Chapter XXX: Biomarkers of Radiation Injury and Medical Countermeasures (MCM) Effects: Predicting Late Effects and Assessing MCM Efficiency

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What are Biomarkers?

Shortly after the discovery of ionizing irradiation emitting isotopes, and cathode ray tube (x-rays), there were reports of tissue damage by this form of irradiation. Physicians and biologists have continually focused on the need to more effectively quantitate irradiation dose sustained, and analyze tissue responses on an ongoing basis. With the need to identify and develop new radiation mitigators (agents that can be given after irradiation exposure, but before the onset of symptoms and/or external signs of irradiation damage), there has been a requirement to define reliable biomarkers of acute irradiation exposure. Acute radiation effects are those detected during or shortly after irradiation exposure. Other chapters in this textbook describe the sensitivity of the bone marrow and lymphatic organs to ionizing irradiation, and the window on these internal organs by sampling the peripheral blood.

One of the first described biomarkers of ionizing irradiation exposure was measurement of the peripheral blood lymphocyte count (1). Measuring lymphocyte numbers per cubic millimeter of peripheral blood at each of two time points after suspected irradiation exposure was used to plot the decrease over time and was a reliable biomarker of radiation dose. Lymphocytes, which die an intermitotic death, do not show the classic cell cycle dependent radiosensitivity during the mitotic and G2 phase, as do other cell types. Thus, two measurements of peripheral blood lymphocyte counts were recognized as a reliable dosimeter for determining what total body dose (or equivalent in the case of partial body irradiation to significantly higher doses than the LD50/30 for total body) had been sustained. However, in mass casualty situations or even in the setting of multiple patients arriving in an emergency room with no external signs of combined injury (thermal burn, concussion, hemorrhage, fractures, and other traumas), the paradigm of sequential peripheral blood analysis would be impractical, as it requires two sampling points. Even finger-stick and processing capillary tubes for white blood cell counts, and automatically counting numbers of leukocytes in hundreds or thousands of patients may be impractical, because two different data points would be required to be able to plot a curve, and showing the rate of lymphocyte count reduction.

Another chapter in this textbook by David Brenner and colleagues describes the RABIT (2), an automated peripheral blood sampling device, which is currently being developed to address the problem of high throughput sampling. However, adapting this automated technology to lymphocyte count decrease over time requires that an individual be present on two separate occasions to get a measurement of the change in peripheral blood lymphocyte count. In a setting of mass casualties, a single sampling for biomarker(s) of radiation exposure is desirable.

Lymphocyte killing by irradiation is dose dependent and can be used as a rapid biomarker. Another use of lymphocytes is their display of chromosome damage markers. After several cell divisions in the bone marrow, spleen, and lymph nodes has occurred in response to the oxidative stress of ionizing irradiation exposure, chromosome abnormalities are detected in peripheral blood lymphocytes, and in other peripheral blood nucleated cells. Ring forms, chromosome translocations, and other abnormal chromosome morphologies are observed as a direct measure of DNA double strand breaks (3). During mitosis when the heterochromatin of the nucleus reshapes and forms chromosomes, these are lined up for cell replication and separation. The DNA double strand breaks, which have not been properly repaired can result in abnormal

separation of the pairs of chromosomes leaving an extra fragment on an arm of one chromosome while depleting this segment from others. Repair of irradiation-induced broken DNA strands can result in an abnormal morphology, and this results in ring form or heterokaryon chromosomes. These abnormal shaped chromosomes can be a reliable marker of radiation exposure. The RABIT and other high throughput automatic measuring devices may provide this service in the setting of mass casualties as well, and the technology is being developed. With chromosome abnormalities, as a biomarker, the timing of the sample appears to be important, as these abnormal chromosomes may appear and then disappear as the cells containing them die. Other data suggest that abnormal chromosome morphologies persist for days to weeks or longer after radiation (Dicentrics, ring forms, and other abnormal structures) (3). For high throughput analysis of radiation exposure, physical biomarkers in peripheral blood cells will require automation of the observations. Physical biomarkers are available, but there is need for a biomarker, which can be rapidly assessed by a chemical or biochemical method.

A major challenge for the development of a reliable biomarker of radiation exposure is the issue of specificity for ionizing irradiation. Ionizing radiation induces activation of stress response genes and genes for inflammatory cytokines, increased RNA transcription levels, suppression of other transcripts, and the production or suppression of proteins (4). The same changes in RNA can occur after exposure to other sources of oxidative stress and/or inflammation including: ultraviolet irradiation, chemotherapy drug administration, exposure to infectious agents including bacterial and viral agents, and physical trauma including thermal burns, penetrating wounds, and over-pressure injuries including concussion and bone fractures. For this reason, it is important to assess the level of combined injury when making measurements for irradiation.

