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MEETING REPORT

Modifying Normal Tissue Damage Postirradiation Report of a Workshop Sponsored by the Radiation Research Program, National Cancer Institute, Bethesda, Maryland September 6–8, 2000

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Late effects that develop in normal tissues adjacent to the tumor site in the months to years after radiotherapy can reduce the quality of life of cancer survivors. They can be dose-limiting and debilitating or life-threatening. There is now evidence that some late effects may be preventable or partially reversible. A workshop, ''Modifying Normal Tissue Damage Postirradiation'', was sponsored by the Radiation Research Program of the National Cancer Institute to identify the current status of and research needs and opportunities in this area. Mechanistic, genetic and physiological studies of the development of late effects are needed and will provide a rational basis for development of treatments. Interdisciplinary teams will be needed to carry out this research, including pathologists, physiologists, geneticists, molecular biologists, experts in functional imaging, wound healing, burn injury, molecular biology, and medical oncology, in addition to radiation biologists, physicists and oncologists. The participants emphasized the need for developing and choosing appropriate models, and for radiation dose–response studies to determine whether interventions remain effective at the radiation doses used clinically. Both preclinical and clinical studies require long-term follow-up, and easier-to-use, more objective clinical scoring systems must be developed and standardized. New developments in biomedical imaging should provide useful tools in all these endeavors. The ultimate goals are to improve the quality of life and efficacy of treatment for cancer patients treated with radiotherapy.

INTRODUCTION

With improvements in health care, including cancer therapy, more elderly cancer patients will be living longer after their treatments. In addition, there are increasing numbers of children and young adults benefiting from recent successes in treating cancer who have the potential for many years of life after treatment. The ability to eradicate a tumor by radiation therapy is limited by the risk of complications in the normal tissues within the treatment field, particularly those termed late effects, developing months to years after treatment. These include fibrosis (which may take several forms), pain, neurological dysfunctions, edema, stricture and obstruction, atrophy, ulceration and necrosis, and fractures. The risk of late effects is a function of the treatment given (radiation dose and schedule, treatment volume, chemotherapy, surgery), characteristics of the tumor and the patient, and the type of the normal tissue. Procedures such as IMRT (intensity-modulated radiation therapy, in which the radiation field is tailored more closely to the shape of the tumor, reducing the volume of normal tissue included in the field) allow escalation of tumor doses, and they will have an impact on the nature of late effects and their incidence, but will not likely eliminate them. Therefore, there will be increasing demand for improvements in quality of life for cancer survivors. What can be done to prevent or treat late effects while maintaining or improving local tumor control?

The Radiation Research Program of the National Cancer Institute sponsored a workshop, ''Modifying Normal Tissue Damage Postirradiation'', on September 6–8, 2000, to ex-

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TABLE 1
Workshop Participants

Name	Affiliation	Expertise
William H. McBride, Ph.D., Chairman	University of California, Los Angeles Medical Center	immunity, cytokines, radiobiology
K.Kian Ang, M.D., Ph.D.	MD Anderson Cancer Center	spinal cord, PAIs, radiation oncology
Mitchell Anscher, M.D.	Duke University Medical Center	TGFB, radiation oncology
Mary Helen Barcellos-Hoff, Ph.D.	Lawrence Berkeley National Laboratory	TGFB, extracellular matrix, radiobiology
C. Norman Coleman, M.D.	National Cancer Institute	radiation oncology, medical oncology, radiation biology
Kevin Connolly, Ph.D.	Human Genome Sciences	FGF10, inflammation, GI, arthritis
Dimitry M. Danilenko, D.V.M., Ph.D.	Amgen, Inc.	FGF7 and other FGFs, integrins
Mark W. Dewhirst, D.V.M., Ph.D.	Duke University Medical Center	vascular physiology, hypoxia, inflammation, radiobiology
John J. Feldmeier, D.O.	Medical College of Ohio	hyperbaric oxygen for late effects, radiation oncology
Kathleen C. Flanders, Ph.D.	National Cancer Institute	TGFB
Zvi Fuks, M.D.	Memorial Sloan-Kettering Cancer Center	FGF2, extracellular matrix, apoptosis, radiation oncology
Dennis E. Hallahan, M.D.	Vanderbilt University	TNFB, cytokines, gene therapy, radiation oncology
Martin Hauer-Jensen, M.D.	University of Arkansas for Medical Sciences	GI radiobiology, thrombomodulin, TGFB
Richard P. Hill, Ph.D.	Ontario Cancer Institute/Princess Margaret Hospital	tumor and normal tissue radiobiology, physiology
Randy Jirtle, Ph.D.	Duke University Medical Center	TGFB, genomic imprinting, M6P/IGF2 receptor, radiobiology
Manuela Martins-Green, Ph.D.	University of California, Riverside	chemokines, angiogenesis., ECM, wound healing
James B. Mitchell, Ph.D.	National Cancer Institute	cellular radiobiology, SMAD, oxygen
John Moulder, Ph.D.	Medical College of Wisconsin	ACE inhibitors, kidney, radiobiology
Sarah J. Nelson, Ph.D.	University of California, San Francisco	MR imaging
Paul G. Okunieff, M.D.	University of Rochester	tissue oxygenation, hyperbaric oxygen, FGF1, radiation oncology
William C. Parks, Ph.D.	Washington University School of Medicine	matrix metalloproteinases, wound healing
Mike E. C. Robbins, Ph.D.	Wake Forest School of Medicine	kidney, skin, polyunsaturated fatty acids, light
Anita Roberts, Ph.D.	National Cancer Institute	TGFB, ECM, morphogenesis
Mark Rosenberg, M.D.	University of Minnesota	nephrology, angiotensin
Philip Rubin, M.D.	University of Rochester Medical Center	late effects of radiation, radiation oncology
Thomas M. Seed. Ph.D.	Armed Forces Radiobiology Research Institute	lipoxygenase inhibitors, hematopoiesis
Helen B. Stone, Ph.D.	National Cancer Institute	radiation biology
Robert M. Strieter, M.D.	UCLA School of Medicine	pulmonary medicine, chemokines, inflammation, angiogenesis
Paul Strudler, Ph.D.	National Institutes of Health, Center for Scientific Review	radiation, grant review
Howard D. Thames, Ph.D.	M. D. Anderson Cancer Center	time–dose relationships, volume effects
Elizabeth Travis, Ph.D.	M. D. Anderson Cancer Center	pulmonary radiobiology and genetics

plore new opportunities and to consider ways to encourage research on this topic. Participants represented basic scientists and physicians with backgrounds in radiation biology and radiation oncology, as well as those with expertise in imaging, pulmonary medicine, nephrology, wound healing, cytokines, chemokines, growth factors, angiogenesis, inflammation, extracellular matrix, and proteases (Table 1).

Acute reactions, such as those in the skin and mucosa, are primarily the result of death of stem cells and an inability to replenish functional cells that are lost from these tissues. Late effects occur in slowly proliferating tissues with long turnover times, where stem cells have been considered to play a minor role, if any, in replenishment. Further, failure of such tissues is thought to occur when functional cells attempt to proliferate to replace cells that are lost. Since they may still have radiation damage, this can precipitate their death, and an avalanche effect can occur. In some situations, early damage obviously has late consequences, for example after denudation of a mucosal surface. The relative lack of repopulation from stem cell pools of late-responding tissues tends to make radiation reactions in these tissues more debilitating than those in acutely responding tissues as well as more chronic, which diminishes the quality of life for those who are afflicted. The disconnect between observed acute and late events is increasingly being challenged by the discovery of subclinical cellular responses during the latent period prior to expression of injury that suggest a continuing, evolving process. It appears that most late effects are complex and involve multiple pathogenetic mechanisms (*1, 2*). The contributions of

vascular and parenchymal damage in initiating the downward spiral to late complications were recognized and their relative importance was hotly debated years ago (*3–5*). Recently, the concept that late effects represent dysregulation of an integrated wound healing process that involves both parenchymal and vascular elements has increased in prominence [see recent review in ref. (*6*)]. There is therefore potentially much to be learned from comparing and contrasting tissue responses after irradiation with those after other forms of injury, both acute and chronic, such as burn injury, surgical wound injury, nonhealing cutaneous ulcers, chemotherapy, and the scarless healing of fetal wounds. However, the kinetics of healing will be different in radiation injury, and in fact, radiation has been used to inhibit certain hyperproliferative healing responses, such as to prevent keloid formation and to block neointimal proliferation leading to restenosis in the treatment of coronary artery disease. Understanding how radiation works in such situations may be valuable in developing new strategies for ameliorating radiation-induced late complications.

