Chapter IX: Acute Radiation Effects: Organ Specific Organs Dose and Species Differences

Section L: Irradiation Effects on the Microenvironment

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Introduction:

Tissue Organoids as a Platform for Study of the Microenvironment:

In recent years, advances have been significant with respect to explant of tissue organoids. An organoid contains stem cell populations, differentiated progeny, and cells of the microenvironment (50). Tissue explant systems have included simple culture techniques, as well as those, which employ a scaffold or biomatrix onto which prints out phenotypes within the organoid can attach. Systems for organoid culture include those with small intestine (51), skin (52), bone and cartilage (58), and solid tumors (59). A system of fetal brain organoid cultures has also been published (60). Tissue organoids represents another potential tool for the study of Radiobiology, however, the organoid system does emphasize the limitations of ex vivo culture. From the time of explant, cells in the organoid rely exclusively upon interaction with other cells in proximity, and the tissue culture medium and/or scaffold. The absence of the circulation in regulated protein, fatty acid, and peptide levels is absent. Therefore, there is inherent limitation in the organoid system.

Organoids can be valuable for comparing the radiobiologic interaction between cell populations to confirm results seen in vivo (For example, if irradiated small intestine (ilium) in vivo at five days after radiation exposure shows a decrease in the length of intestinal villi, breakdown of the mucin protective layer at the lumen and disappearance of lymphoid cells, some of these observations can be confirmed in the organoid. However, it is not possible to replicate the systemic immune response and circulatory delivery of neutrophils and macrophages to the irradiated site, which is seen in vivo. These other cell systems may influence the biology of the intestinal villi from days 2 – 5, and these influences will be absent from the organoid culture. However, if a biomarker for intestinal crypt cells (stem cells) reveals decrease in these cell numbers over the 5 days of irradiation in vivo, similar studies could be carried out on tissue organoid and could confirm the disappearance of the cells with a stem cell marker. Another potential advantage of tissue organoids is that they allow study of cell populations that may be rapidly eliminated in vivo by the same systemic circulatory responses. For example, study of intestinal/enteric neurons, which have been shown to extend from the muscularis layer into the lumen through the villi may be persistent in irradiated organoid culture, whereas these cells may disappear or be undetectable, because of rapid inflammatory changes seen in the tissues in vivo.

In all in vitro organoid culture systems, the microenvironment is disturbed. Changing components of the culture medium including addition or deletion of specific hormone supplements, and even the influence of antibiotics and antifungal agents required to maintain tissue culture may have a profound effects on the mix of cells in the organoid.

The long-term bone marrow culture system represents first successful “organoid” explant system to provide evidence for stable interaction over weeks – months of stem cell population with cells of the microenvironment (1-4) (Fig. 1) The “cobblestone island” of flattened hematopoietic cells or the stromal cells indicates healthy hematopoiesis at one year after 7.5 Gy total body irradiation. Marrow stromal cells grew, but there is no hematopoietic activity (Fig. 2). A caveat is that all cells detected in the bone marrow microenvironment in vivo are not detectable in the organoid system. There has been no description of bone marrow neurons in long-term bone

marrow cultures. Furthermore, the requirement for 17-Hydroxycorticosteroid as a supplement to maintain long-term cultures (1), brings with its use the loss of steroid-sensitive lymphocyte, which are found in the intact bone marrow in vivo. The observation that totipotential hematopoietic stem cells persist in long-term cultures, despite the presence of corticosteroids and absence of other cell populations is itself an interesting observation (6-12). However, investigators using long-term bone marrow cultures understand the limitations of the system. Co-culture of specific cell populations has been a mainstay of cell biology for decades, but the continuous bone marrow culture system is very different from a co-culture. The attachment of specific cell populations to form adherent layers in bone marrow culture, and the observation that a fully formed long-term culture at 4 weeks arises from migration outward in the flask of deposit/clumps of complex bone marrow cell populations represents an extension of an organoid. Each clump of bone marrow cells observed within hours of explant, can be observed to contain hematopoietic, stromal, endothelial cells, and adipocytes. Adipocytes were first described in long-term marrow cultures in both an intrinsic level in horse serum or by adding 17-Hydroxycorticosteroids (1). However, adipocytes are not necessary, as originally proposed, for maintenance of hematopoietic stem cells. In fact, the accumulation of neutral fact in bone marrow stromal cells was shown to be dependent upon high fat levels in the culture medium and simply changing the culture conditions from horse serum to fetal calf serum removed the presence of adipocytes and actually increased the longevity of hematopoiesis in cultures by preventing the sloughing from the adherent layer of cells laden with fat droplets (4-5). Those investigators studying the radiobiology of organoids should review the literature on long-term bone marrow cultures to understand the limitations of an explant system.

