Radiation combined injury is defined as an ionizing radiation exposure received in combination with other trauma or physiological insults. The range of radiation threats we face today includes everything from individual radiation exposures to mass casualties resulting from a terrorist nuclear incident, and many of these exposure scenarios include the likelihood of additional traumatic injury. Radiation combined injury sensitizes target organs and cells and exacerbates acute radiation syndrome. Organs and cells with high sensitivity to combined injury are the skin, the hematopoietic system, the gastrointestinal tract, spermatogenic cells, and the vascular system. Among its many effects, radiation combined injury results in decreases in lymphocytes, macrophages, neutrophils, platelets, stem cells, and tissue integrity; activation of the iNOS/NF-κB/NF-IL6 and p53/Bax pathways; and increases in DNA single and double strand breaks, TLR signaling, cytokine concentrations, bacterial infection, and cytochrome c release from mitochondria to cytoplasm. These alterations lead to apoptosis and autophagy and, as a result, increased mortality. There is a pressing need to understand more about the body’s response to combined injury in order to be able to develop effective countermeasures, since few currently exist. In this review, we summarize what is known about how combined injury modifies the radiation response, with a special emphasis on DNA damage/repair, signal transduction pathways, apoptosis, and autophagy. We also describe current and prospective countermeasures relevant to the treatment and prevention of combined injury.

Key Words: DNA damage, apoptosis, autophagy, radiation injury, combined injury, free radical

Introduction

The potential for harmful radiation exposure has increased dramatically since the development of nuclear weapons during World War II. The number of nations with the capability to produce nuclear weapons is ever-increasing. The potential for nuclear accidents and accidental exposures will become greater with the expected proliferation of nuclear power plant construction to meet growing demands for energy more friendly to the environment. The widespread use of radioisotopes in medicine increases the dissemination of radioactive materials and patient exposures. And of course, the frighteningly real possibility that terrorist groups could use nuclear weapons or other radiological weapons poses a serious risk of mass casualties. The fact that more than 50% of cancer patients receive radiotherapy at some point during the course of their disease (5) represents another significant source of exposure as normal tissues are subjected to radiation injury.

Those charged with responding to such threats have modeled many of these potential exposure scenarios, but for the most part they have assumed radiation exposures alone. It is unrealistic however to assume that accidental radiation injury will occur in the absence of other injuries—especially when considering terrorist incidents. It has become abundantly clear that radiation exposure combined with many kinds of other injuries, ranging from trauma to infection, often results in a negative synergistic response more harmful than the sum of the individual injuries. We have only recently begun to appreciate the practical consequences of combined injury and to understand that the body’s response to combined injury may be different from the responses to radiation or physical injury alone.

In this review we aim to summarize our current understanding of how the physiological response to radiation is modified when other injuries are present. We focus on responses especially relevant to health effects: DNA damage and repair; signal transduction processes; free radical-mediated apoptosis and autophagy; and bacterial infection. We also discuss the potential effectiveness of current radiation response-altering drugs that could also be used to treat or prevent combined injury exposures as well as the potential for new drug development.
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Ionizing radiation is defined as any type of electromagnetic radiation (e.g., X-rays or gamma rays) or particulate radiation (e.g., neutrons or alpha particles) that has sufficient energy to ionize atoms or molecules; that is, to eject electrons from their outer orbits. In considering the effects of radiation on biological systems, it is important to distinguish the different types of ionizing radiation in terms of their linear energy transfer (LET), a measure of the amount of energy transferred to a substance as the radiation passes through it. It is classified into two types of radiation: low linear energy transfer (low-LET) radiations and high linear energy transfer (high-LET) radiations. Table 1 summarizes the types and basic physical characteristics of radiation in these categories. Low-LET radiations include gamma rays, X-rays, beta particles; high-LET radiations include neutrons, alpha particles, and heavy-particle cosmic rays (21). Radiation exposures of concern to health officials cover the full LET spectrum, and exposure could come from external sources as well as internalized radioactive substances (via inhalation, ingestion, or wound contamination).

The most radiation-sensitive organs include the hematopoietic system (16), the gastrointestinal (GI) system (53), skin (56, 58), and the vascular system (63, 68). A dose range (1-7 Gy in human) of ionizing radiation poses a risk of damage to the hematopoietic system, leading to decreases in blood cells and platelet counts and increases in susceptibility to infection and hemorrhage (8, 76) while high-dose whole-body irradiation (≥8 Gy in humans) causes acute GI syndrome in addition to hematopoietic complications. The GI effects manifest as loss of intestinal crypts and breakdown of the GI mucosal barrier. High doses can also induce GI hemorrhage, endotoxemia, bacteremia, anorexia, nausea, vomiting, diarrhea, and loss of electrolytes and fluid (73). There is no clear demarcation between the hematopoietic and GI syndromes; they represent a continuum of damage. There is hematopoietic damage that influences GI damage at higher radiation and there is likely some GI damage even at lower radiation doses.