Late effects were once thought to be inevitable and irreversible, but there is now evidence that postirradiation modification of normal tissue injury is possible. A cell or tissue is likely to have an altered metabolism and phenotype after irradiation, as the injury elicits complex responses in an attempt at healing. The dysregulated physiology leads to late effects that vary in form and severity with time, dose and tissue. Taking these variables into account, the relative contributions of cell depletion, radiation-induced gene expression, pathology-induced gene expression, and the microenvironment have yet to be fully evaluated. The pathophysiological basis for late normal tissue injury is clearly complex, and discovering the processes involved will require a multidisciplinary effort by physiologists who understand the organ systems, molecular biologists to assist in understanding the molecular interactions involved, and radiation biologists and radiation oncologists with their perspectives on radiation effects and clinical aspects. Long-term studies of these radiation-induced alterations are needed that consider the totality of the interactions that are involved and how they result in a diverse range of symptoms.

CLINICAL ISSUES: QUANTIFICATION OF NORMAL TISSUE INJURY

Background

Fundamental to the study of normal tissue response is an accurate assessment of radiation damage. Philip Rubin described the LENT scoring system (Late Effects Normal Tissue), which was developed for quantification of normal tissue injury, particularly the late effects after radiotherapy. A related scoring system, the Common Toxicity Criteria (CTC; see the website http://ctep.info.nih.gov/CTC3/default.htm), was developed by NCI primarily to identify the acute toxicity of chemotherapeutic agents as a tool for drug development. The American and European clinical radiotherapy trials cooperative groups RTOG and EORTC collaborated in formulating the criteria for LENT, which provides a reference system for documenting, quantifying and reporting late effects of cancer treatment. It can be employed in longitudinal studies for evaluating new anticancer therapies, through determination of the therapeutic ratio, as well as treatments for managing normal tissue damage. Briefly, LENT involves grading normal tissue responses using four categories: subjective, objective, management and analytic (SOMA). For each organ or tissue, and for each category, functional and structural symptoms, tolerance doses, and their management are described and assigned scores according to their severity. Subjective symptoms are those reported by the patient, such as pain, numbness and bowel habits. Objective symptoms are those determined by physical examination, radiological abnormalities, and laboratory tests, and include such things as edema, atrophy, ulceration, and reduction of respiration volume. Management indicates treatment approaches, such as pain medication, special diets, or surgical interventions. Analytical criteria include more sophisticated imaging and special laboratory tests. We refer the reader to an entire issue of the *International Journal of Radiation Oncology, Biology, and Physics* devoted to papers on the topic, as well as scoring tables for 16 major organ sites and 35 subsites (*7–30*). The European radiation oncologists simultaneously published a series of articles on LENT/SOMA in *Radiotherapy and Oncology* (*31–35*).

Opportunities

The LENT/SOMA system should be coordinated with the CTC scoring system for acute toxicities, to quantify damage and to provide common guidelines for reporting normal tissue toxicity. The new system should be adopted by both the national and international communities, as has been the case with the TNM system for classifying the extent of disease in cancer patients. This would facilitate both research and patient care. A computerized scoring system should be developed that is easier to use, more concise, quantitative and objective. The challenge is to make it more appealing to clinicians. Some of the new developments in functional, metabolic and molecular imaging may provide useful tools for assessing normal tissue damage, especially for detecting cytokines, chemokines and their receptors (see below). The system also needs to take into account the quality of a patient's life and related measures of outcome after management of the radiation effects, since some late effects are readily treatable, but those that persist can have a major impact on a patient's professional, social and recreational activities for the rest of his or her life.

MECHANISMS IN THE DEVELOPMENT OF LATE EFFECTS: GENETICS OF NORMAL TISSUE TOXICITY

Background

Radiation responses, including late effects, clearly can have a genetic basis, as described by Elizabeth Travis. For

example, patients with inherited diseases such as ataxia telangiectasia are unusually radiosensitive, and they develop normal tissue necrosis at doses of radiation commonly used for radiotherapy of cancer. Similarly, SCID mice are more radiosensitive than normal mice. These two examples involve defects in the ability to repair DNA damage.

In addition to these extreme examples, wide variations in the rate or likelihood of developing pneumonitis and lung fibrosis after irradiation and bleomycin treatment has been observed in humans and in animal models that cannot be accounted for by known risk factors. This suggests a genetic basis of susceptibility, which is confirmed by studies in the classic animal model of fibrosis-prone (C57BL/6J) and fibrosis-resistant (C3Hf/Kam) mice. There are two loci involved in bleomycin-induced lung fibrosis and three in radiation-induced lung fibrosis (*36, 37*). One of these loci, common to both types of fibrosis, is located within the region of the major histocompatibility complex, and it may involve the genes for Tnf or MnSOD. Another, affecting susceptibility to bleomycin only, appears to involve the gene for bleomycin hydrolase. The others have not been identified.

Genetic control of radiation response extends beyond the lung. Irradiation of the colon and anus also elicits different late responses in the two strains of mice. The incidence of hyperplasia, metaplasia and neoplasia of the stratified squamous epithelium is greater in C3H mice, but late responses in the deep glands in irradiated colon and ulceration are more prevalent in C57BL/6 mice and seem to be associated with the fibrosis phenotype (*38*). It is likely that similar differences in radiosensitivity exist in humans and that they have a genetic basis.

There is some clinical evidence that certain subgroups of patients are less able to tolerate irradiation. Co-morbidity can have an impact on late effects (*39*). For example, patients with inflammatory bowel disease tolerate less radiation dose to the intestine (*40*). Patients with certain collagen vascular diseases may also be more susceptible to late effects, but the evidence is not clear (*41, 42*). The etiology of these diseases is complex and may include non-genetic factors as well as single or multiple genes (*43–45*).

Opportunities

Identifying a genetic basis for the risk of developing late complications will require more thorough epidemiological and basic research. This will be a difficult task, because it is not known how many genes are involved in susceptibility to late effects. The genes may vary with the nature of the response, and susceptibility may vary with the combination of genes present. Furthermore, the interaction between genetic and environmental factors, such as diet, subclinical infection (especially cytomegalovirus in the lung), or tumor, may be especially important in the development of late effects in humans.

Fibrosis associated with other genetic and infectious diseases might provide clues to other candidate genes. Among the kinds of studies that might be useful in identifying susceptible individuals are linkage studies to identify polymorphisms in such genes as *TP53, TNF*, fibronectin and *ICAM1*. In general, HLA typing for associations with fibrosis has yielded conflicting data (*46*). Mapping of genetic loci can provide useful information. In addition, gene arrays, differential display, gene trapping, or proteomics with genetically and phenotypically defined model systems provide powerful complementary approaches. For groups of organs with common genetic links for injury, neural network analyses might be useful (*47*). Once the genes causing variation in lung fibrosis in mice are identified, it will be critical to determine their function and their relevance to normal tissue damage in other organs and in other species, including humans. This in turn would suggest candidate target molecules for possible interventions.

PHYSIOLOGY OF TISSUE RESPONSES

Background

Tissue response to radiation is governed by cellular responses, by tissue-based reactions, and by systemic influences (*48, 49*). Discussion of this topic was led by Mark Dewhirst. Cells *in vivo* reside in highly organized tissues and organs that are made up of many different cell types that interact with one another and with their environment through exquisitely regulated pathways. The interactions are regulated by cytokines that act through autocrine, paracrine and endocrine signals, growth factors, cell adhesion molecules, and the extracellular matrix. After traumatic injury, in the early inflammatory phase, bleeding releases platelets, VEGF and TGFB increase, the microvasculature becomes hyperpermeable, fibrin is formed, and inflammatory and endothelial cells infiltrate. During the proliferative phase, angiogenesis and fibrinolysis take place. This is followed by the phase of tissue remodeling and establishment of normal vasculature, in which hypoxia and apoptosis are thought to play a role.

Radiation-induced injury results in the death of cells, which in turn stimulates hypoxia in the tissue (*50*). Cells surrounding the damaged area produce factors that cause blood vessel leakage and attract and activate leukocytes that generate an inflammatory response. These leukocytes produce numerous cytokines and growth factors including VEGF and TGFB, which in turn stimulate cells to proliferate and to produce extracellular matrix (ECM), contributing in this manner to the fibrotic lesion.

There has been an increasing interest in the role of hypoxia in tumor progression and response to treatment, but there have been very few studies on its possible role in the development of normal tissue damage after irradiation. Hopewell's observation of colonies of endothelial cells blocking small vessels 4.5 months after irradiation with 20 Gy suggests one mechanism that could result in focal hyp-

oxia in irradiated tissues (*51*), as could vascular insufficiency. It has been shown that hypoxia can induce apoptosis in normal tissues during the fourth day after surgical wounding in rats, when tissue remodeling and establishment of normal vasculature are taking place (*52*). In the irradiated lungs of rats, hypoxia was found in macrophages 6 weeks after a dose of 28 Gy, before functional or histopathological changes were detectable (*53*). By 6 months, hypoxia was more severe in macrophages, was also present in type II pneumocytes and endothelial cells, and was accompanied by severe fibrosis and an increased breathing rate. The hypoxia was implicated in the development of the lung injury. Hypoxia has also been found in white matter in the spinal cord of rats as early as 4 months after a dose of 16 Gy, increasing in extent and severity with time and dose (*50*). In this study, the precipitating factor was thought to be radiation damage to endothelial cells, with breakdown of the blood–spinal cord barrier, vasogenic edema, and hypoxia.