In a setting of a mass casualty event from irradiation exposure, such as a dirty bomb, or fission bomb, hundreds or thousands of patients, who are evacuated and transported to hospitals, including those irradiated patients walking into an emergency room, and even the unirradiated "worried-well" patient present a challenge for the medical staff attempting to triage patients based on need for medical care including an estimate of level of radiation exposure. Reliable, accurate, and specific biomarkers for radiation exposure are a focus of intense research in the CMCR program of NIAID/NIH. In the absence of a "standard" protocol for assessing radiation dose sustained, hospitals must rely on information that is currently available.

How to Assess Radiation Exposure Level?

Decades of research developing methods by which to determine radiation exposure level have come from analysis of nuclear reactor accidents, accidental radiation exposure, and the military experience. The most reliable information to date comes from historical evaluation of individual patients. Where was the individual at the time of exposure and what was the duration of exposure? Physical assessment of the environment remains the most important information for determining radiation dose. In the case of a single high dose rate (fluence) of photons or neutrons from a fission bomb, the distance of the individual from the exposure point (hypocenter) will be the first determinant of level of dose sustained injury.

Physical assessment of the individual includes information referred to as the "two by two rule" (1). Did the individual experience nausea and vomiting within two hours of the explosion? This

data usually indicates that the total body irradiation (TBI) dose sustained was greater than 2 Gy. However, other causes of nausea and vomiting can confound dose assessment: including fear and a prior medical condition, which could confound analysis of an individual presenting with a history of nausea and vomiting. Those, who may report that they were at a significant distance from the site of the explosion and display no physical evidence of exposure by history, may be sent home; but they may still wish to receive an available radiation countermeasure. The administration of radiation countermeasures may be dependent upon historical and individual preference factors, particularly in the absence of other reliable biomarkers.

In a setting of an emergency room, which is challenged to handle between 10 and 100 potential casualties, it may be possible to obtain two peripheral blood lymphocyte counts separated by 4 hours, and if the counts have dropped significantly below the normal range, this data, when factored into an analysis of: 1) distance from the explosion, 2) history of nausea and vomiting, and 3) absence of combined injury may alert medical professionals to keep a particular patient for observation. If an individual presents with combined injury (thermal burn, concussion, fracture, or other signs of trauma), the biomarker reference information may be less relevant, and the person will receive an available radiation countermeasure or be kept in the hospital.

Status of Current Research in Development of Reliable and Specific Radiation Biomarkers.

Decades of research in animal models and in patients receiving clinical radiotherapy, have provided much information on the reliability of use of specific biomarkers of radiation exposure.

There has been a concerted effort to determine whether easily obtained peripheral blood or other tissue sampling can provide information on the radiation dosimetry. Measuring irradiation-induced signatures in hair, fingernail clippings, skin, and tooth enamel for a history of ionizing irradiation exposure, has been explored as a potential biomarker (5). Potential applications of this technology have been confirmed by data showing: 1) disappearance of the radiation-induced changes within minutes of exposure, making the test impractical for an individual coming to the emergency room 24 hrs after exposure, and 2) the effects of various agents on the measurement including: residue of soap and shampoo in the hair, fingernail polish or other materials on the fingernails, and other chemical contaminants on the skin or tooth enamel (5).

There have been other approaches to developing a biomarker for irradiation exposure using peripheral blood, urine, oral cavity swabs, and even breath analysis. For detection of specific molecules, scientists have searched for a specific increase in levels of a detected molecule after radiation exposure. Ionizing irradiation-induced changes in the metabolism of an exposed experimental animal or human are also well known and include irradiation dose specific elevations or depressions in the metabalome (6-7).

In the case ofsuspected toxic chemical exposure, the analysis of blood and urine, has been the mainstay for quantitative assessment of levels of exposure by hospital emergency departments. In the case of chemical levels of drug or toxin exposure, peripheral blood or urine analysis of levels of exposure by Mass Spec (Mass Spectroscopy) or HPLC (High Throughput Liquid Chromatology) are dependent upon the availability of equipment for these assays. Mass Spec analysis is used in the “toxic-screen” for blood analysis of drug or chemical exposure in

unconscious patients, who is suspected of having a drug overdose or toxin exposure. While these assays are highly specific for detection of specific compounds such as cocaine, narcotics, or levels of other therapeutic or non-medicinal drugs, they may not be specific for determining levels of exposure to ionizing irradiation (with the exception of ingested isotopes), because external sourced radiation leaves no residue.

An exciting new possibility for diagnosis of radiation exposure comes from analysis of the metabolomics of the organisms response to irradiation. By 24 hrs. after irradiation exposure, metabolic functions in an irradiated individual have already changed including those related to chemical and biochemical signatures in the peripheral blood, urine, and feces (7). For example, mice exposed to total body irradiation demonstrate a complex mixture of overexpression and under-expression of protein levels in the peripheral blood (8). Furthermore, levels of metabolites in the urine and feces are significantly altered 24 hrs. after irradiation exposure (7). Whether these irradiation-induced changes are specific, reproducible and reliable given the complexity of human genetics, and also the heterogeneity of the human intestinal and lung microbiome remains to be determined. Furthermore, changes in peripheral blood, urine, and fecal signatures of ionizing irradiation exposure, have not yet been correlated with changes in those signatures after other forms of radiation and chemical exposure, nor have they been related to pre-existing medical conditions, polypharmacy (what drugs is the individual taking), and the presence of an ongoing or recent infection.