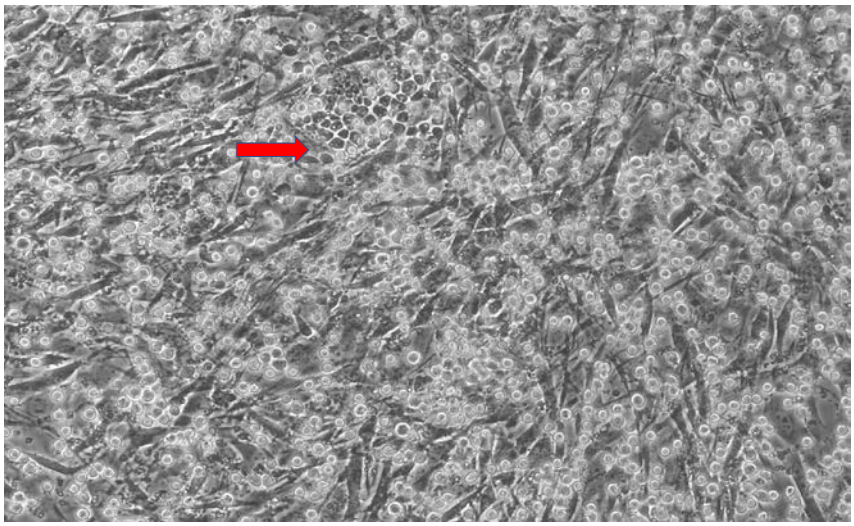

Fig. 1: Long-term marrow culture at week 8. Cobblestone island – red arrow. (x 200)

Fig. 2: One year after 7.5 Gy total body irradiation explanted marrow shows no cobblestone islands (x 100)

Generating Organoid Culture Systems from Embryonic Stem Cells or Inducible Pluripotential Stem Cells

A recent revolution in biology has been the technology necessary to maintain embryonic stem cells. Pioneering studies of Beatrice Mintz and Rudolph Janisch (61) demonstrated the ability to transfect embryonic cell populations with retroviruses and other gene sequences to facilitate implantation of these transduced stem cells into the uterus of pregnant recipient mice and create embryos with specific genetic changes (49). Removing genes and gene functions was also possible through the transduction of embryonic stem cell populations with specific targeted genes (62). These studies required implantation of the genetically engineered embryonic stem cells back into foster mothers to produce genetic mouse strains. Keeping the embryonic stem cells in culture required modification of culture conditions including strict regulation of the growth factors (OP/OP mice). The ability to propagate and expand in vitro embryonic stem cells allowed studies of changing the culture medium conditions and pushing such cells in different directions in a sense of "directing traffic" toward hematopoietic lineage, muscle cell formation, neuron formation, and other lineages. These studies allowed elegant experiments in the radiobiology of genetic limitations induced by irradiation and the radiosensitivity of embryonic stem cells.

Inducible pluripotential stem cells represent yet another revolutionary concept in advance, which has implications for radiobiology. Differentiated cells from adults, transfected with 5 genetic markers including OCT and RAS resulted in dedifferentiation of cells in culture and restoration of markers of embryonic stem cells. These transduced cells could then be induced to differentiate into multiple lineage as or the embryonic stem cells. Expansion of differentiated cells and re-implantation into immunoincompetent mice led to generation of chimeric animals with xenotransplanted (cross-species) cells of different lineages. In the microenvironment of the host rodent, transduced human iPSC or mouse embryonic stem cells (Fig. 3) were shown to enter the microenvironment, and then provide cellular functions that either complemented or reversed disorders in the rodent recipient. The potential for radiobiological studies in these xenotransplant system has recently been recognized. As with the organoid culture systems described above, there are limitations. Prominent is the absence of supportive microenvironment cells from the same species, as was the case with transplant of human bone marrow or cord blood into

immunoincompetent NOD/SCID mice. Human blood cell hematopoiesis in evolution of acute myeloid leukemia could be observed, but the response of these hematopoietic cell populations to the rodent microenvironment in other organs that were not transplanted (spleen, lymph nodes, thymus, and liver) was observed to be different.