Opportunities

Collaboration with individuals who are experts in normal tissue pathophysiology will be essential for studying this aspect of radiation-induced injury. They could provide helpful insights into the response of an organ to various forms of injury, such as surgical and traumatic wounding, and allow the characterization of tissue repair processes that might be aberrantly regulated in irradiated tissue. For example, why is traumatic injury self-limiting but radiation injury is not? What are the molecular differences between the responses to these two types of injury?

It is important to determine the role of the inflammatory response to cell death. A factor contributing to the inflammatory response may be the propensity of the cells to undergo rapid apoptosis or necrosis. The former does not elicit an inflammatory response, whereas the latter does.

The long-standing controversy regarding the relative importance of vascular injury compared to parenchymal injury (*3–5*) should be re-examined with the tools now available. Those for assessing tissue oxygen levels or the histological distribution of hypoxia using hypoxia markers (*54, 55*) could answer several questions. Does hypoxia always develop in irradiated normal tissue prior to the development of late effects? Does tumor hypoxia before treatment alter the physiology and radiation response of the adjacent normal tissue? Blood flow measurements can also provide insights into the physiology of the development of late effects. Hypoxia inhibits cell proliferation and activates TP53-dependent apoptosis, but it also induces cytokines and growth factors. The relative strength of these competing signals for proliferation and cell death at various times could provide clues to mechanisms in the development of late effects. Does hypoxia induce an angiogenic response in irradiated tissue or lead to normal cell apoptosis as it does after surgical wounds (*56*)?

PRO-INFLAMMATORY CYTOKINES AND CHEMOKINES

Background

Cytokines are a class of intercellular signaling molecules, and include interleukins, colony-stimulating factors, interferons, chemokines, and peptide growth factors. They are soluble proteins and glycoproteins. Cytokines regulate cell proliferation and function, are released by injured tissue, and work in conjunction with signals from other sources to integrate programmed responses to injury and other microenvironmental changes. Through signal transduction, they direct cells to grow, differentiate or undergo apoptosis. The primary sources of the various cytokines are epithelial cells, fibroblasts, macrophages and endothelial cells. The action of a cytokine is controlled by cell context (the location and situation in which it is expressed: cell type, differentiation state, and stress response), receptor expression, host condition (hormonal status, acute reactions, chronic reactions), and microenvironmental composition (growth factor milieu, neighboring cells, and ECM).

Robert Strieter's presentation focused on chemokines. Chemokines and their receptors are involved in inflammation, wound healing, angiogenesis, angiostasis, cell recruitment, and metastasis, as well as the development and function of the lymphoid system. These small cytokines are released in response to injury and are produced during the early stages of healing by the cells of the injured tissue. They attract leukocytes to the site of injury and infection and activate them, thereby contributing to the inflammatory phase of healing. Leukocytes, vascular endothelial cells, smooth muscle cells and fibroblasts all have receptors for chemokines. There are four classes of chemokines: CXC, CC, C and CX3C. The CXC class contains two subclasses, the ELR-CXC cytokines, which are mostly angiogenic, and the non-ELR-CXC cytokines, which are angiostatic. Fibroplasia and deposition of the extracellular matrix depend on angiogenesis. Strieter postulated that pulmonary fibrosis may result from an imbalance between angiogenic and angiostatic chemokines (*57*). In the case of idiopathic pulmonary fibrosis, a chronic and often fatal disorder involving exaggerated angiogenesis, fibroproliferation, and deposition of ECM, he and his colleagues found increased levels of IL8, an ELR-CXC (angiogenic) chemokine, whereas in bleomycin-induced pulmonary fibrosis they found decreased levels of IP10, a non-ELR-CXC (angiostatic) chemokine (*58*). Administration of Ip10 to mice with bleomycin-induced pulmonary fibrosis reduced the angiogenesis, deposition of ECM, and fibroplasia (*59*). Whether an imbalance of angiogenic and angiostatic chemokines is involved in radiation-induced pulmonary fibrosis has not been investigated, but Johnston *et al.* (*60*) have shown expression of mRNA for several chemokines in the fibrotic phase after lung irradiation in fibrosis-sensitive C57BL/6 mice, but not in fibrosis-resistant C3H mice. In the pneumonitic phase, no differences were observed.

The role of the influx of inflammatory cells in releasing cytokines that activate resident cells and lead to fibroblast proliferation and matrix protein synthesis is well established in fibrosis (*60*). However, the cytokine-related events occurring early after radiation therapy are not well understood, especially with respect to how they might relate to the development of late damage. In addition to the early production of pro-inflammatory cytokines, including TNF and IL1, within the first 24 h after irradiation, subsequent waves of similar cytokines have been reported weeks and months later (*61–63*). TNF production appears to be protective in the brain, and probably other tissues,[2] but is likely to also cause symptoms of radiation exposure, including vascular responses. While cell death may contribute to perpetuating cytokine responses, T-cell depletion helps to prevent radiation-induced pneumonitis in SPF mice, suggesting a possible autoimmune component (*64, 65*). While inflammatory responses may contribute to the pathogenesis and symptoms of radiation damage, whole-body irradiation, which reduces inflammatory cell infiltration, impairs healing of surgical wounds (*49, 66*), suggesting an essential role for inflammation in tissue repair. However, pneumonitis outside of the irradiated field has been described in humans and is associated with inflammatory infiltrates (*67–69*). Also, irradiation can up-regulate CAM expression and increase inflammatory cell trafficking into tissues (*70, 71*), and blocking CAMs diminishes radiation-induced pneumonitis (*70*). Anti-CAMs are being developed for the clinic for blocking fibrosis in the lung. All this evidence suggests that cellular infiltrates play a role in radiation responses and are a possible target for intervention.

Opportunities

There are two broad approaches to determining the role of specific chemokines and pro-inflammatory cytokines: depletion by neutralizing antibodies, specific peptides, drugs and gene knockouts, or repletion by pharmacological replenishment, transgenic overexpression, or conditional expression.

The interrelationship of angiogenesis and fibrogenesis is an understudied area. It is important to determine if chemokines are induced directly by radiation and whether CXC chemokines or other cytokines may be involved in radiation-induced fibrosis in the lung and other tissues. It may be possible to look at the profile of chemokines, angiogenic factors, etc. of the fibroblasts isolated from the lesions (*72*). Do such changes predict early development of late effects? What is the relationship of these phenotypes to those changes described by Hajenkos after irradiation which appear to be driven by TGFB (*73*)? Do chemokines affect other growth factor responses? Studies with hyperbaric oxygen suggest that increased angiogenesis leads to decreased fibrosis. Is this chemokine-mediated? Again, mechanistic studies are needed, and then, if those are positive, various interventions and their timings should be investigated. CAMs that are up-regulated on endothelial cells may be a particularly accessible target for intervention that should be explored further (*74*).

The temporal relationship of cytokine production after irradiation to the progression of injury and ongoing cellular processes in irradiated tissues needs to be determined. What perpetuates the response—is it cell death, is it the imbalance among cell populations caused by cell death, or is it self-perpetuating? Is an autoimmune response a part of this, and can responses to opportunistic pathogens be important? One approach to evaluating the contribution of immunity to late effects might be to look for markers of autoimmunity, such as antinuclear antigen, antiphospholipid antibody, or other serum markers. The MHC locus could provide genetic influences that regulate a number of these events, including any immunological response to injury and collagen modulation. In the lung, is the appearance of symptoms such as alveolitis and pneumonitis mechanistically linked to cytokine responses? What is their role in the development of fibrosis?

THE ROLES OF TGFB AND THE EXTRACELLULAR MATRIX

Background

Injured tissue releases numerous growth factors in the process of healing (*75*). These orchestrate the responses of inflammatory infiltrating cells, vascularization and proliferation in response to the injury. Anita Roberts introduced the TGFB signaling pathway, which is recognized to be important in tissue maintenance and repair after injury and in the development of fibrotic lesions, as well as in controlling inflammation and immunity (*76*). TGFB is produced by a wide variety of cells, and it acts on a wide variety of cells. It is involved in the production of extracellular matrix, in particular collagen deposition, and has a multitude of other functions. Its actions depend on the type of cell receiving the signal (the cellular context). It plays a prominent role in wound healing (*76*). TGFB binds to receptors in the cell membrane that activate signal transduction pathways involving messengers called SMADs, which induce or repress certain targeted genes. Many of these pathways are involved in embryonic and fetal development and in wound healing. Through the use of Smad3 knockout mice, Anita Roberts reported that loss of this signaling intermediate resulted in accelerated cutaneous wound healing, characterized by an increased rate of re-epithelialization compared with wild-type mice. This was associated with significantly reduced local infiltration of monocytes (*76, 77*). Since exogenous TGFB has been associated with in-

[2] J. L. Daigle, C. S. Chiang, J. R. Sun, H. R. Withers and W. H. McBride, Acute molecular and cellular responses of TNF receptor knockout mice to brain irradiation, p. 478. Presented at the American Association for Cancer Research Annual Meeting, San Francisco, CA, 2000.

creased wound healing, these findings are somewhat paradoxical, but similar events occur in fetal wound healing.