The heterogeneous nature of an individual experimental animal or human to a given radiation exposure dose is also a complicating factor in the use of any specific biomarker. For example, the LD50/30 dose of radiation (dose which will kill 50% of exposed animals or humans within 30 days of exposure), and used as a reliable indicator of the "hematopoietic syndrome", is a condition that can be potentially reversed by bone marrow transplantation. This LD50/30 dose has itself remained a mystery. Why do 50% of genetically inbred and "identical" mice survive to day 30 and others do not? In the case of human radiation exposure from a nuclear power plant or laboratory accidents, it is unclear why one individual survives and another does not, if they were both in the exact same spot at time of exposure.

The response of one individual to the same dose of irradiation relies upon multiple factors including: 1) condition of the intestine with respect to volume of digested food and stage of digestion; 2) heterogeneity of that person's response to irradiation by upregulation or downregulation of stress response genes; 3) age; 4) gender; 5) weight, body surface area; and 6) stochastic (random) nature of specific anatomic positions of critical organs and/or cells within an exposed organ; 7) polypharmacy. Differences in the microbiome are another potential variable.

State-of-the-Art with Respect to RNA Transcript Analysis.

Multiple assay systems have been developed to measure RNA levels. Northern Blot analysis was the first such assay used to determine whether a particular RNA moiety was increased following irradiation. This has been largely replaced by the rt-PCR assay, which can measure transcripts in large numbers. The pattern of RNA transcripts for a variety of stress response genes has been shown to be constituatively induced in cell lines derived from Fanconi Anemia (FA) Fancd2-/- mice. Figure 1 shows the effect on RNA transcripts measured by rt-PCR in

Fancd2-/- compared to the control C57BL/6 Fancd2+/+ IL-3 dependent lines tested at 1 hr, 4 hr, or 24 hr after either 2 Gy (Fig. 1A-C) or 5 Gy (Fig. 1D-F) irradiation. Many differences can be quantitated.

Fig 1A:

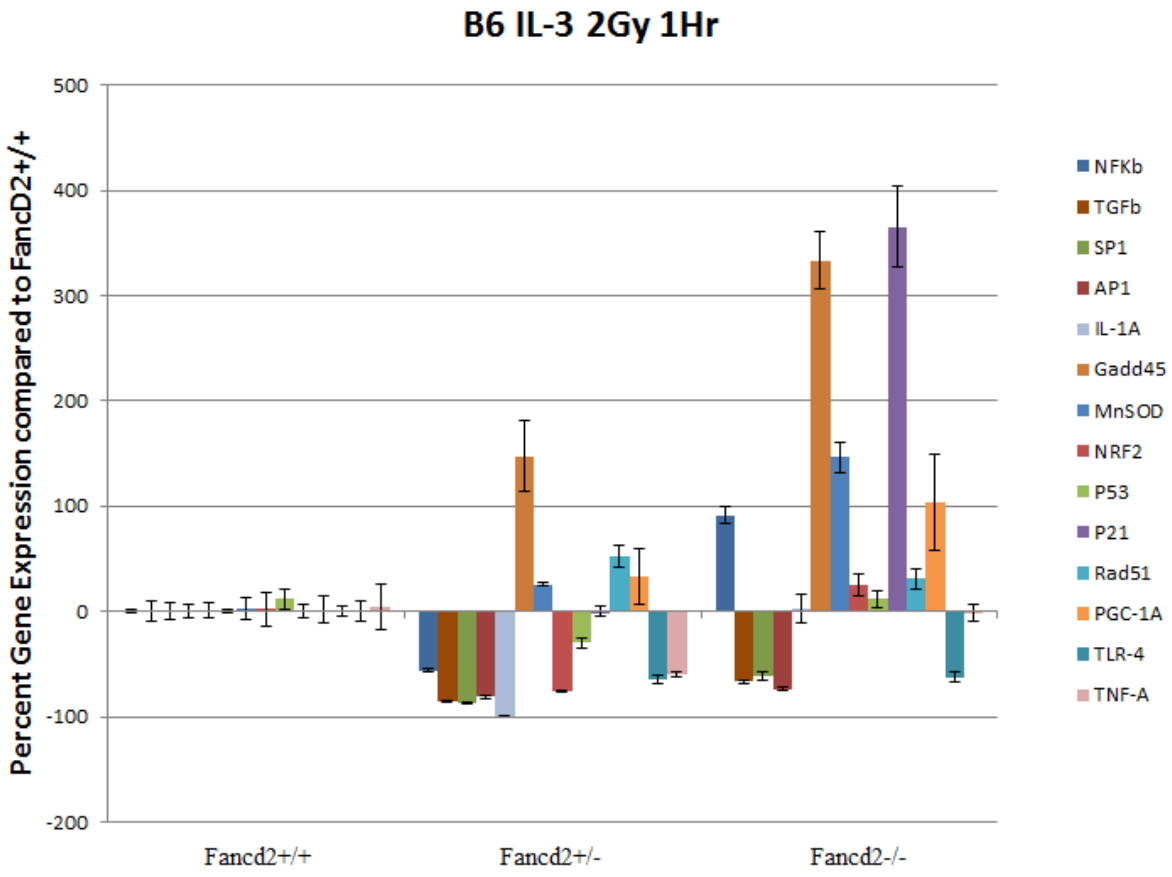
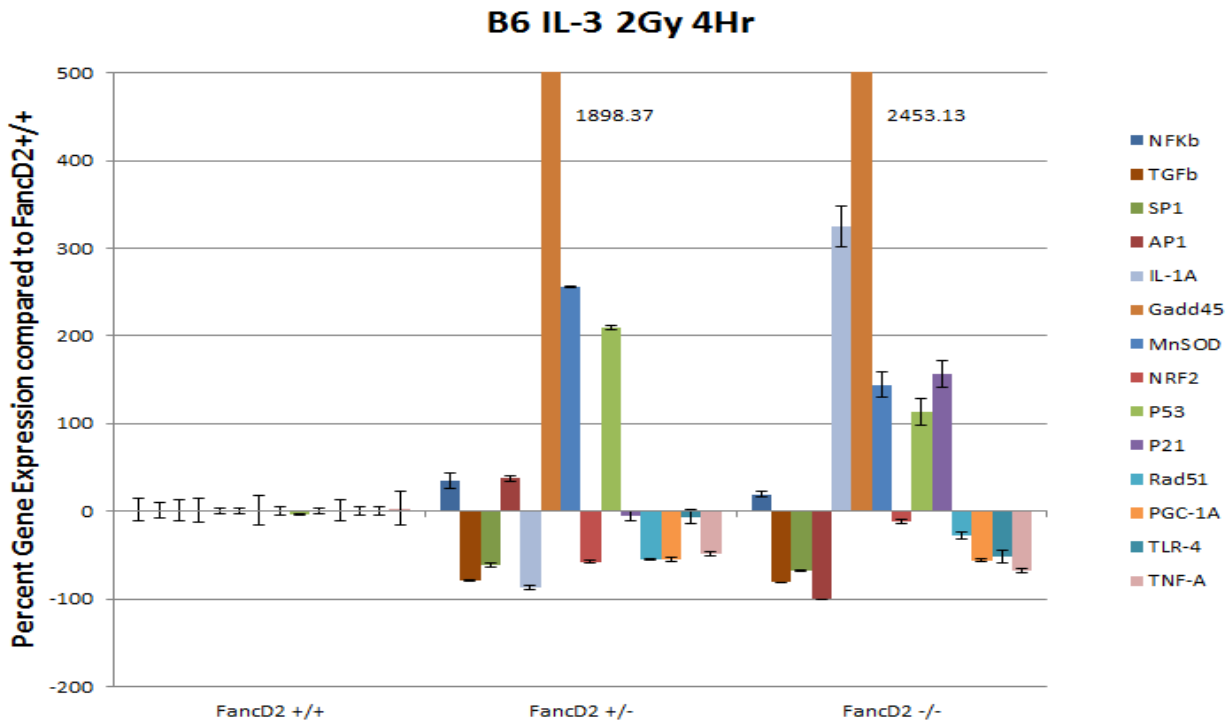


Fig. 1B:


B6 IL-3 2Gy 4Hr
Percent Gene Expression compared to FancD2+/+
500
400
300
200
100
0
-100
-200
1898.37
2453.13
FancD2 +/+
FancD2 +/-
FancD2 -/-
NFKb
TGFb
SP1
AP1
IL-1A
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Rad51
PGC-1A
TLR-4
TNF-A

Fig. 1C:

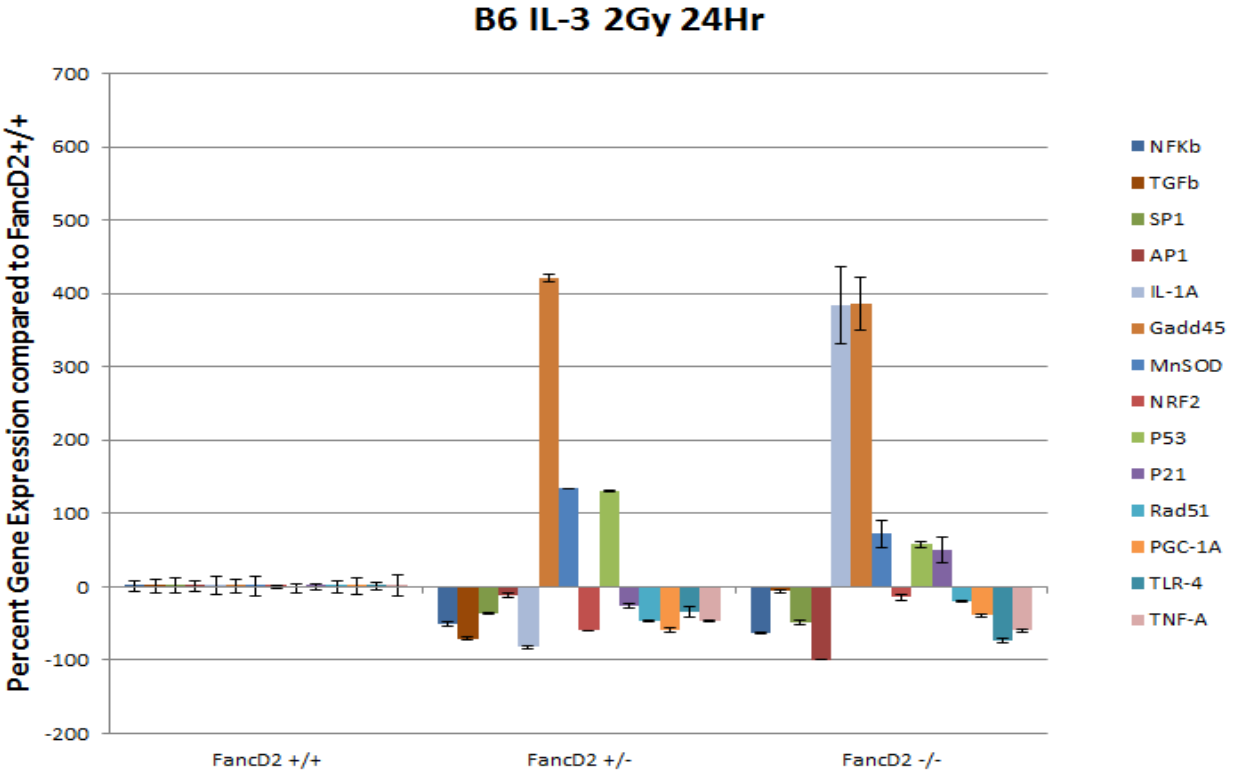
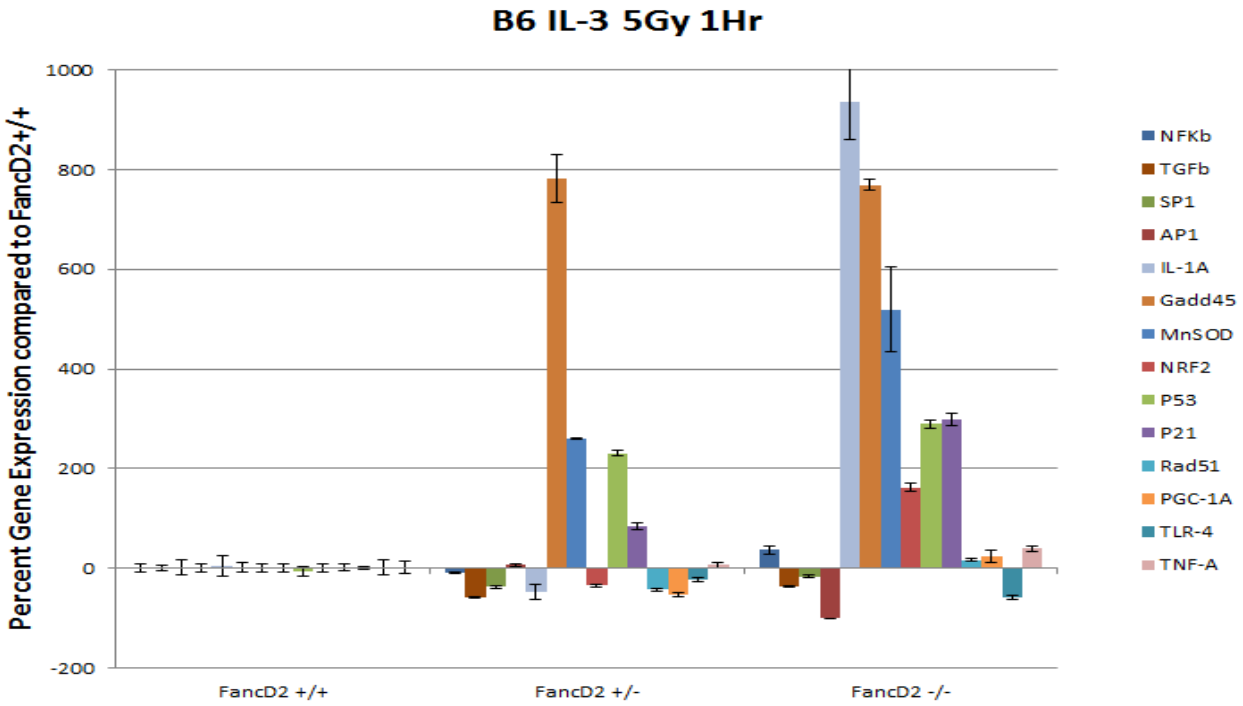