Skin injury from radiation burns is characterized by loss of epidermis and dermis (3, 31), reduction of skin stem cells, and impairment of cell communication and cutaneous integrity, a factor that may trigger the failure of other organ systems (55). Vascular endothelium is also damaged (63). Concomitant and interdependent injuries to various organ systems can lead to multi-organ dysfunction (MOD) and multi-organ failure (MOF) and death can occur as a result.

Combined Injury

Large-scale radiation exposure events in history have shown that irradiated victims are also often subjected to other trauma such as wounds or burns. Combined injuries were observed at Hiroshima and Nagasaki, Japan, where 60-70% of victims received thermal burns concurrent with radiation injury, (26, 35). At the Chernobyl reactor meltdown, 10% of 237 victims exposed to radiation received thermal burns as well (3). In animal models of combined injury including mice (31, 43), rats (1, 71, 75), guinea pigs (37), dogs (6, 66), and swine (4), burns, wounds, and infections usually increase mortality after otherwise non-lethal radiation exposures. In rodents, radiation exposure combined with burns, wounds, or infections decreases survival compared to radiation exposure alone (27,
Radiation Combined Injury Enhances Apoptosis and Autophagy

31, 44, 71). Radiation injury also delays wound closure times (31, 47). Consequences of combined injury include acute myelosuppression, immune system inhibition, fluid imbalance, macro/microcirculation failure, massive cellular damage, and disruption of vital organ functions, which, as is the case with radiation exposure alone, can lead to MOD and MOF, the most frequent causes of death after combined injury (36, 42, 78). Although the mode of combined injury death is fairly clear, the molecular events that lead to combined injury-enhanced mortality remain poorly understood.

It has been well-characterized that a large radiation dose received over a short period of time can trigger a complicated pattern of physiological responses referred to as acute radiation syndrome (ARS). When an ionizing radiation dose sufficient to induce ARS is combined with concurrent additional physical trauma, the response induced by ionizing radiation is sensitized and a non-lethal radiation dose can be transformed to a lethal one. Concurrent trauma exacerbates radiation-induced white blood cell depletion, activates signal transduction pathways, increases cytokine and chemokine production, and increases susceptibility to bacterial infection (31). The changes observed after radiation combined injury appear at various levels—nucleus, cytoplasm, tissues, organs, and system—and at various time after injury. Whether cells survive or die after ionizing radiation alone or ionizing radiation in combination with other trauma depends on the number and severity of lesions, which determines the extent to which signal transduction pathways responsible for triggering cell death by apoptosis and autophagy are activated.

Recent research has identified key molecular intermediaries involved in radiation injury. Among the many molecules activated by radiation injury, inducible nitric oxide synthase (iNOS) and nitric oxide (NO) play important roles in radiation injury-induced apoptosis (32) and autophagy (18). The promoter region of the iNOS gene contains motifs of many transcriptional factors (33). Radiation injury increases iNOS and its transcription factors such as nuclear factor-xB (NF-xB) and Kruppel-like factor 6 (KLF-6) resulting in increased NO production that leads to caspase-mediated apoptosis (32) and protein nitration-mediated autophagy (18). Radiation injury increases concentrations of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in human blood (23); IL-1β, IL-3, IL-6, and G-CSF in mouse blood (31, 64, 72); and IL-6 and IL-8 in CNS of non-human primates (19). Cytokines are responsible for stimulating nuclear factor-IL6 (NF-IL6), which subsequently binds to the consensus motif within the iNOS promoter (ranging from +10 to -300 bp upstream of the TATA box) to activate iNOS gene expression (12). In addition, overproduction of IL-6, NO, or nitrogen reactive species can cause dysfunction of the GI barrier (22, 54, 77), which can allow bacteria to enter systemic organs. Radiation combined injury amplifies these changes (31).

The order in which radiation and wounding is received can affect the lethality of combined exposure. Survival from radiation injury improves when wounding occurs prior to radiation exposure; while wounding that occurs after radiation injury results in decreased survival compared to radiation injury alone (45, 46). However, Reid et al. (66) observed similar lethality regardless of order in a model combining radiation exposure with burn trauma.