Much attention has been focused on the role of TGFB in radiation fibrosis, not only because its activation is induced by radiation (*78, 79*) and its elevation accompanies the development of fibrosis, but also because injection of TGFB causes local fibrosis and systemic production of constitutively active TGFB causes systemic fibrosis (*80, 81*).

Other pathways may predominate in the overall response or contribute to it, depending on the circumstances (radiation dose, time after irradiation, tissue, etc.). For example, TGFB is a relatively late player in radiation nephropathy (*82, 83*). The extent to which TGFB initiates the pathway, as opposed to acting as terminal effector molecule, has yet to be determined. However, Anscher and Jirtle have reported that cancer patients with elevated plasma levels of TGFB prior to radiation therapy are more likely to develop pneumonitis (*84, 85*), pointing to the importance of the interaction between systemic effects and local radiation damage. The source of the elevated TGFB levels is frequently the tumor or the stromal cells that invade it (*86*). Randy Jirtle presented interesting data showing a correlation between *M6P*/*IGF2R* mutation, elevated plasma levels of TGFB, and the development of pneumonitis (*87*). *M6P*/*IGF2R* is mutated in approximately 50% of both lung (*88*) and head and neck tumors (Jamieson *et al.,* submitted for publication), and it is also frequently mutated in tumors of the breast, GI tract, and liver (*87,89–91*). Tumors with a mutated *M6P*/*IGF2R* are likely to be intrinsically more resistant to radiotherapy (Jamieson *et al.*, submitted for publication). Patients whose cancers have mutated *M6P*/*IGF2R* have an enhanced sensitivity to radiation-induced pneumonitis because they have greatly elevated levels of the fibrogenic factor, TGFB, most likely because this receptor functions in one of the pathways for activation of TGFB *in vivo*.

Mary Helen Barcellos-Hoff discussed the important role of the tissue microenvironment and the ECM in modulating the actions of cytokines and in regulating both the cellular phenotype and tissue function. The composition of the ECM varies with its location and function, ranging from the clear gel of the vitreous body of the eye to the dense structure of bone. It is made up of various glycoproteins, proteoglycans, complex carbohydrates, and other molecules. While long overlooked and thought to be merely tissue scaffolding, the ECM is now known to play a dynamic role in tissue function. As a reservoir for storing inactive cytokines, the ECM permits rapid extracellular signaling and has a memory function, recording cytokine action, generating concentration gradients, and stabilizing repair signals. ECM also mediates the action of cytokines, sequestering active forms and modulating signaling. TGFB plays an important role in determining the composition of ECM, in particular regulating collagen deposition, a dynamic process that changes the nature of the ECM and alters the collagen subtypes with time. Decorin, a proteoglycan that plays a role in regulating collagen fiber formation in connective tissue, can inactivate TGFB, while TGFB can induce synthesis of decorin. Its expression is increased in experimental hydronephrosis. Whether decorin can alter the development of fibrosis or reverse fibrosis is not known. Other matrix components can also inhibit TGFB (*92*). Loss of hyaluronic acid and E-cadherin can occur after radiotherapy, and such changes could be important in affecting cell behavior. Also, alterations in and injury to basement membrane and the inability to re-epithelialize after irradiation are very important in setting the stage for fibrosis.

Opportunities

A better understanding is needed of the role of growth factors in the development of radiation fibrosis and of the molecular mechanisms of their effects. Research to discover approaches for preventing or treating radiation fibrosis is also needed. This could be accomplished through basic research on the signaling pathways, identifying specific targets, followed by development of agents directed at those targets. The converse approach could also be used, using a proven agent whose mechanism is unknown to discover the pathways involved.

There are many questions that can be asked. Is radiation fibrosis a consequential late radiation injury (i.e., due to an unhealed acute effect) or a primary effect (*93, 94*)? It is still possible that in some circumstances the presence of TGFB may simply be a marker rather than a cause of the condition, as may also be true for other cytokines. The tools of proteomics, the study of levels of specific proteins as a function of time after a perturbation, will give insights into these questions when combined with functional manipulation in appropriate models. It is essential to know when a growth factor, such as HGF, FGF2 (also known as bFGF) or TGFB, is active, not just that it is present. It is essential to demonstrate proof of principle *in vivo*, to distinguish mere markers from causative factors, and to show that an intervention aimed at a growth factor actually prevents, delays or reverses the development of late radiation injury. Which TGFB isoforms are involved? How important are TGFB activation and the cellular sources for the critical cellular responses? How does TGFB interact with or affect responses to chemokines, pro-inflammatory cytokines, FGF2, FGF7 (also known as KGF), HGF or other cytokines and growth factors that might be influenced by its inhibition? What stimulates increases in active TGFB? Does it occur in waves during the latent period, in response to TNF and IL1? The interaction of TGFB with matrix components might be worth exploring in radiation-induced fibrosis.

Oxidative stress and redox-sensitive reactions are important in the development of fibrosis in the liver (*95*). What roles do these processes play in the development of radiation fibrosis, and in which tissues?

Epidemiological studies are needed to determine the frequency of loss of heterozygosity in the *M6P/IGF2R* gene

responsible for TGFB activation, in the general population, in cancer patients, and in patients who develop late normal tissue complications after therapy. Such correlative investigations must be followed by more detailed studies to verify the relationships and to elucidate the mechanisms and processes involved. The goal is to learn how late effects develop, and to identify potential approaches for preventing or reducing their impact on patients. Possibilities include finding ways to alter plasma levels of TGFB, or blocking M6P/IGF2R.

Is fibrosis reversible? Can TGFB or SMAD pathways be blocked using inhibitors, and if so, must this occur in a sensitive period after radiotherapy to have an effect, or can this be performed at any time? Is SMAD3 a potential target, given that mice null for Smad3 show accelerated healing of cutaneous wounds and reduced fibrogenesis? The relationship of proteases to collagen subtypes and to cytokines during the process of tissue remodeling is not clear. Can the remodeling process be targeted with chemotherapeutic agents that interfere with the fibrin cascade? Deposition of extracellular matrix is a feature of the response to injury by radiation and other agents as well. This may be a good area for mixing radiation biology research with other more ''organ-specific'' research.

The effect of radiation on the structure and function of the ECM needs to be better defined. Does chronic hypoxia or irradiation change the redox status of the ECM, and does this contribute to fibrosis? What roles do the ECM and integrins play in the development of late effects? Are cytokines such as FGF that bind to heparin and other materials in the ECM released from the ECM after irradiation? The contributions to the development of radiation-induced late effects of FGF2 and other growth factors in the ECM need to be determined. The mechanism of the rapid loss of hyaluronic acid and E-cadherin after radiotherapy should be investigated to determine the importance of degradation and production in the process, as well as its relevance in the development of late effects. There are reports of an increase in hyaluronic acid in the lung after irradiation that is not predictive of pneumonitis or fibrosis (*96*). It appears that ECM remodeling is a dynamic process that is driven by proteolysis and cytokines. We know very little about the effects of radiation on this process.

PROTEASES AND TISSUE REMODELING

Background

Tissue remodeling occurs during the process of wound healing and involves changing the composition of the extracellular matrix, as mentioned above. In addition to initiation by cytokines, in particular TNF and IL1, this process is facilitated by proteases, particularly the class known as matrix metalloproteinases (matrixins or MMPs), a topic presented by William Parks. While several members of the MMP family function in the turnover and degradation of ECM, these proteinases typically are not found in uninjured tissue. Rather, they are produced when and where they are needed: in diseased, injured, and inflamed tissue where remodeling is taking place. MMPs require zinc for catalytic activity, and they are inhibited by tissue inhibitors of metalloproteinases (TIMPs), oxidants and α2-macroglobulin. Since many MMPs are expressed by a variety of cell types during wound healing, it is difficult to sort out the roles of individual MMPs in the process. While knockout mice can provide some insights, some defects are lethal. In other cases, the knockout mice are able to survive unless challenged, when they display impaired ability to fight infections, impaired wound healing, and tumorigenesis. A complicating factor in cancer is that cancer cells themselves can be releasing proteases or activating those in adjacent normal tissue (*97, 98*).

