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Chapter XVIII: Specific Techniques in Radiobiology to Approach Specific Challenges

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Goals Specific to Research in Radiobiology Require Specific Approaches or Techniques.

This chapter will address three unanswered questions in Radiobiology: 1) The abscopal effect; 2) Mechanism of the latent period between resolution of acute ionizing irradiation effects and the onset of late effects; and 3) Drift of the LD50/30 total body irradiation dose over time.

Why are these three topics among the most important in radiobiology research, and why do they require novel methodologies and resources?

The Abscopal Effect.

Great radiation biologist, clinical radiation oncologist, and world famous expert in tumor virology, Henry S. Kaplan, M.D., once said "When one is required to aim a beam, one is obligated to have a higher level of intellectual curiosity". Henry Kaplan was an undisputed giant in the history of both radiobiology and Radiation Oncology. His research on the therapeutic management of Hodgkin's Disease is a classic (1), but Dr. Kaplan was also an extremely careful and diligent scientist. He discovered the Radiation Leukemia Virus, which is referenced in the textbook by Ludwik Gross, "Oncogenic Viruses". He elucidated the mechanism of irradiation-induced Lymphomagenesis in the mouse model (2). One of his greatest achievements during a long and productive career, as a clinician, scientist, and teacher was to define questions to be answered by the next generation scientists.

The abscopal effect, is one such interesting phenomenon of radiobiology and its mechanism yet to be elucidated.

Abscopal (away-from) effect describes the observation that patients treated with radiotherapy to a tumor mass in one anatomic location, often displayed an unexpected regression of tumor in another location, distant from the irradiated volume. This effect is often referred to as the "positive abscopal effect". There is another phenomenon often referred to as the "negative abscopal effect", during which patients, who are irradiated to one anatomic area of, for example, the head and neck region may display an unexpected and severe reduction in peripheral blood counts (neutrophils, platelets, red blood cells, and lymphocytes). The observed drop in blood counts is not consistent with the volume of tissue irradiated, certainly with respect to the percentage of bone marrow outside the irradiated head and neck region. Experimental animal studies confirm both the positive and negative abscopal effects (4-6). Animas studies have facilitated research into the molecular and cellular mechanisms involving both phenomena. Mice irradiated to the head and neck region in either single fraction or fractionated irradiation display suppression of distant femur (hind limb) marrow cellularity and marrow function (4-6). This model system has become valuable for research in understanding the mechanism.

Placing orthotopic tumors in two anatomic locations with irradiation to one site has also facilitated studies to determine the mechanism of tumor regression in the non-irradiated orthotopic tumor placed at a distant site. The current consensus for the "positive abscopal effect" is that it represents irradiation-induced immune cell sensitization to tumor, which is extended to immune cell attack on the unirradiated site. Studies with PD-1 receptor targeted drugs support this hypothesis (7). However, the molecular mechanism of distant marrow suppression "negative

abscopal effect" is not yet known. Model systems are available to study both the negative and the positive abscopal effect.

Irradiated tissues release not only the products of dead or dying tumor cells, but also in the case of creating tissue volumes in which there is no tumor (post-operative radiation), there is known upregulation of transcription for stress response genes and genes for inflammatory cytokines (4-6). Many of these protein signaling molecules (cytokines) are known to have effects on distant bone marrow stem cells, and cells of the hematopoietic microenvironment. Ionizing irradiation effects on specific tissues cause release of signaling molecules including nucleic acids from cell breakdown and oxidized lipids. Oxidized lipids have recently been shown to have important signaling roles including stimulation of sterile inflammatory responses (8) and chemotactic functions (9) such as calling neutrophils to sites of radiation-induced inflammation. In past decades, there was much interest in irradiation-induced TGF-β, which was reported to have a negative regulatory role in hematopoiesis (10). Irradiation of a volume of tissue was shown to release increased quantities of TGF-β into the serum and experiments explanting bone marrow from distant sites showed TGF-β mediated inhibition of hematopoietic colony formation (11). However, recent data has shown that the abscopal marrow suppression from head and neck irradiation still occurs in mice genetically altered to resist TGF-β stimulation (6) and also occurs under conditions in which a TGF-β receptor antagonist inhibits TGF-β signaling (11).