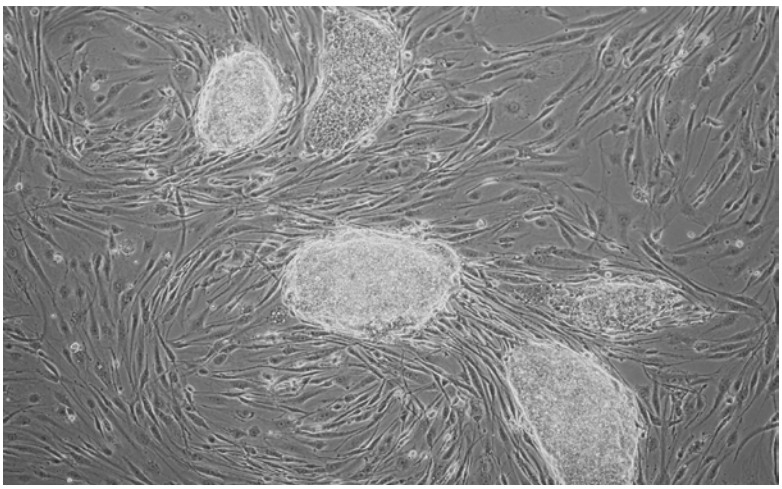

Fig. 3: Embryonic stem cell spheroids growing over stromal cell monolayer.

Research Opportunities Using Organoids, Embryonic Stem Cells, and Long-Term Bone Marrow Cultures

Since the discovery of oncogenes (gene sequences capable of inducing transformation to malignancy of cells in culture or tissues in animals) and repressor genes (genes shown to prevent evolution of malignant cells from cell lines in vitro or in animals), the radiobiology of each of these classes of genes has been a subject of intense investigation. The first studies showing the induction of radiation resistance of cells in culture by an expressed oncogene N-RAS (a human gene analogous to the Kirsten Sarcoma virus oncogene of rats – K-RAS) showed that transduced cells were radioresistant (63). Cell lines in which a specific DNA repair gene or scaffold protein onto which attaches an enzyme critical for repair of DNA double strand breaks, radiosensitive Fancd2-/- cells (47) provided the first evidence of use of these model systems in radiobiology. However, the overexpression of a transduced gene or absence of a gene cannot produce the same radiobiologic effects in all tissues and in all cell phenotypes. For example, absence of a gene product in the Fanconi Anemia pathway (Fancd2) resulted in irradiation sensitivity of mesenchymal stem cells, but paradoxical radioresistance of hematopoietic cells (47).

The relative radiosensitivity of total body irradiated Fancd2-/- mice indicated that the overriding predominant effect was that similar to the radiobiology of the mesenchymal stem cell; however, the multiple other organs and organ systems present in an in vivo total body irradiation experiment are absent, so such a conclusion cannot be reliably derived in the absence of additional information.

Given the complexities of the cell culture systems available including organoids (50-56), embryonic stem cells (49), inducible pluripotential stem cells (50), and genetically manipulated culture systems, the use of any of these systems to study the effects of radiation modifying drugs adds yet another layer of complexity. Administration of a radiation protector (given prior to

irradiation) or a radiation mitigator (given after irradiation, but before the onset of symptoms and signs of radiation illness), a total body irradiated animal may result in an increase or decrease of survival in irradiated animals. All such studies include groups of animals, who receive no irradiation, but the drug being tested. If there is no detectable effect on survival of the drug in unirradiated animals, despite exhaustive testing to identify even a modest change in physiology, biochemistry, or metabolism, one cannot reliably conclude that the drug has a radioprotector or radiation mitigator effect on all cell populations in the animal. As described above with cell lines established from Fancd2-/- mice, some may be radiosensitive and others radiation resistant, while the entire intact animal is radiosensitive. The effect of addition of radiation protector or mitigator drug to these different cell populations in vitro also can produce disparate effects depending on cell phenotype. There are multiple possible explanations for phenotypic differences in response to a radiation dose modifying drug. Some cell phenotypes stay activated, other biochemical systems during differentiation to that phenotype, which counteract the effect of a specific drug on another radiation dose modifying biochemical pathway. Some cell phenotypes may have a rapid response to irradiation resulting in upregulation of biochemical pathways that neutralize the drug. For this reason, mechanistic study of a new radiation dose modifying drug (radioprotector, radiation mitigator) are usually initiated with study of cells in culture from an animal model system, then studies of total body irradiated animals, and ultimately translation to studies in larger animal models (Readers should see the chapter by Manning on large animal models), and ultimately in human clinical trials.