Opportunities

While the role of proteases in wound healing is well established, the patterns of MMP and TIMP expression over time have not been correlated with cytokine expression or with the nature and extent of ECM deposition after irradiation. The possibility that the balance between MMPs and TIMPs is abnormal in irradiated tissues needs to be investigated. There is a large body of research in wound healing and tissue fibrosis that invites comparison to and contrast with the development and healing of radiation injury. The type of delayed and progressive injury that characterizes late radiation injuries is similar to that caused by progressive insults in other chronic diseases (e.g. diabetic nephropathy). Are differences in protease expression in the coagulation cascade responsible for the disconnect between alveolitis and fibrosis in the irradiated lung? This question is raised because the phenotype of alveolitis is different from that of fibrosis and they seem to appear independently. Responses are often tissue- or organ-specific, although the factors that determine such specificity are largely unknown. The importance of MMPs in processing various substrates in radiation-induced super- and dysregulated repair of tissue damage requires further investigation. What activates MMP activity? How are these processes associated with radiation-induced fibrosis? Are tissue proteases involved in angiogenesis? Do MMPs released by irradiated tumor and normal tissue cells contribute to the pathogenesis of late radiation injury?

Some serine and cysteine proteases are also involved in remodeling of the ECM (*99*). What is their role in the processes leading to radiation fibrosis?

The role of TIMPs in the development of late effects has not been studied extensively. The possibility that protease inhibitors might be used prophylactically or therapeutically should be explored. A number of new protease inhibitors have been developed in recent years. To investigate their usefulness, well-characterized model systems are needed that integrate MMP and TIMP expression with molecular,

cellular, and tissue-related events. How does this relate to TGFB activation? What is the ''fingerprint'' of protease activity in irradiated tissues? Are proteases tissue-specific?

MODIFYING LATE DAMAGE

Introduction

The second half of the workshop was devoted to assessment of the state of the field with respect to modification of late effects using agents that are in clinical trials or have potential for early introduction into the clinic. In determining which approach will be best for clinical application, research can start with empirical observations, but these should be followed up by mechanistic studies, so we will better understand how the approaches and agents work and how best to use them. Conversely, mechanistic studies must progress to ideas of how to use the knowledge for the benefit of patients and must lead to better treatments. It is not enough to observe the rise and fall of gene products after irradiation, without determining whether they are affecting the outcome, or whether they are relevant to scheduling of treatments for late effects. Further study of specific blockers/reversal agents is an important area for research in this field. Blockers used to study other types of physiological responses may hold promise, but it is important to determine whether they simply delay the response, as may be the case with corticosteroids (*100*), prevent its progression, or reverse it.

CYTOKINES AND GROWTH FACTORS AS THERAPEUTIC AGENTS

Background

Dimitry Danilenko described how, in radiation-induced epithelial injury, a number of growth factors and cytokines are up-regulated. During the acute phase, a pro-inflammatory response predominates. IL1 and TNF are up-regulated and IL8 is recruited. This is followed by a subacute phase, where repair predominates and EGF (epidermal growth factor), TGFA and the TGFBs are up-regulated, and FGF7 and FGF10 (KGF2) drive epithelial proliferation and migration. Finally, during the chronic phase, fibrosis develops. The TGFBs and PDGF are up-regulated, and contribute to the repair and regeneration of the ECM, while VEGF, PDGF, FGF1 (aFGF) and FGF2 drive angiogenesis and the formation of granulation tissue. Potential strategies for ameliorating this injury can be grouped similarly: prevent or decrease damage by damping the acute phase [anti-IL1/IL1A, anti-TNF antibodies, soluble TNF receptors or IL11], using cytoprotective agents that function by enhancing cell survival when given before irradiation (FGF7, IL11) or enhancing repair and regeneration (EGF, TGFA, FGF7, FGF10, PDGF, etc.). Many growth factors and cytokines are known that provide protection from chemotherapy or radiation in tissues whose epithelial surfaces are damaged by radiation, including IL1, IL11, IL15, EGF, FGF1, FGF2, TGFBs, FGF7 and FGF10.

Much of the research on the use of growth factors for treating radiation injury has centered around hematopoietic growth factors such as G-CSF (Neupogen®), GM-CSF (Leukine®), erythropoietin (Epogen®), and IL11 (Neumega®) that may be given before or after irradiation to accelerate proliferation of hematopoietic cells to hasten recovery. In epithelial tissues, FGF7, which is a specific growth factor for epithelial cells, has similar potential. FGF7 mediates proliferation, differentiation and homeostasis in a wide variety of epithelial cells, including hepatocytes and gastrointestinal epithelial cells, type II pneumocytes, transitional uroepithelial cells, and keratinocytes in all stratified squamous epithelia. It is markedly up-regulated after epithelial injury, such as in epidermal wounds and inflammatory bowel disease. It was originally isolated from human embryonic lung fibroblasts and was observed to stimulate proliferation of keratinocytes. It is expressed by mesenchymal cells and by activated T cells in skin and intestine. It binds specifically to the FGF7 receptor KGFR (now known as FGFR2), which is found in epithelial cells. *In vivo* FGF7 induces many epithelial protective mechanisms, including increasing epithelial thickness, up-regulating expression of antioxidant enzymes in skin, oral cavity and intestine, and inducing goblet cell hyperplasia and increased mucin production in the small intestine and colon.

In preclinical models of oral and lower GI tract mucositis, pulmonary injury and fibrosis, hemorrhagic cystitis, and alopecia, FGF7 has been shown to prevent injury from drugs or radiation when given before or after treatment (*101–104*). Recombinant human FGF7 is currently in clinical trials to determine whether it can reduce the incidence, severity and duration of oral mucositis when administered to cancer patients receiving chemotherapy and radiation therapy. Phase 2 studies in colorectal cancer patients and patients with hematological malignancies have been completed. Phase 2 studies in head and neck cancer patients and phase 3 studies in patients with hematological malignancies are ongoing.

Opportunities

Fluctuations in levels of cytokines and growth factors after radiation treatment may predict late effects and may serve as potential points for interventions. Identifying the critical factors could provide useful tools for selecting patients for treatments to prevent late damage or to reduce its severity. Cytokines also could serve as targets for interventions, provided it was known how early cytokine responses modified downstream late effects. Currently, there is little evidence that decreasing cytokines within the first few weeks after treatment prevents late complications. A good example is steroid treatment, which delays both molecular and symptomatic responses (*105*). COX2 inhibitors are currently being investigated as potential modifiers of inflam-

matory cytokine and prostanoid production (*106*). These may prove more effective than steroids, but it is possible that inhibiting inflammation will prove deleterious to the wound healing process under certain circumstances and that more subtle means of intervention will be needed.

FGF7 clearly has potential for modifying epithelial cell responses to radiation, but more information is needed as to what late effects can be modified by this agent and what is the best schedule and timing of administration. Further knowledge is also required about the possible effects of FGF7 on tumor cell proliferation. Growth factors related to the FGF family that target endothelial and mesenchymal tissue have been identified. These are currently under development as potential therapeutic agents (*107*). The utility of such agents, used singly or in combination, for managing normal tissue injury that follows radiotherapy and/or chemotherapy is yet to be determined. Bioengineered chimeric growth factors have provided a significant paradigm shift in treatment strategies for radiation or chemotherapeutic injuries in hematopoietic tissues (*108, 109*). Similar chimeric growth factors or families of growth factors will undoubtedly be developed for the non-hematopoietic tissues as well.

Sometimes, leads have come from unexpected places. For example, leptin is a hormone produced by adipocytes and is involved in regulation of body weight. It has angiogenic properties and is mitogenic in keratinocytes. Animals lacking the receptor for leptin are obese and have delayed wound healing. Both receptor-deficient and normal mice showed improved and accelerated wound healing with treatment with leptin (*110, 111*). Do leptins inhibit or worsen radiation fibrosis? If so, through what mechanisms?