How should radiobiologists approach study of the "negative" abscopal effect? Explant of cells from the irradiated site and distant site offers an approach toward defining the molecular mechanism of the effect, but does not substitute for critical in vivo studies. Hypothesis driven research is needed in this area. While tools are available for analyzing every molecular signaling molecule in the circulation, measured serially after irradiation, these techniques usually lead to identification of a very large number of molecules in many categories and in many patterns. Simply surveying all proteins (in Heat maps of proteomics assayed data display), peptides, small molecule hormones, nucleic acids, lipids, clotting factors, and other signaling molecules is an approach that will produce more data than can possibly be analyzed even with the power of modern computers algorithms. If one chooses to take this approach ("screening"), then multiple measurements and a time course of appearance or disappearance of specific signaling molecules must be considered. One approach might be to deliver a single fraction 30 Gy irradiation to the head and neck of C57BL/6 mice (an experimental model in which many of the abscopal experiments were carried out (4-6)) and then take plasma (treated to prevent clotting and removal of clotting factor linked small molecules) from the venous outflow of the tumor volume with sampling in real time, starting prior to irradiation, and then immediately after irradiation. A specific approach might be to take the sample every minute for the first ten minutes, and then hourly for the first 24 hrs., and then daily up to day 5. Day 5 is the time in which a profound abscopal effect has been demonstrated. Analyzing plasma samples for "everything" is theoretically possible.

An alternative and perhaps better approach (and one that is recommended), would to be to think critically about the possible signaling molecules that could cause abscopal marrow suppression and generate a hypothesis. The time course, over which, the abscopal effect disappears is also not yet completely elucidated. For example, are the marrow suppression effects still seen 10 days or 30 days after head and neck irradiation? The confounding variable in this experimental

paradigm is that the distant, suppressed bone marrow, may have adapted to the appearance of the abscopal effect signal(s) (molecule or molecules) and become resistant.

Para-biosis experiments have an attractive potential way to elucidate the time course of the abscopal effect. Two animals are connected to share a common circulation. Microsurgical techniques are utilized to merge the arterial circulation from one head and neck irradiated animal to the venous circulation of an unirradiated animal. Similarly, the venous outflow from the unirradiated partner is returned in a circular pattern to the arterial system of the irradiated partner. If the para-biosis experiment is initiated, the animals should be established with the circulatory procedures carried out, and then several days allowed for healing of the surgical wound and adaptation of the physiology. This time delay would be critical for such an experiment, because as described in the chapter in this textbook on "combined injury", radiation plus wound (surgical procedure) represent a significant change in the conditions of the radiobiologic response. The surgery, itself, induces many of the signaling molecules that are being studied in the irradiated environment. The para-biosis experiment does allow confirmation of the existence and the time course of the expression of those signaling molecules that come out of the irradiated volume. Marrow suppression (cell number, total clonogenic hematopoietic cells, day 7 and day 14 CFU-GEMM (6) and competitive repopulation assay of true stem cells (12)) all of which are parameters that can be measured in the non-irradiated partner at serial time points after irradiation of the other partner. If the abscopal effect is demonstrated in the distant femur marrow of the unirradiated partner at 5 days, as has been published in recent experiments of head and neck irradiation suppression of the femur marrow (4-6), then the question arises as to when the abscopal suppression dissipates. If it is gone by 10 days or 30 days, then the search for the mediator of the abscopal effect can be carried out by measurement of plasma at multiple time points.

The Latent Period

Another major challenge in Clinical Radiation Oncology and Radiobiology is an explanation of why there is a latent period between resolution of the acute irradiation effects and onset of late effects. The latent period is a term that was initially used in Clinical Medicine to describe conditions of infection by principally viruses. Viral infection can occur rapidly, and then a patient may present for an interval with no symptoms or signs for weeks to months. There is then reactivation of the virus with onset of the classic viral syndrome including fever, loss of appetite, sleepiness, lethargy, and shaking chills. The latent period has also been termed "incubation period" when referring to transmission of infectious agents.