Species differences in response to any drug, not just those associated with radiobiology, have been known for decades. A radiation dose modifying drug, which shows potent effects on rodent cell lines in vitro, and mice in vivo, may be ineffective with human cord blood stem cells (64).

Therefore, the elegant tools available for the radiation biologist including those described in this chapter may help elucidate mechanisms of radiation effect on organs and organ systems, which may be confirmed by studies in animal models. However, the investigators should be aware of the requirement to confirm these studies in multiple animal species including those which may not facilitate use of all the in vitro culture tools described in this chapter, but which must be analyzed for translation of the work in tissue culture systems to large animal models and humans.

How Can Long-Term Bone Marrow Cultures Can be Used in Radiation Biology?

The radiation microenvironment of the bone marrow is perhaps the most well studied and well delineated of all organs or organ systems in the body. Pioneering studies by E. Donnell Thomas, M.D. first demonstrated that lethal doses of total body irradiation could be rescued by intravenous administration of donor bone marrow from another animal. These studies initially done in dogs have been practically reproduced in multiple rodent models, pigs, and Rhesus Macaque monkeys (65). The first bone marrow transplantation in humans was carried out in the 1960s, and this evolutionary medical breakthrough led to protocols of bone marrow transplantation in a wide variety of patients in whom the delivery of total body irradiation was therapeutic and designed to clear space in the recipient bone marrow by removing hematopoietic progenitor cells and differentiated progeny in recipient's bone marrow.

The experiments of E.D. Thomas (60) at the University of Washington in Seattle and George Santos (61) at Johns Hopkins Medical Center, as well as many other marrow transplant centers documented several strong conclusions from these first experiments including:

1. Peripheral blood cell of all lympho-hematopoietic lineages (T-lymphocytes, B-lymphocytes, myeloid/neutrophil-macrophage progenitors, erythroid cells, and megakaryocytes leading to platelet production) have been shown to be of donor bone marrow origin.

2. There was nearly always chimerism, situation in which residual recipient hematopoietic cells live in co-existence with the predominant donor bone marrow transplanted cells.

3. Bone marrow cells when injected intravenously or intraperitoneally, transited other organs, where they could be identified by radiolabeling techniques, but then left those organs (lung, liver) to "home" to the marrow microenvironment.

4. The histocompatibility markers, which were being elicited in great detail during the same years, revealed that bone marrow transplantation across major histocompatibility barriers often led to the phenomenon of graft vs. host disease, as well as host vs. graft disease. In the former situation, hematopoietic cells differentiating into T-lymphocytes, B-lymphocytes, as well as NK cells and dendritic cells often can recognize the recipient as "foreign", and begin a system of rejection of tissues in the recipient. These rejection phenomenon were observed in the skin, lung, soft tissues, and in the bone marrow itself. The phenomenon of host vs. graft disease had been well described previously with other organ transplant attempts across major histocompatibility barriers. Subsequent experiments showed that irradiation of the thymus in the recipient could greatly reduce graft vs. host disease and led to an array of experiments showing that T-lymphocytes of donor bone marrow origin were "educated" in the thymus and programmed to respond to foreign antigens. In the absence of this thymic education, T-lymphocytes of donor origin produced less graft vs. host phenomenon.

After these additional observations on the success of bone marrow transplantation, another field of transplantation medicine evolved.

A major conclusion from these initial marrow transplant experiments was that above a certain total body irradiation dose bone marrow transplantation could not save the irradiated individual. Thus, the categories of "hematopoietic syndrome" (rescued by marrow transplantation) was described. Other chapters in this web-based textbook describe the phenomenon of the gastrointestinal syndrome, the central nervous system syndrome, and the total body irradiation dose rates associated with each. Perhaps, most significant about these initial marrow transplant studies was the opening of a door to understanding the basic principles of hematopoiesis and bone marrow biology.

Study of Hematopoietic Stem Cells in Yolk Sac, Spleen, and Embryonic Bone Marrow.

The ontogeny of the bone marrow in mammals is studied in multiple experiments of embryogenesis. In the developing mammal, the first hematopoietic cells first detected in the embryonic yolk sac, then in a specific region of the kidney, then in the spleen, and finally in the bone marrow. Species differences in the origin of evolution of sites of hematopoietic cells were reported. Rodents continued to produce hematopoietic cells in the spleen, while primates including humans do not. In humans, the spleen remains a lymphopoietic organ, but does not normally produce red cells, platelets, and white cells. The spleen retains a microenvironment (stromal supportive cells) capable of producing hematopoietic cells, and in specific diseases referred to as myelodysplastic syndromes, the spleen can produce all hematopoietic cell lineages. This phenomenon is referred to as extra medullary hematopoiesis or production of hematopoietic cells outside the bone marrow.