BLOCKING THE RENIN-ANGIOTENSIN SYSTEM

Background

The renin-angiotensin system involves a two-step cleavage of angiotensinogen by the acid proteinase renin, which produces the inactive form angiotensin I (AI). Angiotensin-converting enzyme (ACE) then converts AI to the active form AII, which is a powerful vasoactive peptide that contributes to the pathogenesis of progressive renal disease. Angiotensin II is involved in the development of late-onset renal failure in patients who have received whole-body irradiation (with doses as low as 10–12 Gy) prior to bone marrow transplantation (*112, 113*). An alternative ACE-independent pathway exists in humans in which AI is converted to AII by chymase. Recently, it has been demonstrated that vascular remodeling and endothelial dysfunction of small and large vessels may be normalized by treatment with some antihypertensive agents such as ACE inhibitors, AII receptor antagonists, and long-acting calcium channel blockers (*114, 115*). The renin-angiotensin system is also found in the lung, skin, kidney and heart. John Moulder reviewed this topic at the workshop. The two approaches studied most have been the use of ACE inhibitors such as enalapril and captopril, which may be acting through multiple mechanisms, and blockers of the AII receptor, of which there are several [see review in ref. (*116*)]. These agents have been shown to be effective in the treatment of radiation injury in lung and kidney. They also reduced the incidence of dermal necrosis in rats after radiation treatment. AII receptor blockers were more effective than ACE inhibitors for the prevention of nephropathy induced by radiation and for the prevention of pneumonitis induced by chemo-radiotherapy (*116, 117*). AII blockers were equivalent to ACE inhibitors for treatment of radiation-induced nephropathy (*118*). An advantage of AII inhibitors was that they could be stopped after 3 to 6 months without precipitating injury (*119*). Infusion of excess AII, however, increases injury (*120*). While the renin-angiotensin system is important in the development of cardiac failure and fibrosis after myocardial infarction, captopril failed to prevent functional cardiac damage after a single radiation dose of 20 Gy (*121*). This suggests that different mechanisms may be involved in the development of radiation damage in different tissues and organs, and at different doses.

Opportunities

Mechanistic studies are needed, particularly in relation to the timing and duration of treatments and their tissue-specific effectiveness. It is not yet clear which of the multiple mechanisms of action of captopril are responsible for reducing late radiation damage in lung and kidney, why it must be given for a long time, possibly for the duration of life, or in what tissues this approach might be effective. Such studies could lead to development of better ACE inhibitors or AII receptor antagonists.

The molecular targets that are involved and the best approach to blocking radiation effects have yet to be established. Moulder's studies have clearly shown the importance of AII in radiation nephropathy, since blocking the function of AII with ACE inhibitors or AII receptor antagonists reduces the severity or prevents the development of renal injury. While activation of the renin-angiotensin system causes hypertension, and severe hypertension is a prominent feature of radiation nephropathy, this does not explain the efficacy of AII blockers and ACE inhibitors against radiation pneumonitis (*117*). Is the beneficial action of inhibiting AII observed only in those organ systems that express a renin-angiotensin system? Because of the alternate pathway mentioned above, ACE inhibitors alone would not effectively block AII, which could explain why captopril was ineffective in the heart. AII receptor antagonists would be expected to be effective, however, and studies should be expanded to include such agents.

What are the mechanisms of action of ACE inhibitors and AII blockers in these tissues in relation to radiation injury? There is no evidence that the renin-angiotensin system is activated during the interval after irradiation when

these agents are effective. AII blockers eliminate the radiation-induced increase in TGFB (*83, 122*). How does the renin-angiotensin system interact with the TGFB pathway and other pathways, such as collagen metabolism, tissue remodeling, and the stress pathways? The inhibition of cellular proliferation by AII blockers could be preventing mitosis-linked cell death.

Clinical trials are needed to determine whether either an ACE inhibitor such as captopril or an AII blocker such as losartan can prevent or reduce late complications in lung, in which injury is prevalent and easily measured, or in kidney. While both agents are approved for clinical use, there is far more clinical experience with long-term administration of captopril than with losartan. Studies of captopril in adult and pediatric BMT patients are ongoing at the Medical College of Wisconsin and are expected to be completed as early as 2004. End points of that study are renal function at 6, 12 and 24 months, actuarial incidence of BMT nephropathy, and pulmonary function. If complication rates can be reduced significantly, as predicted by dose modification factors of 1.2 to 1.4, subsequent clinical trials involving radiation dose escalation may be feasible.

HYPERBARIC OXYGEN IN THERAPY OF NON-HEALING INJURIES

Background

There have been a number of anecdotal preclinical and clinical reports and small studies indicating that hyperbaric oxygen, usually in a series of 35 to 45 or more treatments or ''dives'' at 2.0 to 2.5 ATA, can aid in healing late radiation injuries. These include radionecrosis of the mandible (associated with tooth extraction), pelvic bone, larynx, chest wall, brain, and soft tissues, and radiation cystitis, proctitis and enteritis [e.g. refs (*123–129*)]. It has been postulated that irradiated tissues are hypoxic because of hypovascularization and that hyperbaric oxygen increases tissue vascularity (*130*). The mechanism through which this might be working has not been elucidated. John Feldmeier reviewed this topic. The absence of large, controlled clinical trials as well as a lack of experimental evidence defining the mechanism of action has led to skepticism in the radiation community regarding the effectiveness of hyperbaric oxygen. Clinical hyperbaric oxygen facilities are not available everywhere in the United States, and for some patients, access to this treatment modality would be problematic. Furthermore, physicians may be reluctant to recommend that patients undergo a second series of time-consuming treatments. Three animal studies have shown reduced radiation damage with hyperbaric oxygen treatments (*131–133*). A fourth study showed no benefit from hyperbaric oxygen treatment in preventing radiation myelopathy 8 weeks after irradiation, a rather early end point (*127*). A multicenter international clinical trial is under way for hyperbaric oxygen treatment of radionecrosis of soft tissues, mandible, bladder, rectum, colon, vagina and bladder and is to be completed by 2005. It is sponsored by the Baromedical Research Foundation. The trial is randomized and double-blinded, with crossover of the two arms of the study.

Opportunities

Both mechanistic studies, including animal studies, and systematic controlled clinical trials of hyperbaric oxygen are needed. In what radiation dose range is hyperbaric oxygen effective, and in which tissues or organs? How many treatments are needed? What oxygen pressures should be used? When should treatments be started? Does injury progress again after treatments are stopped? Answering these questions would be facilitated by knowing the mechanisms of action of hyperbaric oxygen. The possibilities are: anti-inflammatory action, stimulation of angiogenesis (see above), increasing SOD expression, and changes in signaling mediated by HIF1. Do both hyperbaric oxygen and hypoxia induce HIF1? If hyperbaric oxygen acts through a different mechanism than other treatments for late radiation damage, then it might be combined with them for added benefit. Would carbogen or carbogen plus nicotinamide be more effective than hyperbaric oxygen?

OTHER APPROACHES

Background

A number of other approaches that have been reported in the literature were mentioned at the workshop. Pentoxifylline, alone or in combination with tocopherol, appears to diminish radiation-induced fibrosis in some models (*134–136*) but not others (*137, 138*). Pentoxifylline is a methylxanthine derivative that alters tissue blood flow. It also inhibits production of TNF, but its mechanism of action in reducing late radiation effects is not known. In a clinical study, Delanian found that the combination of pentoxifylline and tocopherol, but neither agent alone, dramatically reversed human chronic radiation-induced fibrosis (*136*).

Cu/Zn and MnSOD reduced radiation-induced fibrosis in pigs and in humans (*139, 140*) and reduced the incidence of radiation-induced cystitis (*141*). While these enzymes eliminate superoxide radicals, their mechanism(s) of action in these cases is ill-defined. Recent studies indicate that exposing human fibroblasts obtained from radiation-induced fibrotic skin to liposomal Cu/ZnSOD led to phenotypic changes. These included enhanced endogenous MnSOD protein and activity and a significant reduction in *TIMP* and *TGFB1* gene expression (*142*). Although these findings require confirmation, they suggest that modulating antioxidant enzymes levels may lead to reversal of a profibrotic phenotype. However, as a note of caution, hypersensitivity reactions have been associated with the administration of MnSOD, highlighting the need for a balance

between pro-oxidants and pro-reductants in cells and tissues (*143, 144*).

Stem cell transfer, although well established for correction of hematopoietic deficiency after whole-body irradiation, is still in its infancy with respect to other tissues. However, the finding of pluripotent stem cells in various organs and the increasing ease with which stems cells can be cultured suggest that this will be a fruitful area of future research. Injection of unirradiated fibroblasts has been shown to strengthen surgical wounds in irradiated skin in rodents (*145, 146*). Injection of fibroblasts does not affect healing to any extent in unirradiated tissue. One potential problem is that injected cells die rapidly and only a small percentage are left alive.

Opportunities

The mechanisms of action of SOD, pentoxifylline and tocopherol in reversing fibrosis need to be discovered so that these agents can be used most effectively to provide a rational basis for combining treatment approaches, and to identify molecular targets for continuing investigation.

Cell transplantation has not been tried in intact irradiated skin or other tissues, nor has it been studied in detail. Improved methods are needed for introducing stem cells into tissue. This may still be the most promising approach. A lot of venture capital is being invested in this approach to plastic surgery.

