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Poloxamer 188 prevents acute necrosis of adult skeletal muscle cells following high-dose irradiation

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Abstract

Acute cellular necrosis occurring minutes to hours after massive ionizing radiation exposure (IR) results from rapid membrane lipid peroxidation, blebbing and membrane breakdown. We have shown, previously, that certain polymer surfactants can restore structural integrity and transport barrier function of cell membranes following high-dose IR. We now investigate, specifically, the efficacy of the amphiphilic surfactant Poloxamer 188 (P188) in preventing acute necrosis of adult rat skeletal muscle cells after high-dose IR. Explanted cells were treated with ^{60}Co IR doses of 10, 40 or 80 Gy and their viability was determined using fluorometric probes at 4 and 18 h post-IR. IR of 10 Gy did not cause acute necrosis. Significant acute cell necrosis was observed after 40 and 80 Gy doses in a dose-dependent manner. Post-IR treatment with P188 significantly enhanced the cells' viability post-IR treatment. By comparison 10 kDa neutral dextran, a hydrophilic polymer, was found to be ineffective. Despite progressive cell death over 18 h after high-dose IR, cells treated with P188 manifested greater survival than media or dextran-treated cells. It appears that use of P188 or similar multi-block copolymers to prolong viability of irradiated cells in vitro through membrane sealing is an important step in development of effective interventional therapy for extreme IR exposure. Not only can repairing the membrane prevent acute necrosis, but it also can provide a critical time opportunity to address other mechanisms of cell death, such as apoptosis or mitotic arrest, which manifest over a longer time frame.

Keywords: Post-mitotic cells; Ionizing radiation; Necrosis; Poloxamer; Membrane sealing

1. Introduction

Today, there is no known effective therapy to treat victims of massive radiation exposure in the range typical of the emergency Chernobyl fire fighters. At such extreme doses, even terminally differentiated cells like nerve and muscle are rapidly killed. Exposure to intense ionizing radiation produces damaging reactive oxygen intermediates (ROI) that rapidly degrade membrane lipids and alter the structure of the bilayer, compromising its ionic barrier function (Fig. 1). This is followed by diffusion of ions across the membrane, including the influx of extracellular calcium, and the onset of acute cellular necrosis. Because cellular metabolic energy is largely expended by providing ATP to membrane ion pumps, loss of lipid bilayer integrity leads quickly to metabolic exhaustion and arrest of cellular enzymatic activity including DNA repair. Loss of cell viability through ROI-induced membrane permeabilization occurs within hours [1–5]. This mechanism is a common cause of cell necrosis in many diseases, including post-ischemic reperfusion injury, electric shock and burn injuries, and traumatic injury [6–9].

It has been shown that several multi-block copolymer surfactants (i.e. poloxamers) can induce sealing of damaged membranes following reperfusion injury, electroporation, heat shock and traumatic injury [9–13]. Recently, we reported that poloxamers can prevent hemolysis of heavily irradiated adult erythrocytes and can arrest leakage of either calcein-loaded skeletal muscle cells or lymphocytes result-

Abbreviations: EH, ethidium homodimer-1; HEPES, *N*-2-hydroxyethylpiperazine-*n*-2-ethanesulfonic acid; IR, ionizing radiation exposure; P188, Poloxamer 188; PEO, poly(ethylene oxide); PPO, poly(propylene oxide); ROI, reactive oxygen intermediates