During the 1970s, there was a significant controversy from experimental hematologists as to whether supportive stromal cells (microenvironment) of the bone marrow was a fixed tissue, or was also dynamic and could be transplanted from a donor to a recipient. Werts and Degowin were first to demonstrate that heavily irradiated sites of the bone marrow (one hind limb of a mouse) could serve as a site for migration ("homing") of bone marrow stromal cells, as well as hematopoietic cells. Prior to this publication, it was generally accepted that hematopoietic cells could migrate from one marrow site to another. Clinical observations of partial body irradiated patients suffering subtotal body irradiation from nuclear accident and animal model experiments in which one hind limb was shielded from total body irradiation, demonstrated that the entire hematopoietic system in all bone marrow sites could be reconstituted from residual shielded marrow, but these observations were limited to the hematopoietic stem cell and its progeny (lympho-hematopoietic cells).

Transplantation of the hematopoietic microenvironment was later confirmed by others (21, 24, 30).

The components of the hematopoietic microenvironment have been shown to consist of many cell edges, not just the marrow fibroblasts, or as it has recently been called the mesenchymal stem cell. Marrow stromal fibroblasts (MSCs) have been shown in tissue culture experiments and in vivo to be capable of differentiation to osteoblasts, chondrocytes, which form bone and cartilage respectively, and also remain as fibroblast appearing cells, which support the attachment of hematopoietic cells. However, other cell lineages have been documented in the marrow microenvironment. These lineages include: endothelial cells, also called reticular adventitial cells, neurons, and pericytes (cells that surround endothelial cells). 3-dimensional structure of the hematopoietic microenvironment has also been studied. Preferential homing of injected labeled donor hematopoietic cells to sites beneath the bone in case of femur, tibia, or other long bones (endosteal area) have been shown to be relatively hypoxic suggesting that the marrow microenvironment is modified by oxygen concentration within specific anatomic site. Other studies have suggested that a specific population of osteoblasts derived from MSCs serves at the specific site for homing of hematopoietic cells. General principles from the last several decades of research with respect to the difference between hematopoietic and stromal microenvironment cells remained unchanged.

Hematopoietic stem cells do not normally differentiate to cells of the microenvironment. Microenvironmental cells do not normally differentiate to hematopoietic cells. Thus, these two general lineages appear to be separate. In the case of malignancy of cells of the bone marrow (acute myeloid leukemia), this normal balance may be disrupted and recently publications indicate that malignant hematopoietic cells may induce a microenvironment, although the origin of such cells that produce this niche for growth of leukemia cells is not yet known.

How Does the Microenvironment Support Hematopoietic Stem Cells?

The ease of transplantation of single cell suspensions of bone marrow and the availability of multiple mouse models facilitated decades of research into the phenotype of the hematopoietic stem cells. While some controversy persists, it is generally accepted that true totipotential lympho-hematopoietic stem cells, are capable of constituting all lymphoid and bone marrow cell lineages is present in adult bone marrow at a frequency of around 1 per 10,000 cells. Fluorescence activated cell sorting (FACS) and availability of monoclonal antibodies with fluorochrome labels specific for each of several cell surface antigens, allowed precise sorting of single cell suspensions, and documentation of the cell surface phenotype of the potential hematopoietic stem cell.

FACS-7 color cell sorting for cells with the presence or absence of 7 different cell surface markers now are routinely used to sort hematopoietic stem cells for research. Controversy still persists as to whether HSCs can change cell surface markers and go back from lineage negative to lineage positive phenotype depending upon cell culture conditions. One reason for the excitement about sorting and identifying the true hematopoietic stem cell has been the goal of developing a system in which to expand such cells. Techniques including bioreactors, microenvironment external scaffold culture systems, and mixtures of growth factors have led to incomplete success. Even in the long-term bone marrow culture system (to be described below), the expansion of true hematopoietic stem cells is limited, and the persistence of the production of committed progenitors in vitro for very long times in excess of one year is attributed to the persistence in these cultures of a small number of true totipotential hematopoietic stem cells.

Genetic markers for cell surface proteins that could distinguish one donor from another using the H2-loci (mouse equivalent of the HLA histocompatibility antigens in humans) facilitated development of assays for the totipotential stem cell.

