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Chapter XXII: Proteomics and Networks

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The first widely utilized signatures of ionizing irradiation exposure were based on studies of serum proteins. Pioneering work by Philip Rubin and colleagues (1-3) first correlated histopathologic evidence of radiation damage with elevated peripheral blood levels of inflammatory cytokines (proteins produced by the organism in response to ionizing irradiation exposure). Each of the many inflammatory proteins discovered by scientists in the 1960s and 1970s, were based on, then standard assays for protein levels. Scientists would purify a protein from the peripheral blood, immunize experimental animals (usually rabbits or goats), and then obtain large quantities of serum from the experimental animal, isolate antibodies, and purify those, which reacted with that human peripheral blood protein. Immunoassays for that protein were carried out on gel electrophoresis plates, and then radiation dose response curves of dilutions of antibody from the rabbit or goat immunoglobulin were used to present a quantitative estimate of the level of protein. The field of antibody production was revolutionized by Cesar Milstein (6, 19) with its discovery of monocloning antibodies, enabled the production of large quantities of a specific antibody by hybridoma cells lacking an immunoglobulin producing plasma cell tumor cell line with an antibody producing β-lymphocyte. Improvements in antibody identification and isolation techniques led to automation, and now the very sophisticated Luminex plate assay in which microbeads containing antibodies to each of a large number of proteins can be assayed and quantitated (4).

This chapter will review currently available technologies for quantitating proteins and also the expanded technology of protein networks (understanding how the level of an individual protein affects many others in secondary, tertiary, and quaternary) interacting that greatly extend the response to a stimuli, which can elevate or depress level of a seemingly unrelated specific protein. Measuring protein responses to ionizing irradiation is extremely critical, and cannot take the place of measurements of RNA (described in the chapter on RNA measurements, as an indication of radiation exposure), because it is the protein levels that often initiate the signaling. The signaling, then produces a response to in-field radiation exposure in clinical radiotherapy, abscopal distant effects in the body, and, of course, the response to total body irradiation.

What Do Protein Levels and Protein Signaling Indicate?

Hundreds of proteins, and their peptide components circulate in the plasma during peripheral circulation at all times. Relative levels of protein are used as the standard analysis by physicians to determine the health of an individual. Elevated levels of a protein can indicate a disease state or a response to an invading pathogen. For example, the elevated antibody level of one immunoglobulin in response to a particular bacterial pathogen such as menningioccus can be detected in the peripheral blood long before agar culture plates of that bacterium in a sample of peripheral blow from that patient grow to a level, where they can be diagnosed by a microbiologist 24 – 48 hrs. later. The antibody response to bacteria is a rapid and more effective indicator of an invading pathogen and can lead to early antibiotic treatment in time to prevent complications of that infection. As another example, low levels of the major serum protein, albumen, indicate a poor level of nutrition of an individual, and the health of that patient's liver, the anatomic site where this protein is synthesized.

There are situations in which measuring serum proteins do not reflect the state-of-health of an individual. Serum is defined as the liquid component of the blood after all cells have been

removed. Isolation of serum is typically carried out by blood centrifugation in which red blood cells, white blood cells are pelleted to the bottom of a centrifuge tube, and the liquid above (serum) is extracted for assay. The problem with this assay is that it usually is carried out using blood that has clotted, and all of the clotting proteins, as well as, other proteins bound to the clotting factors are removed in that pellet. Obviously, measurement of peripheral blood levels of clotting factors such as would be required for the diagnosis of hemophilia A (level of clotting factor VII would be low), and Hemophilia B, (level of clotting factor IX would be low) requires measurement of plasma, not serum. Clotting factors produce a gel-like material in the peripheral blood, and this gel contains not only the clotting factors, but multiple other proteins, which stick to the cascade of clotting factors during the clotting process. The protein clotting factor levels are another measure of health of the liver, as is the serum level of albumin. Clotting factors VIII, as well as serum albumen are synthesized by hepatocytes in the liver. Therefore, radiobiologists may be advised to study plasma with the serum proteins when investigating irradiation effects on the liver.

In the 1980s, scientists appreciated the importance of protein interactions with other proteins. By the current decade, computer software programs became available to integrate and display the data for the multiple downstream effects of elevation or depression of a specific protein. This presentation is termed protein networks.