In Radiation Biology, and in Clinical Radiotherapy, there is a different form of latent period, the explanation for which has continued to elude scientists. Single fraction or fractionated irradiation induces normal tissue damage in the irradiated volume, and these pathologic and histopathologic features have been well described. After healing and recovery, patients in experimental animals demonstrate restoration of normal tissue function, anatomy, and appearance. Depending on the irradiation dose and volume treated, there is then the appearance after some delay of the irradiation late effects, principally, fibrosis, formation of new blood vessels (telangiectasia), and loss of function of the particular irradiated volume (13-14). The

interval between these two phenomena is called the latent period. Recent research has focused on finding the molecular changes in the irradiated tissue during the latent period (14).

C57BL/6J mice, which develop radiation fibrosis, demonstrate a recurrence during initiation of the late effects of the same gene transcripts that are found during the acute irradiation effect. In contrast, C3H/He mice, which do not develop irradiation fibrosis, show an upregulation of the transcripts for inflammatory cytokines and stress response genes that is similar to that in the C57BL/6J mice, but do not return to a baseline level, but show rather a pattern consistent with a continued acute effect (15). However, the C3H/He mice, while not developing fibrosis, do show the same irradiation-induced life shortening and other phenomena of continuous irradiation damage. The upregulation of proteins during the acute effect, is similar to the upregulation during the late effects in the C57BL/6J model (15).

One recent theory to explain onset of late effects has been the suggestion that microvascular endothelial cells in the irradiated volume accumulate signaling molecules such as thrombomodulin (16) during the weeks to months of the latent period, and only when reaching a specific level do those endothelial cells begin to die, express inflammatory cytokines, and induce a secondary reaction. However, the secondary reaction elicits proliferation of fibroblasts in the irradiated volume, and migration of inflammatory progenitor cells from the marrow into the irradiated volume (17), so if endothelial cells do explain initiation of the late effects, there are different signaling molecules involved that are endothelial cell specific.

Another theory to explain the latent period is elucidated in modern textbooks (18), is that a slowly proliferating population of cells in the irradiated volume takes weeks to months or even years to initiate a cell cycle response. Once cycling, then these cells die and produce a secondary inflammatory response. However, the secondary response is one leading to fibrosis, as described above, and must involve different signaling molecule experiments to attempt to define the slowly proliferating stimulation of cells revealed that these probably not endothelial cells, fibroblasts, or inflammatory cells migrating into the irradiated tissue (17). At the present time, it is known that there are genetic determinants of initiation of the late effects, there are molecular biologic changes occurring in tissues both at the transcriptional and translational level, and that some animal models display different durations of the latent period. Also, known is the role of radiation dose, fraction size, and volume irradiated in the likelihood of observation of late effects. The rest of the mechanism is yet to be elucidated. This area of research remains very important for scientists entering the field of Radiobiology.

Radiation Dose "Drift" for the LD50/30

For decades, experimental testing of the effect of new radiation dose modifying agents, has required the use of experimental animal models. Rodent species are most desirable due to general institutional animal experimentation concerns, using the animal model that is most appropriate for the human analogy of the research topic, and also the best model in which to be able to minimize animal suffering, and gain experimental data with the fewest number of animals per test group. Mice have remained the most valuable experimental model for testing the effects of new agents on the total body irradiated subject. There has been a problem with these kinds of studies that plagues, all researchers, namely, that of the drift of the total body irradiation dose,

which is defined as the LD50/30 (a radiation dose, which kills 50% of the animals in the group at 30 days). The LD50/30 is taken to represent acute radiation response, or death from the hematopoietic syndrome. This irradiation dose is one in which transplantation of bone marrow after delivery of that dose will result in survival of all animals. Thus, it is a radiation dose, which is "rescuable" by bone marrow transplant.

The problem has been that the LD50/30 dose has been observed to change from week to week or month to month. Many investigators have confirmed this phenomenon and attributed it to changes in diet, changes in animal husbandry, or variations in groups of animals obtained from the animal breeder or supplier.