Tissue culture systems for counting numbers of hematopoietic cells first demonstrated the feasibility of quantitation of radiation effects on the bone marrow. The first in vivo technique for quantitation of marrow stem cells was that of Till and McCullough (62). The spleen colony assay (CFU_s) in which recipient mice received total body irradiation and then intravenous injection of varying numbers of donor bone marrow cells allowed investigators to count the spots in the recipient mouse spleen at 7 days providing a method to quantitate hematopoietic cell homing to the microenvironment. Studies were also carried out in bone marrow although these were much more difficult to assay since the 3-dimensional structure of the bone marrow was surrounded by solid osteoid. There was no true "surface" of the bone marrow, as there was in the spleen. Subsequent studies have demonstrated that the day 7 CFU_s represented a more differentiated cell and that waiting for 13 – 14 days for arrival on the spleen of a more primitive

cell could be documented by excising the spots and carrying out 3-dimensional evaluation of the number of lineages in the colony forming from the cell that homed to the spleen. Cell lineages in the CFU_s included erythroid (red cell), megakaryocyte (platelet progenitor), and myeloid (neutrophil/macrophage). The spleen colony assay did not document lymphoid lineages within these colonies. Other assays for the hematopoietic stem cell included the serial transfer assay in which lethally irradiated mice were transplanted with a fixed number of done bone marrow cells, and then those recipients used as donors for transplant of cells into a second generation of lethally irradiated mice. Normal adult bone marrow cells were shown to go through two – three serial transfers and at each stage reconstitute all lineages of the recipient mouse at that stage. This assay was particularly useful for determining the effect of leukemia viruses on marrow stem cells since serial transfer could be increased (12). However, increase in serial transfer assays could be accomplished with expansion of committed hematopoietic progenitor cell pools, not containing true totipotential hematopoietic stem cells (48).

At present, the best assay for true hematopoietic stem cells is a competitive repopulation assay developed by David Harrison of Jackson Laboratories. In this assay, two genetically marked and distinct populations of bone marrow are mixed in varying ratios and injected into a recipient mouse. By varying the ratios, and watching the recipient animals for 120 days (very lengthy and obviously quite expensive experiment since it requires animal housing for four months) (48), investigators could determine the relative number of true hematopoietic stem cells in each inoculant.

How to Study the Interaction of Cellular Components of the Bone Marrow's Microenvironment with Hematopoietic Stem Cells in Long Term Bone Marrow Cultures

For decades, scientists studying the interaction of various cell populations in tissue culture utilized a technique called "irradiated feeder layers". Simply stated, a lawn of confluent (cells touching each other) cells were placed on the bottom of the tissue culture dish or flask, and that flask was irradiated to a dose that would prevent cell division of those cells, usually 10 Gy.

It was appreciated that the irradiated cells did produce growth factors, and stable adhesion molecules on the surface of the irradiated cells provided attachment sites for co-cultured unirradiated cells.

This system provided a window on the effects of one irradiated cell population with respect to others. Concept of this co-culture system, as an "organ culture" was not appreciated until the discovery of the long-term bone marrow culture system. Initial studies in the 1970s by several laboratories (Dexter, Eves, Greenberger) demonstrated that flushing the contents of a mouse femur and tibia into a 40 cm. square surface area tissue culture flask resulted in attachment of hematopoietic cell islands in multiple cell lineages including totipotential hematopoietic stem cells. The fact that these islands could be maintained in culture for periods in excess of 20 weeks, and in the case of some mouse strains for over one year, indicated that a stable interaction between multiple cell populations could be studied outside the animal. The fact that these cultures contain true totipotential stem cells was confirmed by using the Harrison competitive

repopulation assay, so the system was and is available for studying the true totipotential hematopoietic stem cell in interaction with its microenvironment.

The impactful step in establishing this system was the addition of 17-hydroxy-corticosteroids critical to establishing the balance between the different cell lineages (1). Hydrocortisone was shown to stimulate production of specific cell surface molecules and growth factors, but cell population most affected have not yet been separated from the system. For example, cloning cell lines of the bone marrow fibroblasts (cloning is the establishment of a permanent cell line from a single cell) led to studies in which clonal bone marrow stromal cell lines were co-cultured with sorted populations of hematopoietic stem cells. These mixing experiments did not lead to the construct of a long-term bone marrow culture.

It is one purpose of this chapter, and textbook in general that methods be described for utilization of specific techniques in radiobiology. Therefore, a detailed description of the establishment of long-term bone marrow cultures is available and has been described in multiple publications (1-10).