B6 IL-3 2Gy 24Hr
Percent Gene Expression compared to FancD2+/+
700
600
500
400
300
200
100
0
-100
-200
FancD2 +/+
FancD2 +/-
FancD2 -/-
NFKb
TGFb
SP1
AP1
IL-1A
Gadd45
MnSOD
NRF2
P53
P21
Rad51
PGC-1A
TLR-4
TNF-A

Fig. 1D:


B6 IL-3 5Gy 1Hr
Percent Gene Expression compared to FancD2+/+
1000
800
600
400
200
0
-200
FancD2 +/+
FancD2 +/-
FancD2 -/-
NFKb
TGFb
SP1
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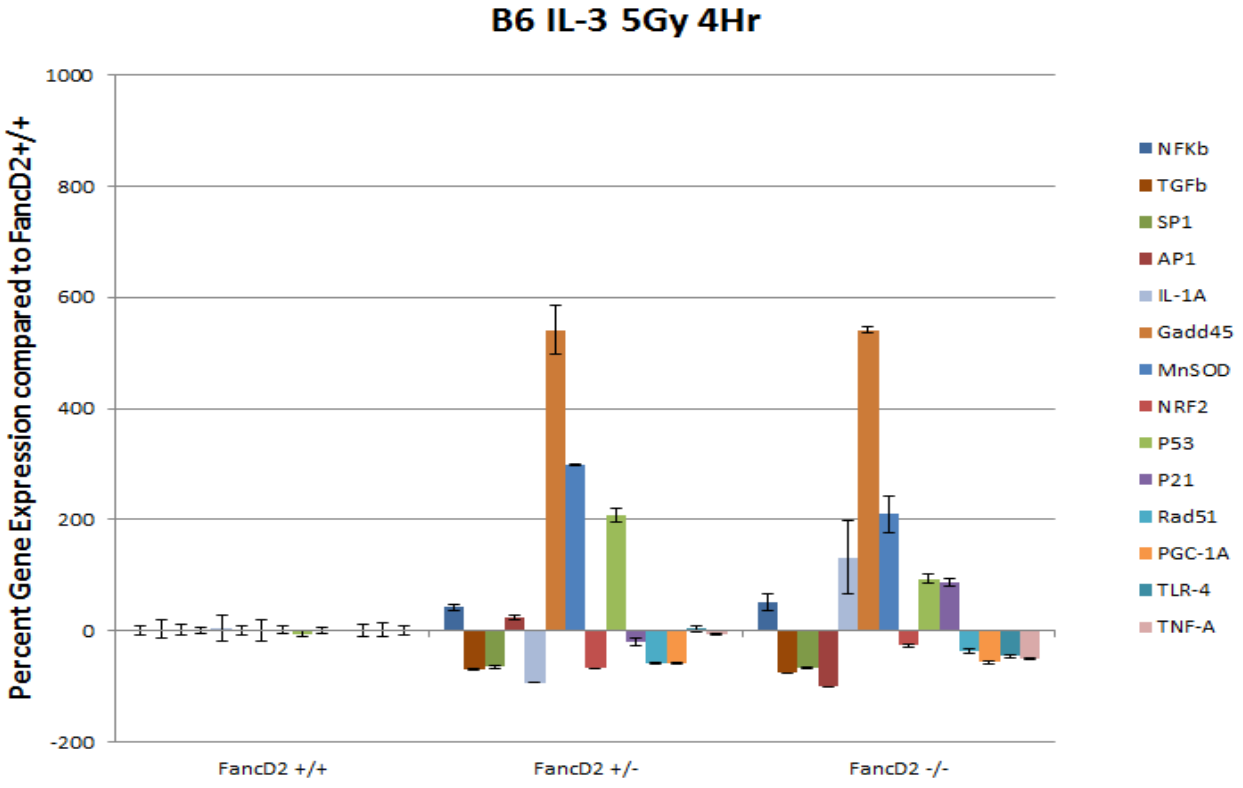
Rad51
PGC-1A
TLR-4
TNF-A

Fig. 1E:


B6 IL-3 5Gy 4Hr
Percent Gene Expression compared to FancD2+/+
1000
800
600
400
200
0
-200
FancD2 +/+
FancD2 +/-
FancD2 -/-
NFKb
TGFb
SP1
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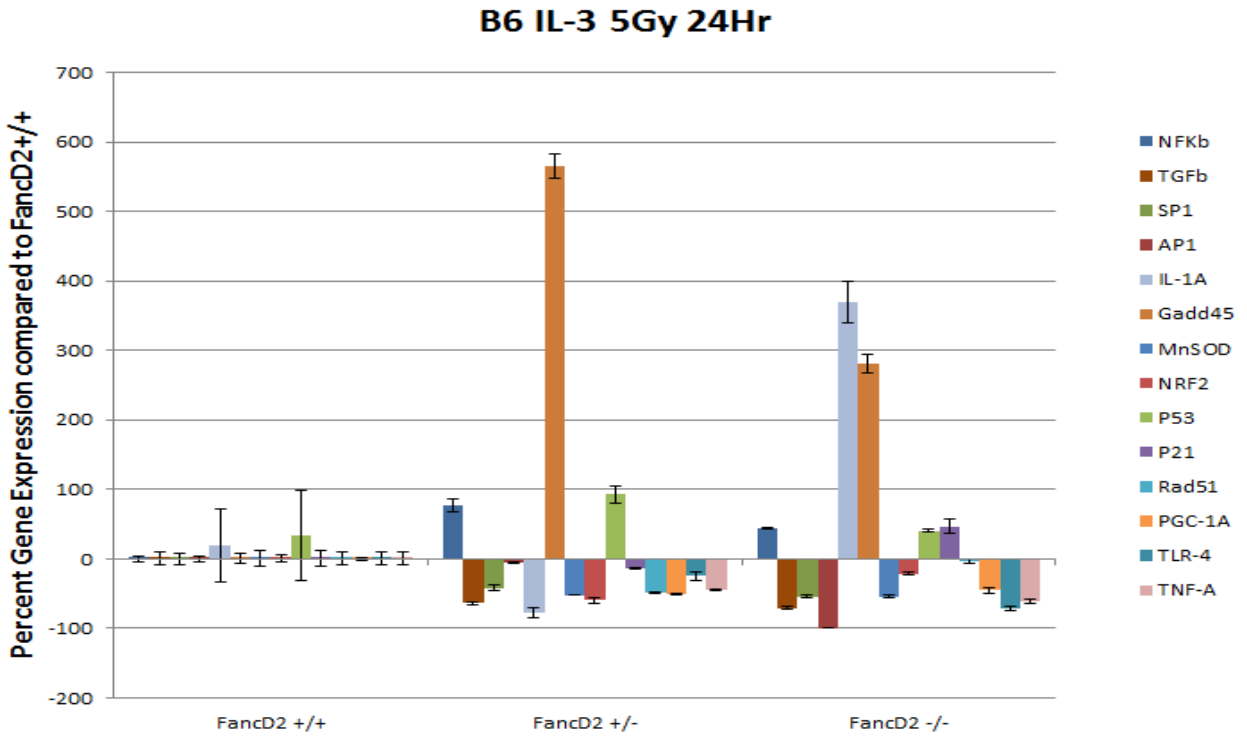
Rad51
PGC-1A
TLR-4
TNF-A

Fig. 1F:



The results in Fig. 1 show that with 2 or 5 Gy irradiation over 24 hrs, there were significant effects of the Fancd2+/- heterozygote or knockout Fancd2-/- genotype in response to irradiation. Across both radiation doses, both genotypes, and all 3 time points, transcripts for TGF-β, SP1, and TLR-4 were consistently decreased compared to Fancd2+/+, while Gadd45 and p53 were elevated. P21 was only increased in Fancd2-/- cells. IL-1A showed variable response dependent on genotype: Fancd2+/- showed significant suppression, while Fancd2-/- showed elevation at 4 and 24 hrs. AP-1 expression was generally suppressed in both genotypes. MnSOD showed relative increases at every time point at 2 Gy, but only showed increases at 1 hr and 4 hrs in the 5 Gy cell lines.

The spectrum of RNA moieties that can be measured include stress response genes, cytokines, promoters for induction of other genes including NFKβ, SP1, AP1, and others. In a DNA repair deficient cell line such as that from a Fancd2-/- mouse, these genes are turned on at a baseline. After irradiation using RNA transcripts to measure changes is a valuable method. The methodologies for these assays are well described (4, 14-16).

State-of-the-Art with Respect to Protein Biomarker Analysis.

Western Blot analysis for proteins using specific antibodies for each protein was the first test available to show that after irradiation, TNF-α, TGF-β, IL-1 were increased (20). The methodology for Western Blot is well described. With the availability of the Luminex Platform Assay for measuring proteins, more sophisticated methods and highly quantitative methods are available to screen for large numbers of proteins before and after irradiation induction. They

serve as valuable biomarkers if appropriately used in the context of organ specific changes (4). Protein analysis is also valuable (8), but organ specific levels will differ from that in peripheral blood (8).

Metabolomic Indicators of Radiation Exposure in Urine, Feces, and Peripheral Blood.

These assays have recently been published in elegant descriptions (7).

Oxidative Lipodomics Analysis as a Biomarker.