DNA Damage and Repair

The degree of chromosomal damage is proportional to the absorbed dose of radiation, and high- and low-LET ionizing radiation produce different types of DNA damage. Low-LET ionizing radiation (gamma and X-rays) causes DNA damage mostly indirectly via formation of free radicals, while high-LET ionizing radiation (neutrons, alpha particles, cosmic ray heavy particles) is more likely to cause direct DNA damage that is more complex and difficult to repair than damage from low-LET radiation (65). Acute exposure to ionizing radiation causes damage to macromolecules as well as increased mitochondria-dependent generation of reactive oxygen species and reactive nitrogen species, with subsequent cell cycle checkpoint arrest, apoptosis, and autophagy (18, 32).

Ionizing radiation induces base damage, single strand breaks (SSBs), double strand breaks (DSBs), and DNA crosslinks. DSBs are the primary lethal lesion (7, 60). Two repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) efficiently repair DSBs. The majority (80%-90%) of DSB repair involves NHEJ (38, 67).

It is not clear if ionizing radiation combined with wound trauma causes a greater amount of DNA damage than ionizing radiation alone. Studies in this area are ongoing in our laboratory to fill in this data gap.

Signal Transduction Pathway Activation in Response to DNA Damage

DNA repair proteins such as RAD50, MRE11, NBS1, RAD17, RAD1, RAD9, and HUS1 bind to ionizing radiation-induced DSBs to form complexes that are detected by ataxia telangiectasia mutated (ATM) kinases. DSBs stimulate ATM phosphorylation within minutes and the phosphorylated ATM is stable for many hours. MDC1, 53BP, BRCAl, and TopBP1 mediate the phosphorylation of CHK2 by ATM and
related kinases. The phosphorylated CHK2 then phosphorylates p53 and CDC25. Phosphorylated p53 arrests the cell cycle at G1/S and phosphorylated CDC25 arrests the cell cycle at both S and G2/M to allow DNA repair [see review (25) and Fig. 1].

Phosphorylated ATM can also induce phosphorylation of the histone variant H2AX at serine 139, generating γ-H2AX (15). Immunocytochemical assays with antibodies recognizing γ-H2AX have become the gold standard for detection of DSBs because there is close to a 1:1 relationship between the numbers of DSBs and γ-H2AX foci formed. Furthermore, the rate of DSB repair correlates with the rate of loss of γ-H2AX foci (69). γ-H2AX triggers the CHK2 signal transduction pathway that activates p53 and CDC25. It should be noted that phosphorylated ATM also directly phosphorylates p53, which transcriptionally activates the CDK inhibitor p21 and arrests the cell cycle at G1/S (41).

Recent evidence demonstrates DSB-dependent ATM phosphorylation activates NF-κB (20, 28). Phosphorylated ATM binds to and phosphorylates IKKγ in the nucleus. The complex exits the nucleus and associates with IKKα and IKKβ. The IKK complex releases NF-κB from its inhibitors, IκBα and IκBβ, and unbound NF-κB is then free to move into the nucleus and regulate target genes. The NF-κB signaling network includes DNA repair, cell cycle check regulation, mitochondrial antioxidants, survival and apoptosis, and cytokine and chemokine expression in response to ionizing radiation-induced damage (31).

Additional trauma such as wounding potentiates gene expression induced by ionizing radiation. Table 2 shows that 60Co γ-irradiated mice display increases in expression of p21, Bax, DDB2, and Gadd45α genes. Mice treated with 60Co γ-irradiation and wound trauma exhibit further increases in p21, Bax, and DDB2, but not Gadd45α. The mechanisms underlying this enhancement in radiation combined-injured mice remain unclear. Studies of DSBs, ATM, γ-H2AX, and p53 in radiation combined-injured mice should help define the mechanisms involved.

**Free Radical-Mediated Apoptosis**

In mammalian cells, low-LET ionizing radiation but
not high-LET ionizing radiation generates free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), via mitochondrial mechanisms (13, 24). Consistent with this observation, free radical scavengers or hypoxia treatment can help prevent low-LET ionizing radiation injury. Free radicals are required for the physiological function of cells, but overproduction of free radicals damages cellular components (Fig. 2). ROS are formed from hydrolysis of water in the nucleus and the cytoplasm. ROS in the nucleus cause DNA damage while ROS in the cytoplasm activate multiple signal transduction pathways involved in growth, apoptosis, and autophagy (18, 31, 32, 57). These injuries can lead to cell-cycle arrest, transformation, and cell death.

