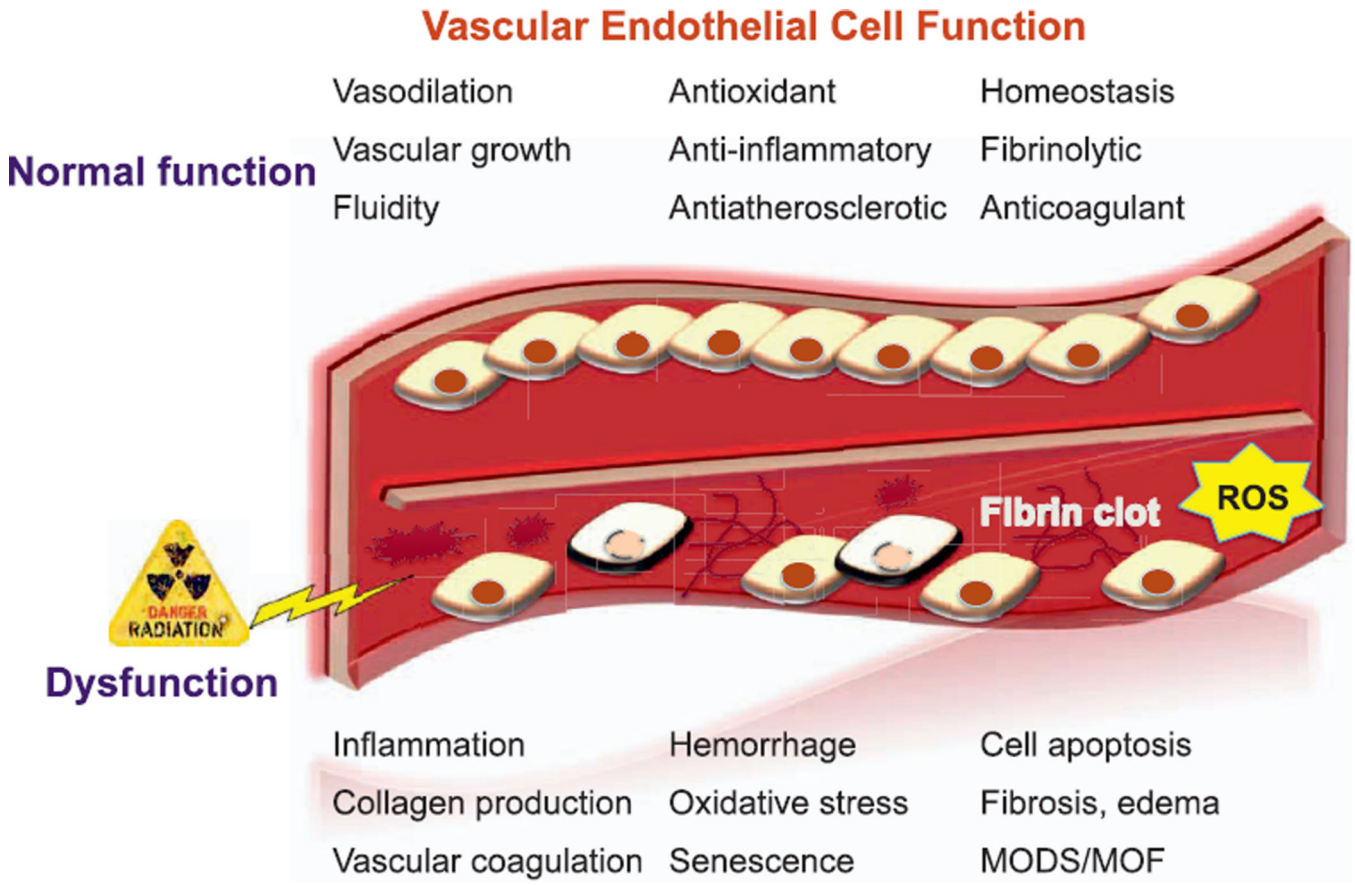

FIG. 1.

Overview of radiation injuries to the vascular endothelial cells. ROS = reactive oxygen species; MODS = multiple organ dysfunction syndrome; MOF = multiple organ failure.

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TABLE 1

Invited Presenters and Areas of Expertise

Name	Affiliation	Expertise
Carney, Darrell	Chrysalis Biotherapeutics, Texas	Thrombin biology, wound healing
Chute, John	University of California, Los Angeles, California	Hematopoietic stem cell regeneration, vascular niche
Haimovitz-Friedman, Adriana	Memorial Sloan Kettering Cancer Center, New York	Microvascular injury, apoptosis, signaling pathways
Hauer-Jensen, Martin	University of Arkansas for Medical Science, Arkansas	Thrombmodulin pathway, drug development
Hlatkey, Lynn	Tufts University School of Medicine, Massachusetts	Proton radiation, VEGF pathway, aging and radiation
Kennedy, Ann	University of Pennsylvania, Pennsylvania	Space irradiation, oxidative stress, radiation-induced coagulopathy
Kolesnick, Richard	Memorial Sloan Kettering Cancer Center, New York	Ceramide pathway, GI-ARS mitigation, platform development
Natarajan, Mohan	University of Texas Health Science Center, Texas	Cardiovascular injury, vascular homeostasis, eNOS pathway
Rafii, Shahin	Weill Cornell Medical College, New York	Stem cell differentiation, angiogenic factors, vascular niche
Sung, Anthony	Duke University, North Carolina	Nanotechnology, platelet biology
Zhou, Daohong	University of Arkansas for Medical Science, Arkansas	Senescence biology, targeting aging pathways, radiation ARS