The work of Tyurina, et al. (9) and Kagan, et al. (10) have demonstrated the cascade of oxidative lipid changes, which follow the initial oxidation of cardiolipin in a mitochondria, and which are associated with the onset of apoptosis. Cardiolipin oxidation induced by cytochrome C, which evolves to a peroxidase after irradiation has been shown to lead to a signaling cascade of multiple lipids (11). Readers should focus on the chapter in this textbook by Tyurina, et al. related to oxidative lipidomics as a biomarker.

Other Novel Approaches to Assessing Irradiation Damage Using Biomarkers.

There are other small molecules including aerosol released markers from exhalation have been suggested as potential methods by which to quantitate irradiation exposure (12).

Biomarkers for Assessing Late Effects and the Duration of the Latent Period.

In the study of late effects of irradiation, there are numerous publications suggesting that late onset of radiation damage (fibrosis) can be measured by a recurrence of the elevation of TGF-β, IL-1, and TNF-α (13). Studies in C57BL/6J mice confirm this phenomenon (7). However, not all mouse strains demonstrate late irradiation effects as evidenced by a biomarker change (14).

Important Role of Genetics.

Using biomarkers may be not only species specific, but also related to the expression of certain genes. Mouse strains, which are intrinsically radiosensitive may have a different baseline set of biomarkers compared to those control animals. This data is shown above in the rt-PCR analysis of transcripts at baseline in Fanconi Anemia mouse Fancd2-/- cells compared to Fancd2+/+ cells (15-16). Individual genetic variation in humans as an outbred species will complicate the use of biomarkers until it is clear how they can relate to the general population rather than specific subsets with specific genetic proclivity to a particular radiation response.

Reliability and Reproducibility in Assessment of Biomarkers of Radiation Exposure.

One of the observations from current efforts to produce a series of tests for reliable indicators of irradiation exposure has been the variation in the assay systems. In laboratory research triplicate or quadruplicate experiments are usually required at each radiation dose to assess the colony forming assay or induction of a specific biomarker. This approach may not be practical in the

case of availability of serum or plasma specimens from a large number of irradiated victims in a terrorist event. Therefore, reproducibility and reliability is a critical indicator of the success of development of a biomarker. Recent studies have shown that a spectrum of proteins, RNA, or metabolic moieties must be assayed in order to show a pattern specific to irradiation exposure (7, 17, 21). Another major concern is that of polypharmacy described below.

Methods for Studying Biomarkers (Acute and Late Radiation Effects).

Assay systems have been developed for measuring DNA strand breaks, DNA chromosome abnormalities, RNA transcript elevation or depression, and protein elevation or depression (4). Studies of oxidized lipid changes, and the time course of onset and disappearance of these changes is another approach (10). All of these methods have been published, and each is addressed in separate chapters in this textbook.

The Relevance of Biomarkers to Development of Radiation Countermeasures.

For a radiation countermeasure to be interpreted for use in experimental animals, and then moving into human testing, the pharmacokinetics of the drug must first be determined. How the drug disappears when injected intravenously, intramuscularly, topically, or orally must be assessed. However, pharmacodynamics is also a measure. This term describes the target of the radiation countermeasure, and how that target is being affected by administration of the agent. Pharmacodynamics indicates that there is a biomarker for radiation effect and how this countermeasure will alter the countermeasure effect. A recent study of irradiation-induced changes in protein levels in intestine compared to bone marrow compared to plasma has shown profound differences in the pattern of induction or suppression of proteins (8). A biomarker for radiation damage in these studies was shown to be granulocyte colony stimulating factor (G-CSF) (8). However, G-CSF is also elevated in a wide variety of toxic and infectious exposures. Therefore, G-CSF elevation cannot be said to be specific. Combining the G-CSF levels with multiple other protein, DNA strand break, RNA, and other protein levels might be the approach to testing the effect of a radiation countermeasure. The best biomarker, of course, is increased survival. Another excellent biomarker is the lack of symptoms or signs from irradiation, as evidence of the effectiveness of the countermeasure. However, objective measures involving laboratory tests must be developed and are not yet ready for deployment.

Concern for Polypharmacy in Assessing Radiation Damage.

In the human population, individuals are taking medications, and sometimes multiple medications for a variety of indications. Furthermore, individuals have different baseline levels of stress response at the time of a radiation event, some will have infectious disease, some will be recovering from surgery, some will have trauma from a sport injury, some may have ultraviolet irradiation damage (sunburn), and there are multiple other baseline differences between individuals. How irradiation will affect those pre-existent biomarker levels is not known. The real concern is that of polypharmacy. Anywhere from 10 – 30% of individuals in a population may be taking medications for some condition, ranging from treatment of hypertension, diabetes, osteoporosis, or simply taking multivitamins and anti-inflammatory agents, and other over the counter medications. How these will affect assays for the biomarkers

of irradiation exposure is not known. Research in this area is lacking, and investigators seeking to make contributions in the area of biomarkers development could well consider studies of polypharmacy effects of new radiation mitigators (16-19).

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