While ROS are short-lived and extremely reactive, RNS are longer-lived and more specific in the reactions they undergo (57). NO reacts with superoxide to form the peroxynitrite anion, resulting in oxidative stress (31). This results in the release of cytochrome c from the mitochondria to the cytoplasm and the subsequent conjugation of the cytochrome c with caspase-9 and Apaf-1 to form apoptosomes that activate caspase-3 and caspase-7. Activated caspase-3 then activates caspase-2, -6, -8, and -10, resulting in apoptosis (33).

Because exposure to ionizing radiation combined with wound trauma enhances iNOS gene expression and iNOS protein levels, due to activation of both NF-κB and NF-IL6 and increases in serum cytokines (31), greater production of peroxynitrite anion and more protein nitration is anticipated relative to that seen after radiation exposure alone. Apoptosis can thus be expected to occur to a greater extent after radiation combined injury. Peroxynitrite anion also leads to more LC3-mediated autophagy (see below).

Ionizing radiation activates PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways (10). The PI3K/AKT pathway activates anti-apoptotic proteins (17, 30). The MAPK pathways include extracellular signal-regulated kinase 1/2 (ERK1/2) activity (2), JNK (48), and p38 (34). The former is anti-apoptosis, whereas the latter two are pro-apoptosis. It is not clear if radiation combined with wound trauma enhances these pathways.

**Free Radical-Mediated Autophagy**

A growing body of evidence suggests that ionizing radiation induces programmed cell death mediated not only by the Bcl-2 family of proteins and caspase proteases (type I cell death) but also autophagy-dependent programmed cell death type 2 (PCDT2) (50). The role of ionizing radiation-induced autophagy in normal cells, especially in the cells of dose-sensitive tissues such as small intestine, is a subject that requires attention.

Autophagy (or autophagocytosis) is a lysosomal mechanism of degradation of self-constituents that is evolutionary conserved and occurs in various eukaryotic cells (14, 40, 70). Three forms of autophagy have been distinguished, based on how intracellular material is delivered to lysosomes: chaperone-mediated autophagy, microautophagy, and macroautophagy (49). Macroautophagy (mAG) is the most common form of autophagy; under normal conditions mAG is responsible for the routine bulk degradation of redundant or defective organelles, long-lived proteins, large macromolecules, and pathogens. mAG thus provides a homeostatic balance between biosynthetic and biodegradative activities and innate immunity. mAG is characterized by the formation of autophagosomes (phagophores), in which portions of cytoplasm are sequestered, cargo packaged within a double
membrane-enclosed vacuole, and transported to lysosomes or late endosomes for biodegradation (29, 59).

One of the crucial steps of this multistage process is conversion of light chain protein 3 type I (LC3-I) (also known as ubiquitin-like protein Atg8) to type II (LC3-II) either by a redox sensitive Atg4 serine protease or by E-1 and E-2 like enzymes Atg7 and Atg3 (9, 62, 74). LC3 protein is considered a marker for autophagosomes (9, 62).

mAG is induced in response to certain conditions including exposure to ionizing radiation. Induction of mAG in response to cytotoxic stress can be either protective or detrimental. It has been recently shown that PCDT2 is related to the damage-regulated autophagy modulator (DRAM), the death associated protein kinase (DAPK), autophagic massive elimination of apoptotic mitochondria, and oxidative activation of Atg4 serine protease, which can occur via free radical mechanisms activated by ionizing radiation. Although the free-radical species produced by ionizing radiation have short-term effects, the subsequent activation of pro-oxidant pathways, such as the iNOS cascade, can potentiate and prolong oxidation and thus extend up-regulation of mAG.

LC3-II is identified in host small intestine-defense cells such as Paneth cells, which are considered to be relatively resistant to radiation and can therefore help maintain the GI barrier after otherwise lethal insults. We assessed the dynamics of LC3 protein to track mAG in ileal crypt cells after ionizing radiation or radiation combined injury. We found that there is a larger increase in LC3-II in CD15-positive Paneth cells at day 7 after radiation combined injury than after radiation injury alone (Figs. 3 and 4). The increase is correlated with iNOS activation, NO production, lipid peroxidation, and protein nitration. The up-regulation of autophagy is accompanied by a decrease in protein-protein interaction between LC3, heat shock protein 70 kDa, and Bcl-2-associated anfhanogene-1 (18).

**Bacterial Infection Activates Signal Transduction Pathways**

It is evident that overproduction of IL-6, NO, or nitrogen reactive species can cause dysfunction of the GI barrier (22, 54, 77), resulting in bacterial entry into the systemic organs. In our laboratory, we collected heart blood and liver tissue from recently deceased or euthanized sham, wounded, radiation-injured, or radiation combined-injured mice and cultured the tissue to determine if facultative bacteria had entered the circulation. Since tissues from healthy animals are normally sterile (except for occasional, transient bacteremia), the presence of bacteria in detectable numbers is indicative of systemic infection.