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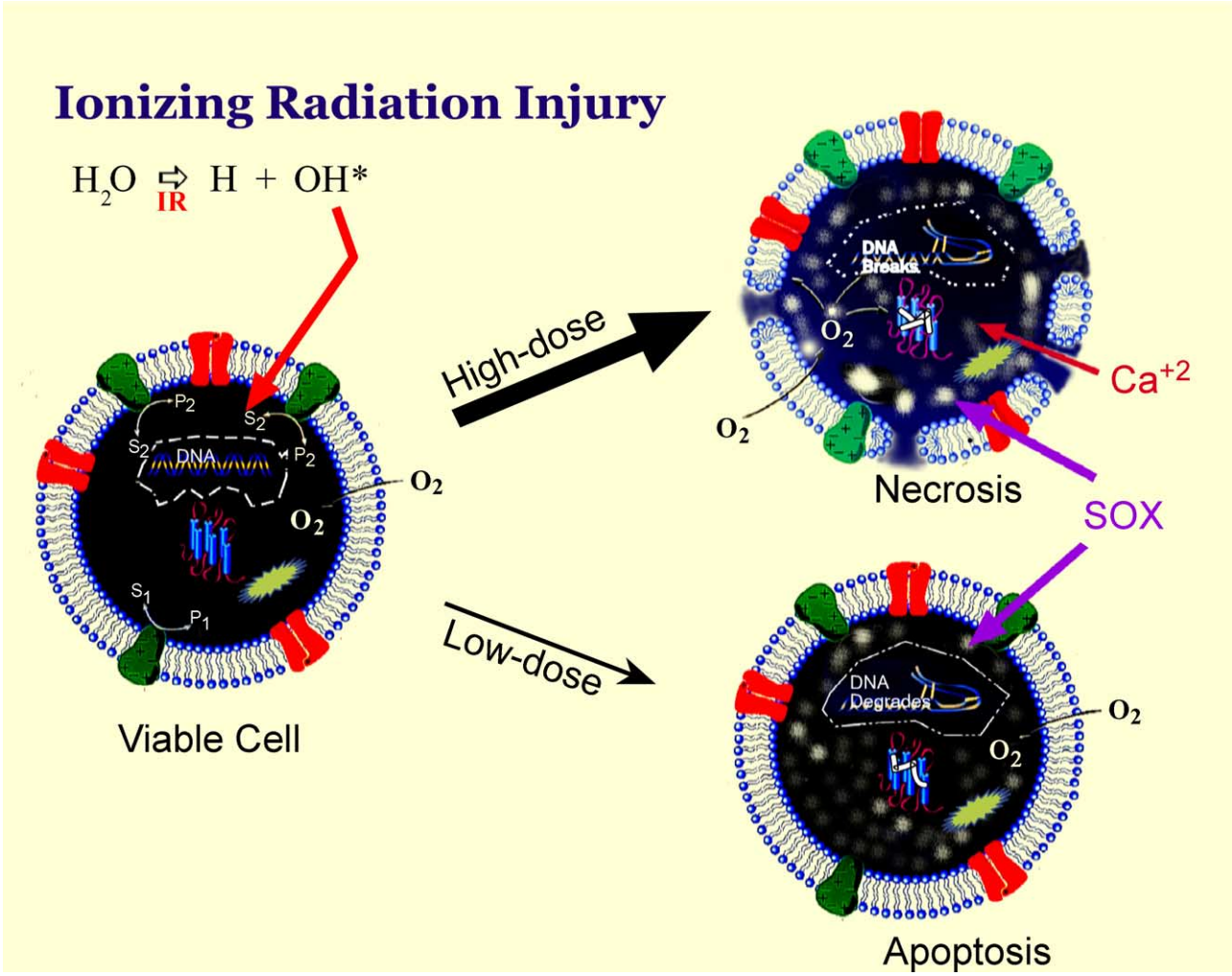

Fig. 1. Illustration to distinguishing modes of cell death resulting from ionizing radiation (IR) exposure. To clarify terminology used in this report, "High-dose" IR generates sufficient hydroxyl radicals (OH*i) to disrupt cellular membranes, resulting in loss of transmembrane ionic gradients, calcium influx effects, metabolic exhaustion, intense superoxide (SOX) generation, necrosis and tissue inflammation. "Low-dose" IR references IR induced cell death mediated by signaling to apoptotic genes with minimal SOX production and production of inflammatory mediators. Protein and nucleic acid damage is a common feature of both.

ing from acute ionizing radiation-induced membrane permeabilization [14–16]. In addition to stabilizing a disrupted membrane, certain poloxamer polymers may reduce the secondary ROS elevation, as suggested by Marks et al. [12].

The purpose of this study was to explore, directly, whether poloxamer induced membrane sealing would actually lead to enhanced cell survival following such severe radiation expose that acute cellular necrosis would be expected. Today, this essential question has remained unanswered. To address this question, we used non-proliferating, terminally differentiated adult rat skeletal muscle cells. The range of ^{60}Co gamma irradiation was selected because of its known ability to induce acute cellular necrosis via membrane permeabilization. Poloxamer 188 (P188), a tri-block copolymer composed of two poly(ethylene oxide) (PEO) chains separated by a poly(propylene oxide) (PPO) chain, was chosen as it is well characterized among the various available poloxamers. Dextran (10 kDa), the purely hydrophilic polymer, was chosen as a control treatment because its molecular weight is similar to P188, it tends to adhere to cell surfaces and is commonly used in burn care to alleviate edema formation.

2. Materials and methods

2.1. Cell culture and viability assay

Flexor digitorum brevis skeletal muscle cells were harvested from 4-week-old female Sprague–Dawley rats (Harlan–Sprague–Dawley Inc., Indianapolis, IN) through the University of Chicago Carlson Animal Facility. The protocol for using laboratory animals was approved by the Institutional Animal Care and Use Committee. The muscle was dissected out within 20 min of sacrifice via asphyxiation. It was soaked for 18–20 h in a solution of 0.3% collagenase type III and 0.35%

trypsin (Worthington Biochemical Corporation, NJ) in phosphate-buffered saline containing calcium and the pH buffer *N*-2-hydroxyethylpiperazine-*n*-2-ethanesulfonic acid (HEPES, Sigma, St. Louis, MO). Individual cells were separated by incubation at 37 °C for 32 min in this solution to allow for enzymatic digestion of the binding structure. After digestion, the cells were washed, separated by a brief, gentle trituration and distributed into tissue culture dishes (Falcon, Cambridge, MA) at 250–350 cells per dish. One muscle cell isolation procedure allowed seeding of 11–15 culture dishes. To let the cells recover from the mechanical stress of the trituration, the cells were left untouched in culture for 3 days at 37 °C and 95% relative humidity in Minimum Essential Medium (Gibco BRL, Grand Island, NY) inside a water jacketed incubator (ThermoForma Scientific, model 3326, Marietta, OH). The medium was supplemented with 25 mM HEPES, 10% Nu-serum (Collaborative Biomedical Products of Becton Dickinson, Bedford, MA), 50 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL).