The time course of changes requires multiple sampling events and putting together the kinetics of the changes. However, protein network analysis facilitates analysis of the complexity of protein level responses to ionizing irradiation.

The protein network responses to ionizing irradiation are vividly displayed by an example using that of the small protein promoters for gene activation.

Multiple promoter proteins, which are called gene promoters, were first discovered in the molecular biology of bacteriophages (viruses that infect bacteria). These proteins are nuclear DNA binding proteins that bind to specific DNA sequences and activate gene transcription into RNA, then allowing translation of proteins. Examples of promoters include NFKβ, Nrf1, SP-1, and AP-1 each of which have promoter binding sites in DNA for specific gene transcripts. The promoters have been shown to profoundly influence the radiation response. For example, mice genetically altered to abrogate the Nrf-1 promotion, have an altered survival response to total body irradiation and radiation fibrosis response (altered acute and late effects) (7, 20-21). The subsequent cascade of protein network events, which follow Nrf-1 or NFKβ binding to specific gene promoters illustrate the complexity of the protein network response. For example, irradiation induction by Nrf-1 binding to the promoter of the gene for TGF-β (transforming growth forming factor beta) may lead to elevation of this serum protein. Continued elevation of TGF-β leads to elevated levels of other inflammatory response proteins including Interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) (5). The elevation of these other inflammatory response proteins then leads to downstream effects for each of those proteins. TNF-α may bind to its receptor on cells in the intestine and induce phosphorylation of RIPK-1, then RIPK-2, and induce necroptosis (8). In a sustained injury response such as is observed after total body irradiation exposure (as well as, seen after thermal burn, traumas resulting in blood loss, and other total body stressful events), production of elevated levels of TGF-β and other inflammatory

cytokines may initiate some, but also blunt other responses. For example, sustained elevation of TGF-β can suppress bone marrow response to stimulation of hematopoietic growth factors such as granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSI), and others.

Each irradiation-induced or suppressed protein has a specific mechanism by which its actions are extended to biologic effects in cells and tissues. However, not all proteins work by a single mechanism of action. For example, TGF-β, was originally determined to have a binding capacity for specific TGF-β receptors on the surface of nearly all cells. Signal transduction through binding of the protein to its receptor elicited specific cellular responses. However, multiple events after TGF-β protein binding to its receptor were shown to be mediated by at least five different signaling pathways. These intra-cellular pathways define a complex protein network occurring inside cells. The TGF-β binding to its receptor induces two different direct pathways of response, one through the activation of SMAD3, and then through PHOSPHO-ERK activation, both of which lead to a direct signaling to the cell nucleus. There are three other pathways by which TGF-β elicits an intracellular signaling response (RHO-Kinase, PI-3 kinase, and JNK kinase pathways) (9). Each of these 5 signaling pathways have a complex response system including action through other proteins. The reference to these 5 different TGF-β signaling pathways is very well described (9). This example represents the complexity of protein response networks inside cells. To complicate this issue, there are multiple forms of TGF-β itself (10, 16-18).

Extracellular protein networks are just as complex. There are multiple collection systems for extracellular fluids. The cell membrane contains "pumps" or transmembrane channels, which maintain homeostasis within cells by regulating sodium and potassium levels, as well as, levels of other ions. Failure of these pumps, as in specific diseases, such as Cystic Fibrosis in which the sodium transporter pump is defective, leads a measurable change in the composition of the extracellular fluid. In the case of Cystic Fibrosis, the normal pumping of water outside of cells in the lung, is defective, and the mucus secretion in the lungs by other cells becomes very thick and a fertile environment for growth of bacteria such as pseudomonas aeruginosa (11). The thick extracellular fluid is also difficult to clear from the airways inducing the fibrotic and cystic lesions that describe the disease. The extracellular fluid in each organ also contains proteins that adjust trafficking across the cellular membrane from the outside in, as well as inside-out. As in the intracellular protein networks, the extracellular protein networks describe the homeostasis of the organism, and how the homeostasis is disturbed by ionizing irradiation.

Enzymes are proteins with specific biochemical functional assays. Cascades of protein enzymes interact with each other, as in the TGF-β signaling pathways described above. Kinases are those proteins, which remove phosphate from other proteins. Removal of phosphate from one enzyme can lead to its activation as a kinase to remove phosphate from another enzyme. Kinase signaling in a cascade is a perfect example of the formation of protein networks, and occurs in the extracellular environment, as well as, inside the cell. With respect to the ionizing irradiation response, the extracellular enzyme, copper/zinc superoxide dismutase-3, (extracellular SOD) represents a rapid response to the free radical accumulation in the extracellular space after exposure to ionizing irradiation. One of the free radical moieties induced by ionizing irradiation, which can cause damage to all components of the cellular metabolic structure is superoxide.