In recent years, the Center for Medical Countermeasures Against Radiation Consortium has focused much effort on this problem. The Physics Core, Ke Sheng, Ph.D., P.I. at the UCLA CMCR has been established and careful dosimetry have been carried out on animal irradiators used between centers, as well as other outside research institutions. Careful attention to radiation beam flatness over the groups of animals being treated, dose rate, concern for confinement of animals in a small area during the irradiation, and other parameters of the physical constraints have been addressed. The physics of delivery of total body irradiation and uniformity between experiments must be established before any research into the mechanism of the radiation dose drift affect can be carried out. Recent publications have focused more on precise dosimetry for TBI of mice (19). Once the physics of the radiation delivery are analyzed and normalized, one then must turn attention to the condition of the animals.

The breeder/supplier of animals should be the same between experiments. For example, mice obtained from Taconic Farms, or Harlan Sprague Dawley, or Jackson Laboratories can't all be the same, because of genetic drift, nor may they be maintained under the exact same conditions. Therefore, attention to this detail should be included. Assuring that the gender, weight, and age of mice is identical between experiments must be done. Consistency of the animal quarters and testing of animal facilities for pathogens, a reliable Institutional Animal Care and Use Committee (IACUC) of all animal experimentation must be in effect. There can be no change in the level of screening for potential infectious agents. The LD50/30 of animals can be affected by infectious agents for rodents that have been described including mouse parvovirus, fur mites, and others. Studies with male mice that are known to be more radiosensitive (20) also must include attention to the issue of animal crowding, fighting, and wounds. Male mice are known to have wounding on the tail region and some of the back, and this produces a "combined injury" effect and can greatly increase the number of animals dying at 30 days after irradiation.

Radiobiology research is currently focusing on the microbiome of the mouse intestine and lung and how this can be altered by changes in diet. Many animal feed suppliers are required to provide information on nutrition, but not constituents of the feed.

References:

1. Kaplan HS. Prognostic significance of the relapse-free interval after radiotherapy in Hodgkin's Disease. Cancer, 22(6): 1131-1136, 1968.

2. Haran-Ghera N, and Peled A. Induction of leukemia in mice by irradiation and radiation leukemia virus variants. Advances in Cancer Research, 30: 45-87, 1979.

3. Morgan WF, and Sowa MB. Non-targeted effects induced by ionizing radiation: mechanisms and potential impact on radiation induced health effects. Cancer Lett, 356(1): 17-21, 2015.

4. Berhane H, Shinde A, Kalash R, Xu K, Epperly MW, Goff J, Franicola D, Zhang X, Dixon T, Shields D, Wang H, Wipf P, Li S, Gao X, and Greenberger JS. Amelioration of irradiation induced oral cavity mucositis and distant bone marrow suppression in Fancd2-/- (FVB/N) mice by intraoral JP4-039/F15. Radiat Res, 182: 35-49, 2014.

5. Shinde A, Berhane H, Rhieu BH, Kalash R, Xu K, Goff J, Epperly MW, Franicola D, Zhang X, Dixon T, Shields D, Wang H, Wipf P, Parmar K, Ferris R, Li S, and Greenberger JS. Intraoral mitochondrial-targeted GS-nitroxide, JP4-039, radioprotects normal tissue in tumor-bearing radiosensitive Fancd2-/- (C57BL/6) mice. Radiat Res, 185: 134-150, 2016.

6. Willis J, Epperly MW, Fisher R, Zhang X, Shields D, Hou W, Wang H, Li S, Wipf P, Parmar K, Guinan E, Steinman J, and Greenberger JS. Amelioration of head and neck irradiation-induced mucositis and distant marrow suppression in Fanca-/- and Fancg-/- mice by intraoral administration of GS-nitroxide (JP4-039). Radiat Res, 189: 560-578, 2018.

7. Vendetti FP, Karukonda P, Clump DA, Teo T, Lalonde R, Nugent K, Ballew M, Kiesel BF, Beumer JH, Sarkar SN, Conrads TP, O'Connor MJ, Ferris RL, Tran PT, Delgoffe GM, and Bakkenist CJ. ATR kinase inhibitor AZD6738 potentiates CD8+ T cell-dependent antitumor activity following radiation. J Clin Invest, 2018 Aug 13. Pii: 96519. Doi: 10.1172/JCI96519 (Epub ahead of print).