In preparation for post-IR viability testing, an initial viability measurement was taken on the third day after cell harvesting. Ethidium homodimer-1 (EH) dissolved in 1:4 DMSO/water and calcein-AM (Molecular Probes, Oregon) dissolved in dry DMSO were added from stock solutions to stain the cells at final concentrations of 10 and 3.3 μM, respectively. EH fluoresces with a red color ($\lambda_{ex} = 528$ nm; $\lambda_{em} = 617$ nm) after binding to DNA within the cell; observing its fluorescence indicates cell death since the cell membrane has become permeable to this molecule of M_r 856.77. Calcein-AM fluorescence occurs only after the uncharged dye molecule has penetrated the cell membrane and is cleaved into calcein by an ATP-dependent process. Observing its green fluorescence ($\lambda_{ex} = 494$ nm; $\lambda_{em} = 517$ nm) indicates that the cell retains metabolic capacity, as well as an intact membrane that can keep the charged calcein within the cell. For the purposes of this experiment, cells showing enough permeability to admit any visible accumulation of EH were deemed non-viable, even if they also showed some residual metabolic activity (some cells appeared both green and red under the fluorescence microscope). Fifteen minutes after the dyes were added to the dishes, their fluorescence was observed under a Nikon Diophot inverted microscope with fluorescent optics. The excitation wavelength was $\lambda_{ex} = 480$ nm.

If the initial cell viability of one cell batch tested in two to three dishes was at least 70% of the original cell count, the cells obtained from this muscle cell isolation procedure were considered healthy and suitable for IR experiments. Then, the remaining dishes were labeled according to the experimental parameters as follows: (1) three dishes for non-IR sham-exposed controls; (2) three dishes for IR but untreated controls; and (3) three dishes for post-IR treatment with either P188 or dextran control. Occasionally, a higher preparation yield allowed a direct comparison of P188 and dextran on the same batch of muscle cells.

During the transport to the IR chamber the culture dishes were placed on top of a 37 °C heating pad in an insulated box to minimize their temperature variation. The non-IR sham-exposed control cells were taken to the irradiation source along with the IR-designated cells, in order to be subjected to the same temperature and motion stress caused by handling and transport. If the viability of sham-exposed cells in 18 h experiments decreased by more than 20%, the cell culture was considered defective and the experiment was discarded. This occurred in four experiments.

2.2. Effect of polymer on cell viability

To rule out a possible independent effect of polymer supplementation on cell viability, we performed a separate set of experiments to measure the viability of polymer-treated non-IR cells. For this purpose, cell culture dishes were treated exactly as a non-IR sham-exposed control but they received either 1 mM P188, 1 mM dextran or regular culture media supplementation. Viability of the cells was then assessed after 18 h. Results of these experiments indicate that neither polymer has any independent effect on the viability of non-IR cells (Table 1).

2.3. Gamma radiation exposure protocol

Cells were exposed to ^{60}Co gamma radiation provided by a Gammacell® 220 (AECL, Chalk River, Ontario). Three IR levels were chosen for this study: 10, 40, and 80 Gy. Exposure time was calculated from a dose-rate calibration table (approximately 1.5 Gy/s) furnished by the Laboratory for Radiation and Oncology Research, University of Chicago. Dishes containing cells to be exposed were placed into the Gammacell® unit and exposed for the time necessary to receive the appropriate dose.

2.4. Polymer treatment of irradiated cells

After exposure, cells were returned to the tissue culture laboratory. At 10 min post-IR, P188 or dextran control treatment was added from a stock solution in culture medium to the medium in the three dishes that had been exposed to

Table 1
Effect of different polymer supplementations to culture media on the mean percent viability (±S.E.M.) of primary isolated rat skeletal muscle cells after 18 h

Polymer supplement	Initial viability	18 h post-supplementation		N^a
		Media control	Polymer	
1 mM dextran	71.7 ± 3.5	76.7 ± 2.9	75.1 ± 2.9	4
1 mM P188	76.4 ± 4.2	71.9 ±3.8	71.9 ± 2.8	4

There is no statistically significant difference between all experimental groups ($P = 0.777$, ANOVA).

[a] Indicates the number of independent exposure experiments each in triplicate.

IR. The other three IR and three non-IR sham-exposed culture dishes received a supplement of regular culture media to match the increased media volume resulting from polymer administration. Cells were then returned to the incubators and allowed to rest for 4 or 18 h. The following combinations of different levels of IR exposure and subsequent treatments were performed:

2.4.1. 4 h post-IR viability

Cells exposed to 10, 40 and 80 Gy received either no post-IR treatment or Poloxamer 188 (M_r 8400, BASF, Parsippany, NJ) at a final concentration of 1 mM. At the 40 Gy level, where the strongest effect of P188 treatment was observed, experiments were also conducted with 1 mM 10 kDa dextran as a purely hydrophilic control polymer treatment.

2.4.2. 18 h post-IR viability

Cells exposed to 40 Gy IR received either no post-IR treatment, 1 mM P188, 2 mM P188 or 1 mM 10 kDa dextran (Sigma).