FUNCTIONAL IMAGING

Background

Powerful tools for both research and patient diagnosis that are being developed for diagnostic and functional imaging were presented by Sarah Nelson. Traditional ''X rays'' provide little definition of soft tissues. CT provides additional soft tissue definition and adds a third dimension. MRI and MRS can provide information on tumor volume, cerebral blood flow, cerebral blood volume, vessel permeability, tissue water diffusion, blood oxygenation, pH and cellular metabolites. Functional imaging can provide information about tissue states, such as fibrosis, about activity of proteins, such as activation of TGFB, and about specific cell events, such as inflammation. SPECT and PET can provide information on glucose metabolism, cell proliferation, angiogenesis, cerebral blood flow, capillary permeability, hypoxia, drug delivery, and reporter gene expression. While anatomical imaging continues to be the mainstay of diagnosis and treatment planning, physiological imaging can be used to determine macroscopic blood flow (angiography, MRI), blood volume or permeability (dynamic CT or MRI, PET), and tissue structure (diffusion-weighted MRI, MT-weighted MRI). Metabolic imaging using MRS, which may be performed with MRI, measures cellular metabolites, commonly with ^{1}H, ^{31}P, ^{19}F and ^{23}Na, whereas SPECT and PET use radiolabeled tracers. These techniques have been used to study changes in response to irradiation of the brain [e.g. refs. (*147–149*)], but they have been used in few other tissues. Molecular imaging is a rapidly evolving field which provides the capability for observing specific molecular events, such as monitoring drug delivery and gene expression, using SPECT, PET, optical imaging, or MRI with contrast agents. Combination whole-body imaging systems are being built to visualize both anatomy and function simultaneously, e.g. with CT/SPECT and CT/PET. Imaging devices are being built specifically for studying animal models. New contrast agents and imaging probes that target specific metabolic pathways are being developed, as are probes which offer the capability for imaging reporter gene constructs as markers for delivery and expression of transgenes.

Opportunities

Most of the focus in imaging research has been on the tumor, and the primary benefits to normal tissue have occurred through more precise definition of tumor margins and minimization of the dose delivered to normal tissues. The new capabilities will further aid in distinguishing tumor recurrence from radiation effects.

These techniques now need to be extended to the study and quantification of normal tissue toxicity, as mentioned above. Serial, noninvasive studies are needed for spatial and temporal mapping of the development of normal tissue toxicity and its response to treatment. It might become possible to predict which patients are likely to develop normal tissue complications, those who might benefit from treatment, or when such treatment should be started and for how long treatments should be given. Non-invasive imaging would also be useful for the spatial mapping of the development of normal tissue toxicity. Some imaging methods are available or are being developed for small animals. For example, there are now methods for imaging protease function and apoptosis noninvasively and for imaging changes in endothelial receptor expression and angiogenesis (*150, 151*). The National Cancer Institute has solicited research applications in this area. Funding opportunities are presented in the web page of the Biomedical Imaging Program: http://cancer.gov/bip/NCI-DIPini.htm. Collaboration between radiation oncologists, radiation biologists, and experts in biomedical imaging would aid progress in this field.

MODELS FOR STUDYING LATE EFFECTS

Richard P. Hill discussed the choice of appropriate models for studying the mechanisms and treatment of late effects. He pointed out that the choice of the appropriate model depends on the question being asked. Cultured cells can be useful models, particularly for examining humoral influences, but have limitations. They are usually exposed to nonphysiological concentrations of oxygen, deprived of paracrine and endocrine signals, in monoculture, and grow-

ing in a two-dimensional world. They may not be in contact with their neighbors or with the ECM that would surround them and control their behavior if they were in a tissue *in vivo*. It is difficult to mimic the metabolism and pharmacokinetics that govern drug concentrations in the intact organism. There is a need to develop tissue-based models that include cytokines, stroma and inflammatory cells. Three-dimensional culture models involving coculture of the relevant human cells need to be introduced in this field. For studies of molecular mechanisms, cell lines with the appropriate genetic profiles must be used, but even different cell types from a single individual can have different responses. For example, lymphocytes are much more radiosensitive than fibroblasts from the same individual, a reflection of differing pathways of cell death.

Studies must be extended to *in vivo* models to verify whether the findings apply there as well. Considerations for choosing an *in vivo* model include similarity to humans with respect to dose–response range, tissue structure, radiation pathology (mechanism of development of late effects), patterns of gene expression, and fractionation and volume effects.

In general, mice are not very good models with which to study late effects because of species differences in life span, physiology, biochemistry, radiosensitivity, anatomy and size, although they have clear advantages in cost and genetic information, particularly for the ability to obtain chimeras and transgenics. For example, mice are resistant to radiation-induced nephropathy, requiring higher doses than other species (*152*). Because the wild-type mice are already so radioresistant to nephropathy, studies involving increased resistance, by use of either drugs or transgenics, might be difficult to carry out unless the adjacent tissues are able to tolerate the increased radiation doses.

Species differences in imprinted genes should also be considered in the choice of animal models, a topic incorporated into the presentation by Randy Jirtle. For example, the gene *M6P/IGF2R* is a tumor suppressor gene that is involved in TGFB activation, and LOH predicts for radiation pneumonitis (see above). In animals below primates, including rodents, this gene is imprinted, which means that during gametogenesis, the paternally derived allele is inactivated by the methylation of CpG sequences (*153*). The presence of only one functional allele for this gene in rodents and two in humans suggests that mice would in general be more susceptible to cancer induction than humans and less susceptible to fibrosis formation, as is seen (*154*). Other genes involved in normal tissue responses may have different imprinting patterns that could affect responses differently in humans and in rodents. As these studies suggest, we need to select animal models that share a common genetic profile with human subjects, at least with respect to the pathway under study.

Larger animals have some advantages over smaller animals. For example, the skin and heart in pigs and the brain in nonhuman primates are similar to those organs in humans. Larger size allows treatment volumes that are more relevant to patients and allows the use of techniques such as lung lavage to look for subclinical toxicity. However, the cost of larger animals would appear to restrict their use to proof-of-principle studies. Veterinary radiotherapy might be used to perform preliminary ''clinical trials'' of modifiers, as was done in hyperthermia and intraoperative radiotherapy research. For these studies, with the consent of their owners, pet animals bearing spontaneous tumors are entered into research studies, much as are human participants. After treatment, the owners bring their pets back to the clinic for regular follow-up visits. This veterinary radiotherapy model has the advantages of studying animals with a greater size and longer life span than rodents and of studying spontaneous tumors. The understanding gained can be carried forward into clinical trials in patients.

New models are needed for identifying the various stages leading to fibrosis and other late effects. These should be designed to answer questions generated by observations made in humans, not in mice, rats, pigs, etc. This is not to say that animal models should not be used, but to say that they should be used to answer questions relevant to the pathology in humans. For example, if a patient demonstrates specific molecules that can be recapitulated in an animal model, then a variety of strategies can be employed to demonstrate proof of principle.

The tumor itself may play an important role in the response of normal tissues to radiation. Tumors invade, displace and destroy tissues as they grow. They also produce proteases, cytokines and growth factors and elicit the development of a vascular supply. Paraneoplastic syndromes are evidence of abscopal effects of tumors. Tumors contain varying numbers and types of leukocytes, and they may contain hypoxic cells, or perhaps create hypoxic regions in the adjacent normal tissues. Tumors express varying amounts of extracellular matrix. During and after therapy, injured and dead tumor and normal cells are undoubtedly releasing substances that affect the surviving normal cells. Whether this contributes to healing of normal tissues or to the development of late effects is not known, and it is imperative that comparisons be made between tumor-bearing and normal animals, and among specific tumor types. Studies should therefore be carried out in both appropriate tumor-bearing and non-tumor-bearing animals. Any treatment that decreases the tumor cure rate will not be useful clinically. Even more important, the incidence and growth rate of metastases outside the radiation field must not be increased. This could be a greater risk, because the goal of normal tissue treatments is to enhance cell survival and proliferation and tissue recovery.

Mathematical models will need to be developed as new principles are discovered. These models can be use to generate testable hypotheses. Howard Thames presented preliminary data at the meeting that indicated that late effects in the clinic occur at random with time after treatment, suggesting that they are precipitated by external forces,

such as injury or infection, acting on a radiation-damaged tissue. Further research on such possible interactions is needed.

GENERAL ISSUES

There is a need to study late tissue responses using clinically relevant radiation doses and treatment schedules: of the order of 2 Gy per fraction and 30 to 40 fractions, totaling 60 to 80 Gy. Large single doses may induce damage through somewhat different mechanisms than fractionated treatments, although either of these could be appropriate for addressing certain questions. With the development of 3D CRT, IMRT, proton therapy, and brachytherapy, treatment fields can be made to conform more snugly to the shape of the tumor. However, normal tissues will always be included in the treatment fields, which must be large enough to accommodate uncertainties in positioning of the patient, movement of the patient during treatments, and tumor motion as a result of organ motion and breathing, and to include tumor extensions that are likely to be present but are not detectable by imaging techniques. The benefits to normal tissues of using smaller treatment volumes may be counterbalanced by the increased doses employed. Low-dose hypersensitivity may contribute to late effects, especially with IMRT, where larger volumes of normal tissue will receive low doses because more beam angles are employed (*155*). Thus studies must be performed at doses and volumes of irradiated tissues likely to be used both now and in the future. New schedules may be needed for administering radiotherapy if it becomes possible to ameliorate late normal tissue damage. The studies described and proposed here might explain the mechanism for the relative sparing of late-responding normal tissues that is achieved by administering radiotherapy in many small fractions (*156*).