Briefly, the contents of a mouse tibia and fibula are flushed with a 21 gauge needle from the excised bones into a 40 cm. square tissue culture flask. Fisher Scientific has produced a plastic formula that facilitates long-term maintenance of these cultures and was the first system utilized. However, many other plastic companies including Corning and Glass culture vessels have also been shown to facilitate establishment of the hematopoietic islands that produce long-term marrow culture. It is common to all of these glassware/plastic ware resources is the requirement for $10^5 – 10^6$ M17-Hydroxy-corticosteroid (1). Cultures are observed with an inverted microscope weekly. Healthy culture forms by growth out from clusters of attached cells to a confluent monolayer. The parameters used to compare different culture conditions in different mouse strains in the long-term marrow culture system have been described in great detail (47). This culture system has been used to demonstrate the effect of mouse genotype, addition of chemical toxins, retroviruses, and irradiation on the different cell populations within the bone marrow.

Additional experiments can be carried out using long-term marrow cultures or the co-cultivation system that preceded it (Irradiated feeder layers and a second cell population either in attachment or in a semi-solid medium co-cultured above the irradiated feeder layer.). One such example of utilization of a co-culture technique is shown in a publication in which a lead block is placed over a linear accelerator for irradiation of ½ of a culture flask. Then, fragments of the culture flask with a number of hematopoietic cell colonies forming over that particular area was calculated by placing the hematopoietic cells in semi-solid medium over the irradiated bone marrow stromal cell layer. Cells from the hematopoietic cell over-layer were not able to move, so their stimulation of growth would be a measure of the diffusion of any growth factors or inhibition of such growth factors by irradiation of half of the flask. These studies were published previously (Fig. 1 reproduced from Greenberger, et al., IJROBP, 1985). Long-term bone marrow cultures have been utilized to stimulate differentiation to multiple lineages by adding specific growth factors to this organ culture. Baseline production of cells have been shown to be along the granulocyte/macrophage lineage, however, culture conditions can be modified to stimulate lymphocyte differentiation, erythroid differentiation, or production of megakaryocytes

by altering the growth factors. Radiation biology experiments can be carried out in this culture system varying genotype and also varying the additional of growth factors with a combination of factors.

Genetic Transduction of the Hematopoietic Microenvironment.

While multiple publications demonstrate successful gene transduction of hematopoietic stem cells, embryonic stem cells, and inducible pluripotential stem cells, relatively little research has been directed toward understanding the role of introduced transgenes, or genetic manipulation of genes within cells of the hematopoietic microenvironment. Bone marrow stromal cells represent multilineage cells of the narrow microenvironment and are capable of differentiation to multiple lineage including osteoblasts, chondrocytes, and cell supportive of hematopoietic stem cell homing using promoters specific for individual cell phenotypes, investigators have selectively knocked out (recombinant deletion negative) or overexpressed specific genes. Another technique of interest and value in recent years has been the use of conditional knockout or knocked in gene regulatory techniques. These studies will link the transgene of interest to an inducible promoter such as that activated by Tetracycline or Estrogen. The advantage of this technique is that the gene of interest is not affected until after gestation, birth, weaning, and in many cases, growth to adulthood. Placing mice on drinking water containing Tetracycline/Doxycycline or Estrogen activates (in the case of TET-On) or inactivates (in the case of TET-Off) the transcription of mRNA for that gene, and the production of relevant protein (63).

An example of conditional regulation of a gene relative to radiation biology is that of Manganese Superoxide Dismutase (MnSOD, SOD2). Bone marrow stromal cells, derived from the hematopoietic microenvironment of long-term bone marrow cultures, were established from mice in which the MnSOD transgene was regulated by the TET promoter. Establishment of bone marrow stromal cell lines from these mice showed regulation in tissue culture of the radiosensitivity of the cells by TET-controlled expression of the MnSOD protein. MnSOD is the mitochondrial targeted Superoxide Dismutase, which represents the “first line of defense” against irradiation, as it neutralizes an irradiation-induced superoxide at the mitochondrial membrane. MnSOD levels in the mitochondria downregulate mitochondria membrane permeability, leakage into the cytoplasm of cytochrome-C, activation of the Caspase cascade of cell death, and apoptosis. Thus, higher levels of expression of MnSOD are associated with relative radiation resistance in cell lines, tissues, organ systems, and controlled expression of other stress response genes. The studies with constitutive (always expressed) high levels of MnSOD, in transgenic mice compared to the recent studies with TET controlled levels of MnSOD demonstrate the importance of the latter technique for studying acutely changed levels of this important protein.