Cu/ZnSOD is an enzyme, which dismutates superoxide into Hydrogen Peroxide. Another enzyme Catalase or its partner Glutathione Peroxidase, neutralizes Hydrogen Peroxide into water. This antioxidant system represents an example of extracellular protein networks, which act to restore homeostasis after total body irradiation. In some situations, the protein networking occurs simultaneously inside and outside cells. While extracellular SOD acts to dismutate superoxide in the extracellular space, two other enzymes act to carry out the same function intracellularly, MN-SOD (SOD2) is an intracellular superoxide dismutase, located in the mitochondria. A third SOD functions as a Cu/ZnSOD (SOD1) of cells in the cytoplasm. Each of these protein enzymes has a specific function, but also is a component of a cascade of proteins in the protein network for a specific enzymatic activity.

How Do Protein Networks Influence the Ionizing Irradiation Response?

Proteins signal many pathways that activate both positive and negative reactions to irradiation. The pathways should (ideally) balance each other to return cells or organisms to homeostasis (the condition at baseline prior to irradiation). As described in other chapters in this textbook, the ionizing irradiation response, depends on irradiation dose, dose rate, and beam quality (protons, neutrons, electrons, and photons). Radiation exposure, while time-limited, may initiate continual signals of the irradiation damage of hours, days, or permanent, and limit the ability of cells, tissues, organs, and the entire organism to return to homeostasis. Protein network analysis provides a window into understanding how irradiation damage response cause acute and chronic damage.

The complexity of protein networks differs between sampled tissues. Protein signatures (levels that correlate with a particular event) have been described as different between the small intestine and bone marrow (5). Two critical organs that regulate the acute response to irradiation are the bone marrow and the intestine. Other chapters in this textbook describe the radiation damage to these tissues and how such damage leads to the severity of the irradiation injury. However, sampling of tissues to determine radiation response and the level of irradiation sustained may not be possible with bone marrow or intestine, therefore, peripheral blood is often the most efficient sampling source. Because the peripheral blood contains proteins, which accumulate from the events in all organs in the body (blood circulates through the entire body), a serum (or plasma) level of a particular protein may not reflect events going on in a particular organ in the body. For example, elevation of the hematopoietic growth factor proteins granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor (G-CSF and GM-CSF, respectively) is dramatic within 24 hrs. after total body irradiation; however, such elevated levels are not detected in the intestine of those same animals (5). Furthermore, administration of a pharmaceutical agent designed to mitigate radiation injury such as JP4-039 (17), may alter the levels of serum/plasma proteins, and alter indirectly their protein signaling events in the intestine and marrow (12). Because the peripheral blood is a window on all somatic organs in a total body irradiation response, current research is focused on how to use the peripheral blood assay system to accurately determine the level of radiation exposure, the effect of delivering a mitigative drug, and the expectations for outcome, and need for medical intervention.

Measurement of Protein Networks

Several computer-based software programs are available to synthesize and display data on protein networks (13, 14-15). Scientists seeking to enter this field of investigation should research the available tools for such studies. As more data become available from greater numbers of investigations, the complexity of protein networks are displayed in continually modified and upgraded software packages. The situation is even more complex, because proteins influence levels of other non-protein signatures of radiation exposure. The enzymes, which modify lipids and phospholipids are proteins. An elevation of a specific enzyme can lead to a specific lipid synthesis and the lipid causes the profound radiobiologic change. For example, calcium independent phospholipase A_2 gamma is critical for the synthesis of the oxidized lipid hepoxillin-A3, a mediator of neutrophil accumulation in the intestine, the actions of which can exacerbate radiation intestinal injury. Thus, the language of oxidative lipidomics (described in a separate chapter in this textbook) overlaps with the language of proteins, proteomics, and protein networks.