Radiation dose–response curves will be needed to indicate the range of radiation doses within which a given treatment for late effects is effective: Prevention or reversal of late effects may not be possible at higher doses. Dose modification factors will be necessary for prioritizing the various treatment approaches for clinical trials.

Long-term studies need to be performed. Even for standard cancer treatments, little is known about the development of normal tissue complications more than 5 years after treatment. It is possible that waves of cellular and molecular responses after irradiation may continue for years, even for the life of the patient. For studies of interventions in both animals and patients, is an earlier end point, such as 1 year, a good predictor of results at 5, 10 or 20 years? Is a 6-month end point in a mouse equivalent to a 5-year end point in a human? Progress would be more rapid if shorter-term end points could be used, but such end points must be shown to be relevant. Do late effects continue to progress for the life of the patient, as has been suggested for breast tissues (*157*), or do they reach a plateau after a number of years in some tissues? Does the kinetics vary from tissue to tissue? Are older patients more susceptible to developing late effects? Is it necessary to wait until the tumor has completely disappeared before initiating treatments for late effects? Does an intervention only delay the progression of late effects, or does it arrest or reverse them?

Tissue tolerance to re-treatment should be evaluated as a part of mechanistic studies, since clinicians need to know the risks in treating recurrent tumors. Is the timing of post-irradiation intervention critical for obtaining the best responses? Would a second course of radiotherapy timed in relation to ''waves'' of molecular and cellular responses give a better therapeutic outcome? Will imaging help to assess such alterations in patients? Are the normal tissue end points valid in the studies that are used to justify re-irradiation? Does reirradiation subject the patient to the risk of different, even later late effects?

How is irradiated tissue perturbed by subsequent injury, such as surgery or dental extraction? Can anything be done to prevent or reduce the risk of tissue breakdown in such cases? Does the susceptibility of irradiated tissue to breakdown after trauma remain constant throughout a patient's lifetime? It will be essential to use appropriate control groups to evaluate properly the potential benefits of the kind of post-therapy treatment of late effects discussed at this workshop.

While the focus of this workshop was on the effects of radiation alone, most patients treated with radiotherapy also receive surgery and chemotherapy. Little is known about the long-term effects of chemotherapy alone or in combination with radiation. The Stanford group, reporting their experience over 40 years in treating patients with Hodgkin's disease, showed an increased relative risk for heart disease in patients who received mediastinal irradiation in addition to chemotherapy (*158*). The risk was lower with improved techniques that involved less radiation to the heart area (*159*). The picture is complicated by the number of chemotherapeutic agents available, and because two or more may be used in a given patient. Collaboration with medical oncologists will be needed for this research.

Carcinogenesis is a late effect of cancer treatment that was mentioned only briefly at the workshop. If carcinogenesis and the other late effects of radiation share common pathways, then treatments to reduce or prevent fibrosis, etc. might also reduce the incidence of second cancers. On the other hand, cell loss and carcinogenesis are generally inversely correlated, and treatments that encourage proliferation in irradiated cells may therefore carry an increased risk of cancer induction. It will be important to distinguish between the outcomes and to ensure that treatments do not increase the incidence of second cancers.

RESOURCES NEEDED

There is a need to support observational research in humans that is not necessarily hypothesis-driven, but that is

TABLE 2
Key Recommendations of the Workshop

Long-term support is essential for long-term preclinical and clinical studies of late radiation effects and their treatment, because late effects develop months to years after therapy, and may continue to progress throughout the life of a cancer survivor.
Multidisciplinary teams will be needed to address the complex research problems in the field of late effects. Radiation biologists, physicists and oncologists will need the assistance of pathologists, physiologists and geneticists, as well as experts in such fields as functional imaging, wound healing, burn injury, molecular biology, and medical oncology.
The **LENT/SOMA scoring system** is an essential tool for assessing late effects in patients and for comparing treatments. It must be improved by replacing subjective scoring systems with objective ones as these are developed and validated. The system should be computerized and made easier to use.
Tissue sharing and a repository of irradiated and unirradiated normal tissues could be useful resources.
Mechanistic studies will identify potential targets for interventions and will suggest how they might be used most effectively in the clinic.
Dose modification factors for potential treatments for late effects must be determined from radiation dose-response studies in clinically relevant dose ranges and treatment schedules. This will assist in prioritizing therapies for clinical trials.
Models for studies of late effects and their mechanisms must be chosen carefully, and in some cases, new models should be developed.

critical to the definition of the issues *in vivo*, so that hypotheses can be generated that are relevant to human pathophysiology. Research in this area would be facilitated by establishing a repository of irradiated normal tissues, starting with a limited sample, with records on radiation dose and schedule, tumor type, size and response, time after treatment, and some measure of tissue response. While sampling irradiated tissues from patients for research purposes will not be possible, tissues removed as part of treatment for complications could be stored for study. Tissue sharing is another way to meet these needs. Once the tissue bank has been established and is of sufficient size, these tissues would be valuable resources for researchers looking for genetic or molecular bases for late effects.

Adaptation of current mechanisms or new mechanisms of funding may be needed to provide long-term support for all phases of research on late effects: for preliminary data, for preclinical research, and for clinical research, for which follow-up periods of 10, 15 or 20 or more years may be necessary. While grantees must be held accountable for being productive, the standards that should be applied need to account for their long-term nature, as in clinical studies, where a single trial usually covers several years. Investigators might be judged periodically on their progress, i.e. past accomplishments, and on the ongoing accumulation and analysis of data. Targeted funding might be a partial answer.

A special funding mechanism, the P50 grant, supports Specialized Centers for multidisciplinary research on a specific disease entity or biomedical problem area, from basic to clinical research and development. Among the types of activity supported are protracted patient care. Applications for these grants must be in response to a Request for Applications or program Announcement issued by an Institute or Division within NIH.

It was a feeling of the workshop participants that young investigators could contribute much to this field but will need encouragement and support from mentors and institutional administrators who recognize both the importance of their research and its long-term nature. The contributions of young investigators to collaborative projects must be credited so they can receive promotions. More seasoned investigators might be able to sustain their careers by having other research projects that result in more frequent publications.

CONCLUSIONS

The key recommendations of the workshop are summarized in Table 2.

Late effects are clinically and biologically important. They are not necessarily an ''end process'' but may represent a ''chronic-active process'', and therefore may be partly reversible. Mechanistic studies will require input from an array of experts within and outside the field of radiation biology, as indicated by the scope of this workshop. Clinical studies that will be needed will require funding mechanisms that support carefully designed long-term studies on well-selected patient populations. Modifications of the current funding mechanisms or special funding mechanisms may be necessary to obtain high-quality long-term data. Evaluation of grants and investigators requires consideration of the long time span for research on late effects. Despite the complexity of the biological processes that lead to late effects, the current efforts to prevent or reverse them are encouraging. There are many opportunities in this understudied field. The greatest is the opportunity to improve the quality of life of cancer survivors.

GLOSSARY

3D CRT	three-dimensional conformal radiotherapy
ACE	angiotensin converting enzyme
AI	angiotensin I
AII	angiotensin II
BMT	bone marrow transplantation
CAM	cell adhesion molecule
COX2	cyclo-oxygenase 2
CT	computerized tomography
Cu/ZnSOD	copper/zinc superoxide dismutase
ECM	extracellular matrix
FGFs	fibroblast growth factors
FGF1	acidic fibroblast growth factor, also known as aFGF

FGF2	basic fibroblast growth factor, also known as bFGF
FGF7	fibroblast growth factor 7, also known as keratinocyte growth factor (KGF)
HGF	hepatocyte growth factor
HIF1	hypoxia-inducible factor 1
HLA	human leukocyte antigen
ICAM1	intercellular adhesion molecule 1
IFNG	interferon gamma
IMRT	intensity-modulated radiotherapy
IP10	interferon gamma-inducible protein 10
LOH	level of heterozygosity
MHC	major histocompatibility complex
MMPs	metalloproteinases, matrixins
MnSOD	manganese superoxide dismutase, orgotein
MRS	magnetic resonance spectroscopy
MT	magnetization transfer
SMAD	messenger in TGFB signaling
SPECT	single photon emission computed tomography
SPF	specific-pathogen-free
TIMPs	tissue inhibitors of metalloproteins
TNM	T = tumor extent, N = regional lymph node involvement, M = metastases
VEGF	vascular endothelial growth factor

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