2.5. Fluorescence viability assay

Either 4 or 18 h after IR or sham exposure, cells were loaded with fluorescent dye for viability testing using the same assay as described for the initial viability testing. In addition, either fluorescence or phase-contrast photomicrographs of representative cells were taken using a 35 mm SLR camera (Nikon F3) to allow for monitoring the IR-induced morphological changes.

For the purposes of photomicrography, a subset of the cells was cultivated on a 1.5 mm cover slip inside a culture dish. After IR, the cover slip was immediately placed on the microscope stage and the cells were observed continuously for up to 4 h. To avoid temperature effects in the culture dish during this period, the microscope stage was enclosed with an incubation chamber kept at 37 °C. A mineral oil layer floating on the media prevented the culture from drying out. The cells were photographed every 0.5 h. In addition, a representative series of photos of a cell's contraction due to IR-induced death was taken.

2.6. Data analysis

The results of the viability assays from each experimental culture dish were recorded as both the number of cells exhibiting any visible red EH fluorescence and the number of cells with only calcein-AM green fluorescence. The percentage of cells that were viable at the time interval after exposure was determined as the percentage of cells exhibiting calcein fluorescence alone (percent viability). This value was the prime experimental parameter for statistical analysis. Statistical analysis considered the mean percent viability of the multiple samples for each parameter (various IR exposure levels or sham exposure, with and without P188 or dextran). If data obtained were outside of the 95% confidence interval, i.e. deviated by more than 1.96 × S.D. from the mean, the results were excluded. This happened only in one 40 Gy/18 h experiment where media volume supplementation alone yielded an exceedingly high viability of 64.5% compared to 77.6% in the non-IR sham-exposed control.

A repeated measure ANOVA analysis of the data was used to test for the presence of an effect due to IR and an effect due to either polymer treatment. When the ANOVA analysis indicated a statistical difference between the test groups, multiple pair-wise comparisons between test conditions were made using Bonferroni's *t*-test. Statistically significant differences were required to have $P < 0.05$. Tests were run using the SigmaStat Statistical Analysis Program (SPSS Inc., Chicago, IL).

3. Results

3.1. Cell culture viability

Skeletal muscle cells were typically 1–2 mm in length and 25–50 μm in diameter. They were quite vulnerable to damage by mechanical trauma. After several months of practice, it was possible to achieve typical levels of survival. The average initial viability of the cultured skeletal muscle cells after 48 h in culture 79.4 ± 5.2 (mean ± S.D.). These cells were used in the IR exposure experiments.

3.2. Viability post-irradiation

Fig. 2 presents several skeletal muscle cells, viable and non-viable, as observed under the microscope. Fig. 2A is a fluorescence picture of the cells taken at 18 h after non-IR sham exposure, as they appear during the fluorescence-based viability counts. The viable cells exhibit calcein fluorescence (green) only, whereas the non-viable cells demonstrate either calcein (green) and EH fluorescence (red) together or EH only (red). In other words, cells exhibiting any fraction of EH fluorescence were considered non-viable. Fig. 2B is a phase-contrast photomicrograph of the same cells. It shows the normal cell shape and morphological changes in the contracting, non-viable cells. These results demonstrate that the viability assay used in our study is adequate for its purpose.

Fig. 3A–H demonstrate the process of 40 Gy IR-induced cell contraction through a series of phase-contrast photomicrographs. Generally speaking, at approximately 1–2 h after 40 Gy IR, at least one bleb somewhere along the cell membrane could be observed. The cells appeared to contract or collapse at the site of this initial bleb. The full contraction and death of the cell rapidly followed. In the final stage of cell death, the bleb was enlarged to a bubble and often appeared as a halo surrounding the cell. The entire process, from the first visible change in membrane structure to the full contraction, lasted from 15 to 30 min.