8. Tyurina YY, Poloyac SM, Tyurin VA, Kapralov AA, Jiang J, Anthonymuthus TS, Kapralova VI, Vikulina AS, Jung M-J, Epperly MW, Mohammadyani D, Klein-Seetharaman J, Jackson TC, Rochanek PM, Pitt BR, Greenberger JS, Vladimirov YA, Bayir H, and Kagan VE. A mitochondrial pathway for biosynthesis of lipid mediators. Nature Chemistry, 6(6): 542-552, 2014.

9. Samhan-AAK, Ji J, Demidova OM, Sparvero LJ, Feng W, Tyurin V, Tyurina YY, Epperly MW, Shvedova AA, Greenberger JS, Bayir H, Kagan VE, and Amoscato AA. Oxidized phospholipids as biomarkers of tissue and cell damage with a focus on cardiolipin. Biochim Biophys Acta, 1818(10): 2413-2423, 2012.

10. Boerma M, Wang J, Sridharan V, Herbert JM, and Hauer-Jensen M. Pharmacological induction of transforming growth factor-beta1 in rat models enhances radiation injury in the intestine and the heart. PloS One, 8(7): E70479, 2013.

11. Hauer-Jensen M, Richter KK, Wang J, Abe E, Sung CC, and Hardin JW. Changes in transforming growth factor β1 gene expression and immunoreactivity levels during development of chronic radiation enteropathy. Radiat Res, 150(6): 673-680, 1998.

12. Epperly MW, Zhang X, Fisher R, Franicola D, Shields D, Quickel M, Hankey-Giblin P, Wang H, and Greenberger JS. Reduced competitive repopulation capacity of totipotential hematopoietic stem cells in the bone marrow for Friend Virus infected Fv-2-resistant mice. In Vivo, 31(3): 313-320, 2017.

13. Langberg CW, Sauer T, Reitan JB, and Hauer-Jensen M. Relationship between intestinal fibrosis and histopathologic and morphometric changes in consequential and late radiation enteropathy. Acta Oncologica, 35(1): 81-87, 1996

14. Kalash R, Epperly MW, Goff J, Dixon T, Sprachman MM, Zhang X, Shields D, Cao S, Wipf P, Franicola D, Berhane H, and Greenberger JS. Amelioration of irradiation pulmonary fibrosis by a water-soluble bi-functional sulfoxide radiation mitigator (MMS350). Radiat Res, 180: 474-490, 2013.

15. Kalash R, Berhane H, Au J, Rhieu BH, Epperly MW, Goff J, Dixon T, Wang H, Zhang X, Franicola D, Shinde A, and Greenberger JS. Differences in irradiated lung gene transcription between fibrosis-prone C57BL/6NHsd and fibrosis-resistant C3H/HeNHsd mice. In Vivo, 28: 147-172, 2014.

16. Richter KK, Fink LM, Hughes BM, Sung CC, and Hauer-Jensen M. Is the loss of endothelial thrombomodulin involved in the mechanism of chronicity in late radiation enteropathy? Radiotherapy and Oncology, 44(1): 65-71, 1997.

17. Epperly MW, Sikora CA, Defilippi S, Gretton JE, and Greenberger JS. Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. Am J Resp Molecular Cell Biology, 29: 213-224, 2003.

18. Denham JW, Hauer-Jensen M, and Peters LJ. Is it time for a new formalism to categorize normal tissue radiation injury? Int J Radiat Oncol Biol Phys, 50: 1105-1106, 2001.

19. Steinman J, Epperly M, Hou W, Willis J, Wang H, Fisher R, Liu B, Bahar I, McCaw T, Kagan V, Bayir H, Yu J, Wipf P, Li S, Huq MS, and Greenberger JS. Improved total-body irradiation survival by delivery of two radiation mitigators that target distinct cell death pathways. Radiat Res, 189(1): 68-83, 2018.

20. Epperly MW, Smith T, Wang H, Schlesselman J, Franicola D, and Greenberger JS. Modulation of total body irradiation induced life shortening by systemic intravenous MnSOD-plasmid liposome gene therapy. Radiat Res, 170(4): 437-444, 2008.