The MnSOD transgenic mice are not intrinsically radioresistant (65). This observation is consistent with downregulation of other antioxidant and radiation resistance pathways in these transgenic mice during gestation and adjustment of levels of other radiation defense pathways such that animals did not show natural radiation resistance. In contrast, the TET-regulatable levels of MNSOD, which could be acutely modulated in adult animals, were consistent with rapid modulation of radiation resistance in the acute setting. Of interest, animals genetically

knocked out for MnSOD are radiosensitive, as are cell lines derived from their bone marrow microenvironment. These data suggest that MnSOD is not easily replaced by upregulation of other radiation defense pathways such as the animals are intrinsically radiosensitive (64). Reduction of the MnSOD transgene into tissues and cell lines of these mice did induce radioresistance indicating that the baseline levels in such knockout animals were intrinsically low. Of interest, the TET-regulatable MnSOD levels could be modulated in vitro by placing Doxycycline in the drinking water, and these studies led to modification of expression of antioxidant and stress response genes in an acute setting (63).

Tissue Specific Examples of Difference in Transgene Expression and Function in Different Cells of the Microenvironment.

While much research has been carried out with respect to the microenvironment of the bone marrow, recent studies have focused on cells of the microenvironment and other tissue with self-renewing stem cell populations. Slowly proliferating (or non-proliferating) tissue in the adult animal including cardiomyocytes of the heart, neuronal stem cells in the brain, and alpha/beta stem cells of the pancreas interact with cells of their microenvironment, principally epithelial cells and stromal fibroblasts. In the brain, astrocytes, and glial cells modulate and control damage response to vascular ischemic injury, and in neurodegenerative diseases including Parkinson's disease and Amyotrophic Lateral Sclerosis. A transgene introduced into the embryo of the endothelial cell specific promoter for Von Willebrand factor resulted in expression of the product of the marker gene, attached to the vWF promoter in all cells of the embryo, but only in the brain of the adult (66). These data demonstrate that endothelial cells in the adult brain are differentially regulated by other components of the microenvironment compared to the regulation of endothelial cells in other organs including bone marrow, liver, spleen, and muscle.

Do Cells of the Microenvironment Regulate Organ Specific Radiation Damage?

Radiation biologists have studied possible mechanism for the different response of specific organs to total body irradiation. As described in other chapters in this web-based textbook, hematopoietic organs including bone marrow and spleen and stem cells in the small intestine (principally the ilium) show the most rapid and greatest response to total body irradiation and result in lethality at lower doses than that required for acute damage to the brain, lung, and spinal cord. Generally accepted explanation for organs has been the presence of rapidly self-renewing stem cells in bone marrow/spleen and small intestine; however, more complex tissue and organ responses has been considered as also relevant in the explanation of differing organs responses to ionizing irradiation.

Those organs, which do not express rapid damage after total body irradiation doses that are lethal to bone marrow/spleen and intestine, do demonstrate induction of changes that lead to profound, but delayed tissue and organ specific effects. For example, managing radiation effects on the lung, principally on cells of the microenvironment for pulmonary stem cell (which are slowly proliferating) results in stable alterations in the magnitude and diversity of gene transcripts induction or suppression. These changes are persistent for months in the mouse model and years in irradiated humans. Recent evidence suggests that endothelial cells in the lung display a slow accumulation of thrombomodulin, which when reaching a threshold at a later time after

irradiation induces proliferation of intrinsic lung fibroblasts and also migration into the lungs of bone marrow origin fibroblast progenitors, both of which contribute to the late pathology of radiation fibrosis. While endothelial cells removed from lung tissue may not be distinguishable from those removed from bone marrow (reticular adventitial cells), while the radiation response in vitro may be very similar from the interaction of endothelial cells with stem cell and differentiated cell populations in their respective organs may be quite different in the setting of total body irradiation.

The microenvironment of each organ also contains immunocytes, and cells capable of further modulation of the radiation response. These cells include tissue macrophages, and neutrophils, which line the endothelial surface of blood vessels. The response of these cells to irradiation includes expression of cytokines that may regulate endothelial cell and stem cell populations in that organ. In hours to days after irradiation, migration into the irradiated tissue of other classes of immunocytes including T and B lymphocytes, NK cells, and dendritic cells adds a further layer of complexity to the organ specific radiation response.

All of these factors should be considered when attempting to explain the difference of radiation responsiveness of cell lines in culture from the same cell phenotypes when in the context of a slowly renewing tissue such as the bone marrow or slowly proliferating tissue such as the brain.

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