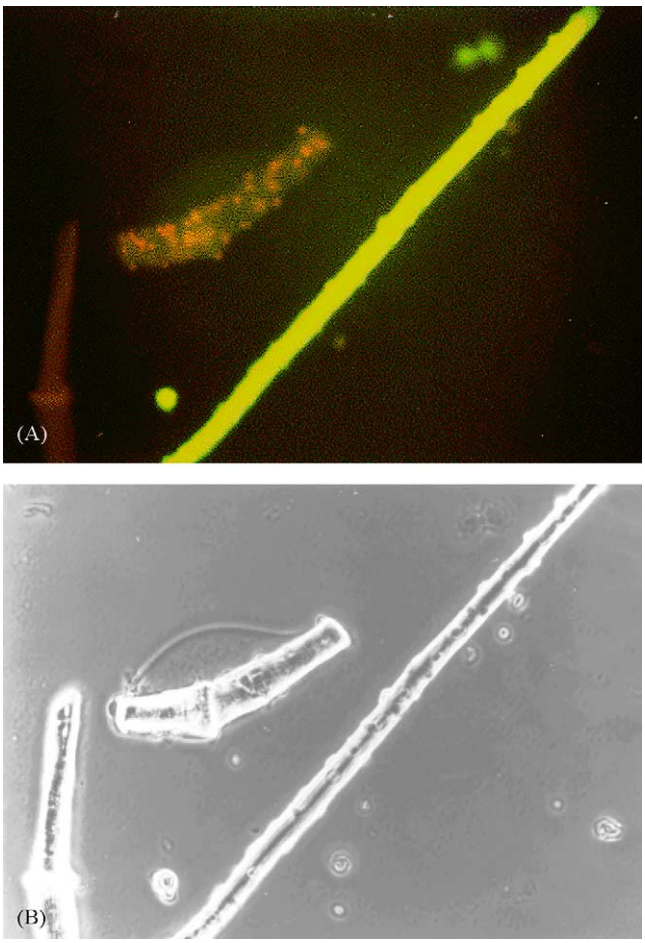

Fig. 2. Photomicrographs of primary isolated rat skeletal muscle cells taken at 18 h after non-IR sham exposure. Sham exposure is defined in Section 2.1. Fluorescence image (A) and phase-contrast (B) of viable and non-viable cells. Fluorescence speed was 400, photo contrast speed 200, blue filter, no cube. Green fluorescence indicates active cell metabolism through calcein-AM cleavage and red fluorescence indicates the permeation of ethidium homodimer-1 through the cell membrane of non-viable cells. The bar indicates 50 μm.

3.3. Cell viability at 4 h after irradiation

Fig. 4A shows the mean percent viabilities (±S.E.M.) of the primary isolated skeletal muscle cell cultures before IR treatment, 4 h after the application of 10, 40, and 80 Gy of gamma irradiation and after P188 treatment (1 mM, added 10 min after IR). ANOVA analysis indicates a statistically significant effect on viability due to IR dose (10 Gy versus 40 Gy versus 80 Gy) ($P < 0.001$), as well as a statistically significant effect on viability due to P188 administration ($P < 0.001$). It also indicates a significant interaction between these two effects ($P < 0.001$).

Multiple pair-wise comparisons between the data in Fig. 4A for the various exposures (non-IR sham exposure or 10, 40, and 80 Gy IR) and treatments (P188 or no treatment) indicated the following. After 10 Gy IR, cells exhibited essentially no change in viability, whether they were P188-treated (75.3±4.3%) or untreated (72.2±4.5%), compared to sham-exposed controls (79.2 ± 2.6%, $P \geq 0.05$). Cells exposed to 40 Gy showed significantly greater viability with P188 than without (67.0±6.9% versus 10.7±3.0%; $P < 0.01$). The cells' viability following P188 treatment was not significantly less than that of sham-exposed controls (82.2 ± 2.1%; $P < 0.10$). At 80 Gy, the viability of cells treated with P188 was significantly greater than that of untreated IR cells (18.8 ± 5.5% versus 0.4 ± 0.3%; $P < 0.05$), although significantly fewer treated cells remained viable compared to sham-exposed controls (74.2 ± 3.1%; $P < 0.01$).

Fig. 4B shows the mean percent viabilities (±S.E.M.) at 4 h after exposure to 40 Gy for experiments using 1 mM of 10 kDa dextran (to be compared with the 40 Gy/P188 data in Fig. 4A). Statistical analysis indicates that dextran treatment had no effect on cell viability, and there was no significant difference between dextran-treated and untreated cells (9.2 ± 3.5% versus 3.1 ± 1.2%; $P \geq 0.05$). There was a statistically significant difference between P188 and dextran treatment post-IR (67.0 ± 6.9% versus 9.2 ± 3.5%; $P < 0.01$).

3.4. Cell viability at 18 h after 40 Gy

Fig. 5 shows the effect of each of three different treatments on the mean percent viabilities (±S.E.M.) in the primary isolated skeletal muscle cell cultures at 18 h after 40 Gy IR. One of each of the three different polymer treatments was added 10 min post-IR: 1 mM P188, 2 mM P188 or 1 mM 10 kDa dextran. ANOVA calculations indicate statistically significant effects due to the IR and P188 treatments (both with $P < 0.001$), but not to the dextran treatment ($P \geq 0.10$). They also indicate a statistically significant interaction between the IR and the P188 treatments ($P < 0.001$).

Multiple pair-wise comparisons of the cell viability between non-IR sham-exposed control, untreated IR cells and the different post-IR treatments indicated the following. The cells did show greater viability when treated with 1 mM P188 than without treatment (18.1±3.7% versus 2.2±0.9%; $P < 0.01$). The fraction of viable cells, however, was significantly smaller compared to non-IR sham-exposed controls (77.5 ± 2.4%; $P < 0.01$). The cells treated with 2 mM P188 demonstrated similar improved viability compared to no post-IR treatment (14.0 ± 3.5% versus 3.5 ± 1.1%; $P < 0.01$) and lower viability compared to non-IR sham-exposed controls (78.2±2.1%; $P < 0.01$). There was no statistically significant difference between the effects of 1 and 2 mM P188 ($P \geq 0.05$).