In sham-treated mice no bacteria were found in the tissues tested. In wounded and radiation-injured mice *Enterococcus sp.* and *Staphylococcus sp.* were only occasionally detected. However, in radiation combined-injured mice, *Enterococcus sp.*, *Staphylococcus sp.*, *Bacillus sp.*, and *Lactobacillus sp.* were common, and the same bacterial species were also isolated from ileum. Bacteremia in mice receiving wounds alone was transient and present only until day 3 after wounding. On the other hand, systemic infection was demonstrated in radiation combined-injured mice through day 17 and sporadically in radiation-injured mice through day 25. In radiation combined injured-mice, *Bacillus* and *Lactobacillus* were isolated within the first 8 d after radiation combined injury. The data imply that mice receiving wounds alone were able to resist infection. While systemic infection occurred in both radiation combined-injured mice and mice receiving radiation alone, it occurred several days sooner in the radiation combined-injured mice.

Bacteremia induced increases in serum cytokine concentrations, which further promoted iNOS over-expression and activation in radiation combined-injured mice (31). It is important to note when interpreting these data that luminal microbiota composition may influence the host’s intestinal response to radiation and may change in those developing postirradiation diarrhea (61). For this reason, it is not surprising to observe variations in the intestinal response either to radiation or radiation combined with wound trauma.
Radiation Combined-Injury Countermeasures

A synergistic effect between radiation and traumatic injury has been reported in mice (31, 43), rats (1, 71, 75), guinea pigs (37), dogs (6, 66), and swine (4). Key features of radiation combined injury include: (a) shock, which occurs earlier and is more frequent and severe compared to simple radiation injury, often becoming the main cause of death at times soon after injury; (b) dramatic suppression of hematopoiesis and the immune system, which negatively affects prognosis after radiation combined injury; (c) extensive and severe GI damage, such as mechanical and immune barrier breakdown, which leads to dysfunction in absorption and secretion and increased risk of infection; and (d) delayed wound healing—often double the healing time of wounding alone.

Since the mechanisms of radiation combined injury appear to be more complicated than the mechanisms of the individual injuries alone, it can be expected that the treatments are also not as straightforward. DiCarlo et al. (11) suggests that the complexity of the response makes them pessimistic that any effective treatments amenable for use in a mass casualty scenario can be found. However, the search for pharmacological countermeasures for radiation combined injury has shown some promise.

Zou et al. (78) reports that cervical sympathetic nerve block once a day for 14 d after radiation combined injury significantly decreases mortality (51). Ledney and Elliott (43) reported that the nonspecific immunomodulator S-TDCM given i.p. immediately after radiation combined injury, along with systemic and topical application of gentamicin, improves survival. They also reported that syngeneic bone marrow transplantation increases the survival of mice with combined injury. Shah et al. (71) reported that human ghrelin attenuated organ injury and improves survival in a rat model of radiation combined with sepsis.

The medical response to a mass casualty scenario will likely always be different from how a small number of first responders to a radiation-contaminated area or radiation therapy patients are treated. It is clearly unrealistic in mass casualty situations to undertake cervical sympathetic ganglia blocks, bone marrow transplants, or even the intravenous administrations of drugs. Intramuscular injections, orally administered drugs, and perhaps subcutaneous injections (39) may be the most complex treatments available to mass casualty victims. Countermeasures for radiation attacks or nuclear accidents that must be given prior to radiation exposure are impractical since it is unlikely such events will occur with adequate warning; however, they could prove useful if radiation exposures are likely or if they are planned, as for radiation therapy. Successful countermeasure development must therefore address the requirements of a variety of very different scenarios.
Fig. 5. Radiation combined injury attenuates the normal defenses. Various interventions to treat radiation combined injury may be used alone or in combination to improve the chance of survival in severely injured patients.

Conclusion

Radiation combined with wound trauma results in decreases in lymphocytes, macrophages, neutrophils, platelets, cell adhesion molecules, tissue integrity, and stem cells, but increases in activity of the iNOS/NF-κB/NF-IL6 and p53/Bax pathways, TLR signaling, cytokine concentrations, bacterial infection, cytochrome c release from mitochondria to cytoplasm, and DNA single and double strand breaks. These alterations lead to apoptosis and autophagy, resulting in mortality. Radiation injury combined with burns, infection, or fractures may be mediated by mechanisms similar to those observed after radiation injury combined with wound trauma. Countermeasures available for radiation combined injury are currently very limited (Fig. 5), so the development of agents for prevention, mitigation, and treatment remains a pressing need.

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