Heat-Maps for Proteins

As is described in the chapter on RNA and Transcriptomics, there is a vast capacity to display hundreds of proteins in graphic presentations called heat-maps (5). In these assay systems, elevated levels of protein are usually displayed as red, and depressed levels as green. The availability of rapid plate readers to take a single serum specimen and display relative levels of change of hundreds of proteins makes available this technology. Methodologies for protein heat-map, display and interpretation are available in a wide variety of publications. Investigators interested in this area should consult these references.

References:

1. Anscher MS. Targeting the TGF-β1 pathway to prevent normal tissue injury after cancer therapy. Oncologist, 15(4): 350-359, 2010.

2. Johnston CJ, Williams JP, Okunieff P, and Finkelstein JN. Radiation-induced pulmonary fibrosis: examination of chemokine and chemokine receptor families. Radiat Res, 157(3): 256-265, 2002.

3. Vanpouille-Box C, Diamond JM, Pilones KA, Zavadil J, Babb JB, Formenti SC, Barcellos-Hoff MH, and Demaria S. TGFβ is a master regulator of radiation therapy-induced anti-tumor immunity. Cancer Res, 75(11): 2232-2242, 2015.

4. Kingsmore SF. Multiplexed protein measurement: Technologies and applications of protein and antibody arrays. Nat Rev Drug Discov, 5: 310-320, 2006.

5. 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.

6. Marks LV. A healthcare revolution in the making: The story of Cesar Milstein and monoclonal antibodies: Making monoclonal antibodies. **http://www.whatisbiotechnology.org/exhibitions/milstein/monoclonals**

7. Leung L, Kwong M, Hou S, Lee C, and Chan JY. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. The Journal of Biological Chemistry, 278: 48021-48029, 2003.

8. Huang Z, Epperly M, Watkins SC, Greenberger JS, Kagan VE, and Bayir H. Necrostatin-1 rescues mice from lethal irradiation. Biochimica at Biophysica Acta, 1862: 850-856, 2016.

9. Zhang YE. Non-smad pathways in TGF-β signaling. Cell Research, 19: 128-139, 2009.

10. Massague J. TGFbeta in cancer. Cell, 134: 215-230, 2008.

11. Dar HH, Tyurina YY, Mikulska KA, Shrivastava I, Tyurin VA, Krieger J, St. Croix C, Watkins S, Bayir E, Ting H-C, Mao G, Ogunsola AF, Flitter BA, Freedman C, Gaston JR, Holman T, Pilewski J, Greenberger JS, Mallampalli R, Bahar I, Bomberger J, Bayir H, and Kagan VE. Theft-ferroptosis by pseudomonas aeruginosa pLoxA via AA-PE oxidation occurs in cystic fibrosis and persistent lower respiratory tract infections. J Clinical Investigation, (in press).

12. Rwigema J-CM, Beck B, Wang W, Doemling A, Epperly MW, Shields D, Franicola D, Dixon T, Frantz M-C, Wipf P, Tyurina Y, Kagan VE, Wang H, and Greenberger JS. Two strategies for the development of mitochondrial-targeted small molecule radiation damage mitigators. Int J Radiat Oncol Biol Phys, 80(3): 860-868, 2011.

13. Anderson NL, and Anderson NG. Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis, 19(11): 1853-1861, 1998.

14. Blackstock WP, and Weir MP. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol, 17(3): 121-127, 1999.

15. Ideker T, and Sharan R. Protein networks in disease. Genome Res, 18(4): 644-652, 2008.

16. Derynck R, and Miyazono K. TGF-beta and the TGF-beta family. In: Derynck R, Miyazono K, eds. The TGF-beta Family. New York: Cold Spring Harbor Laboratory Press, 2008: 29-43.

17. Massague J, Blain SW, and Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. Cell, 103: 295-309, 2000.

18. Drynck R, and Akhurst RJ. Differentiation plasticity regulated by TGF-beta family proteins in development and disease. Nat Cell Biol, 9: 1000-1004, 2007.

19. Riechmann L, Clark M, Waldmann H, and Winter G. Reshaping human antibodies for therapy. Nature 332(6162): 323-327, 1988.

20. Sekhar KR, and Freeman ML. Nrf2 promotes survival following exposure to ionizing radiation. Free Radic Biol Med, 88: 268-274, 2015.

21. Travis EL, Rachakonda G, Zhou X, Korhonen K, Sekhar KR, Biswas S, and Freeman ML. Nrf2 deficiency reduces life span of mice administered thoracic irradiation. Free Radic Biol Med, 51: 1175-1183, 2011.