Dextran (10 kDa, 1 mM) had no statistically significant effect on cell viability compared to no post-IR treatment (5.3 ± 1.1% versus 1.9 ± 1.1%; $P \geq 0.05$). In each experiment, when we performed a direct treatment comparison on the same skeletal muscle cell isolation batch, 1 mM P188 preserved the viability of the cells at a level similar to 2 mM

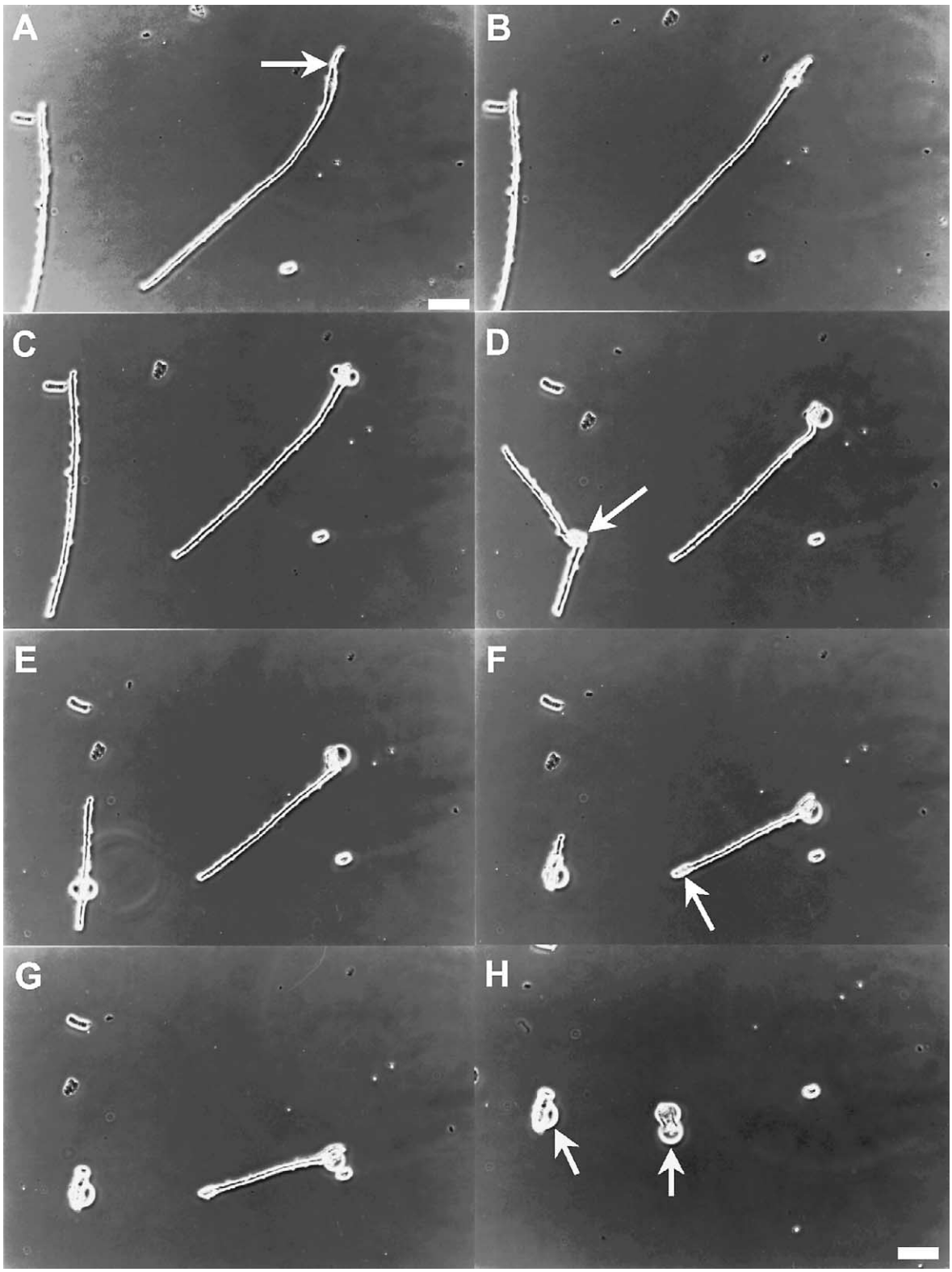

Fig. 3. Phase-contrast photomicrograph series (A–H) of unstained primary isolated rat muscle cells showing the bleb formation (arrows in panels A and D) in the membrane and subsequent collapse and death of the untreated control cells (arrows in panel H) after IR with 40 Gy. The first photomicrograph was taken at 1 h post-IR and the following ones at 2 min intervals. The bar indicates 100 μm.

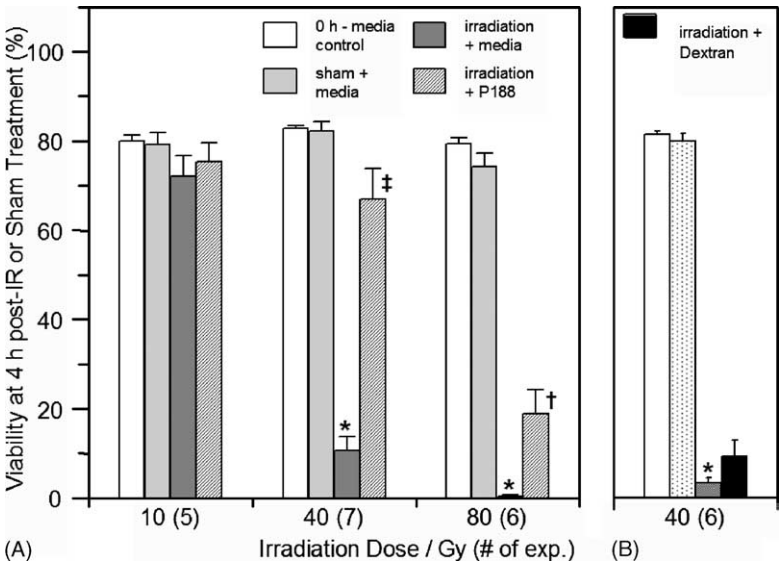

Fig. 4. IR dose and post-IR P188 supplementation effect on the mean percent viability (±S.E.M.) of primary isolated rat skeletal muscle cells after 4 h (panel A). Sham exposure is defined in Section 2.1. Cell necrosis increases with IR doses and is significantly reduced by 1 mM P188 treatment added at 10 min post-IR. Panel B shows the absence of a significant protective effect of 1 mM dextran at 4 h post-40 Gy. There are no statistically significant differences between experimental groups (ANOVA, Bonferroni's *t*-test) except: (∗) statistically significant differences vs. respective initial viability and 4 h sham exposure control ($P < 0.01$); (‡) statistically significant differences vs. respective untreated IR group ($P < 0.01$); (†) statistically significant differences vs. respective non-IR sham-exposed controls and (‡) ($P < 0.01$), as well as vs. respective untreated IR sample ($P < 0.05$).

P188 ($n = 2$ batches), while dextran was not effective ($P < 0.01$) ($n = 5$ batches/dextran versus 1 mM P188, and $n = 6$ batches/dextran versus 2 mM P188).

In the interpretation of these experiments, the definition used for cell skeletal muscle cell viability was both the presence of an intact plasma membrane and active intracellular ATP production. We considered all cells that exhibited leaky membranes (i.e. any visible EH fluorescence), despite sufficient ATP existed to activate calcein, to be non-viable. This approach was justified by our observation that at 18 h post-IR with 40 Gy, the percentage of green fluorescent cells (calcein stained) was much lower than the percentage of cells that labeled with both EH and calcein found at 4 h. This suggests no recovery red/green cells occurred.

In this experimental model, we did not explicitly control the culture media oxygen levels, although at 40 and/or 80 Gy, oxygen depletion in the media might have occurred. In a published high-dose gamma IR study (45–600 Gy at a lower 0.25 Gy/s), Kamat et al. investigated the oxygen effect on the formation of immediate and final products of lipid peroxidation in isolated rat liver mitochondria membranes [17]. They found a significant increase in lipid peroxidation under normoxic conditions compared to either radiation alone or under anoxic conditions. The results of this study suggest that the post-IR viability under oxygen saturated media conditions will be lower than the one we observed in both P188-treated and untreated irradiated samples. With the higher level of peroxidation products under normoxic conditions, however, P188 would still be expected to produce significant improvements.

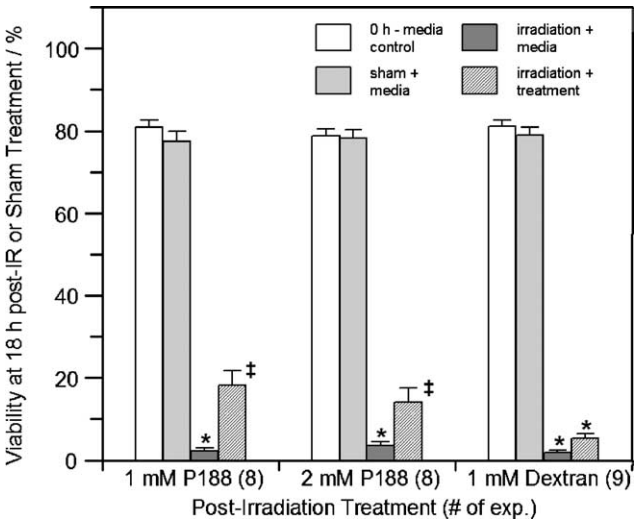

Fig. 5. Effect of different polymer supplementation to culture media on the mean percent viability (±S.E.M.) of primary isolated rat skeletal muscle cells at 18 h post-40 Gy IR. Sham exposure is defined in Section 2.1. Treatments applied at 10 min post-IR were: 1 mM P188, 2 mM P188, and 1 mM dextran. Statistical differences between post-IR treatments are as follows: (∗) no statistically significant differences vs. each other ($P \geq 0.05$); (‡) statistically significant differences vs. (∗) ($P < 0.01$) but not vs. each other ($P \geq 0.05$).

4. Discussion

Cell death following intense IR exposure can proceed via a number of mechanisms other than disruption of the

structural integrity of the membrane, such as induction of apoptosis from effects on cellular signaling pathways or cell death secondary to DNA and RNA damage [18,19]. However, these mechanisms of cell death progress less rapidly than IR-induced disruption of membrane integrity followed by acute cellular necrosis. The primary objective of this research is to develop a strategy to preserve cell viability after massive ionizing radiation exposure. Because acute cellular necrosis destroys heavily irradiated cells rapidly, preventing necrosis is required first to allow an opportunity to therapeutically intervene in the other injuries that could subsequently lead to cell death. Therefore, restoring membrane integrity is a prerequisite for therapeutic success.

We reported previously that after 160 Gy IR, loss of the red blood cell membrane integrity occurs within less than 1 h [15]. In the present study, we observed a dose-dependent progression of acute cellular necrosis in the range from 10 to 80 Gy ^{60}Co IR. While 10 Gy had did not cause acute cellular necrosis, 40 Gy exposure triggered clear evidence of membrane disruption within 1–2 h after IR, with only about 15–30 min elapsing from a first visible membrane defect to the full cellular collapse. The fraction of viable cells 4 h post-IR decreased with increasing IR dose level. The IR dose-response effect on the short-term viability suggests that the ROI-induced lipid peroxidation, and the degree of subsequent membrane permeabilization, is strongly accumulative in the range tested (10–80 Gy).

Although, at 18 h post-IR, about eight times as many cells were viable with P188 treatment compared to untreated cells (18.1% versus 2.2%), the observed post-IR P188 treatment effect in terms of fraction of rescued cells was significantly less marked at 18 h than at 4 h post-40 Gy exposure (18.1% versus 67.0%). This can be seen by comparing the 40 Gy "irradiation + P188" bar in Fig. 4 with the 1 mM "irradiation + polymer" bar in Fig. 5. This decrease in treatment effect could be due to the degradation of the poloxamer molecule over time or loss of its effectiveness before cell repair mechanisms could complete their tasks. This hypothesis is currently being tested in our laboratory by investigating the effect of media supplements that counteract P188 degradation. However, because the effect on cell viability of the 2 mM P188 concentration was no better than 1 mM, a diminishing protective effect of the surfactant over time may not be the only reason. Another likely explanation is that membrane sealing only blocks acute necrosis, while the slower apoptotic processes progress unimpeded, and the metabolically exhausted cells cannot recover from the insult, even after the cell membrane integrity is partially restored. An alternate explanation, is that there are toxic small molecular weight fragments released from P188 degradation which reaches significant levels at the 2 mM concentration.

We postulate that the efficacy of P188 in decreasing the extent of acute IR-induced necrosis is caused by its membrane sealing capability. The mechanisms by which poloxamers accomplish this effect are under investigation. Marks et al. reported clear evidence that poloxamer inserts into the lipid bilayer of living cells and decreases lipid peroxidation [12]. Other experiments have shown that while the hydrophilic PEO tails remain in contact with the aqueous phase or at the interface, it is the hydrophobic PPO core, specifically, that inserts into the acyl-chain portion of a lipid film, suggesting that the amphipathic nature of the poloxamer is responsible for its behavior [20–23]. Additionally, experimental evidence from Maskarinec and Lee suggests that poloxamer interacts exclusively with membrane sites that are damaged, and not with intact membrane layers [24].

The P188 dose in our in vitro experiments is in the range used in multiple safety and efficacy clinical trials on P188 for myocardial infarction and sickle cell crises [25,26]. Only rough comparisons can be made, however, since most studies use continuous P188 infusions over an extended period of time (up to 72 h) rather than bolus injections. In a pharmacokinetic study with infusions of 90 mg/(kg h) over up to 48 h by Jewell et al. average steady-state plasma P188 concentrations of 1.31 ± 0.25 mg/ml were well tolerated in human subjects up to a cumulative dose of 2.5 g/kg (ca. 27 h of infusion) [27]. This plasma P188 concentration is equivalent to 0.16 mM, about one-sixth of our in vitro dose.

Experiments with 10 kDa dextran did not produce a decrease in acute cell necrosis after IR. These results indicate that P188, and possibly other similar amphiphilic molecules, have important physicochemical properties that make them effective in reducing the membrane damage while the hydrophilic dextran does not. One plausible explanation is that the hydrophobic domain of the P188 colocalizes to exposed hydrophobic areas of disrupted cell membranes or hydrophobic areas of exposed proteins. This would concentrate the P188 at the site of injury. Of course, this effect would not occur with a pure hydrophilic polymer like dextran. In previous experiments quantifying hemoglobin leakage from irradiated isolated human red blood cells showed an effective reduction of hemoglobin in the supernatant after post-IR incubation with 1 mM Poloxamine 1107, a larger amphiphilic block-copolymer (M_r 15,400), but not with P188 or 10 kDa dextran at the same concentrations [16]. It remains to be investigated whether particular surfactant sealing effects are cell membrane specific.

The results shown have implications for the development of an effective comprehensive therapy for high-dose IR exposure victims. While reversing membrane injury will not prevent cell death from IR-induced apoptosis or DNA damage, any therapeutic efforts to defy apoptosis or DNA damage will be useless if the cell membrane is disrupted. Clearly, restoring cell membrane integrity soon after injury is essential to prevent necrosis that will manifest within minutes to hours [28]. Repairing the membrane can eliminate the immediate cause of death and allow cell viability so that other potentially lethal or pathological effects of irradiation can be addressed.

Acknowledgements

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