

Evidence for factors modulating radiation-induced G2-delay: potential application as radioprotectors

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Abstract

Manipulation of checkpoint response to DNA damage can be developed as a means for protecting astronauts from the adverse effects of unexpected, or background exposures to ionizing radiation. To achieve this goal reagents need to be developed that protect cells from radiation injury by prolonging checkpoint response, thus promoting repair. We present evidence for a low molecular weight substance excreted by cells that dramatically increases the duration of the G2-delay. This compound is termed G2-Arrest Modulating Activity (GAMA). A rat cell line (A1-5) generated by transforming rat embryo fibroblasts with a temperature sensitive form of p53 plus *H-ras* demonstrates a dramatic increase in radiation resistance after exposure to low LET radiation that is not associated with an increase in the efficiency of rejoining of DNA double strand breaks. Radioresistance in this cell line correlates with a dramatic increase in the duration of the G2 arrest that is modulated by a GAMA produced by actively growing cells. The properties of GAMA suggest that it is a low molecular weight heat-stable peptide. Further characterization of this substance and elucidation of its mechanism of action may allow the development of a biological response modifier with potential applications as a radioprotector. GAMA may be useful for protecting astronauts from radiation injury as preliminary evidence suggests that it is able to modulate the response of cells exposed to heavy ion radiation, similar to that encountered in outer space.

KEYWORDS: Checkpoints, G2-delay, ionizing radiation, radioprotectors.

1. Introduction

It is well established that exposure of human cells to ionizing radiation leads to growth delays mediated by arrests in the progression of cells through the different phases of the cell cycle. These arrests result from regulatory mechanisms leading to the activation of checkpoints [1-3]. Delays in the progression through the cell cycle, corresponding to the activation of checkpoints in G1, S, and G2, have been described in cells exposed to ionizing radiation [4-6]. These delays in cell cycle progression are probably exploited by the cell to perform DNA repair, and as a consequence to reduce the adverse effects of radiation, such as cell killing and induction of mutations. As a result, one might expect that any modulation of radiation-induced cell cycle delays, either by external manipulations or by genetic alterations, would alter cell killing and mutation induction.

When one examines the durations of radiation-induced delays in G1, S, and G2, delays in G1 and G2 figure as more prominent lasting in the order of hours per Gy, as opposed to minutes per Gy measured for S-delays. Therefore, delays in G1 or G2 are often considered as suitable targets in the development of strategies to modulate cell radiosensitivity to killing [4-6].

Although the molecular mechanism of the G1 delay has been delineated to a considerable degree [4-6], the relationship between a postirradiation arrest in G1 and cell radiosensitivity to killing is not always clear [7-9]. In contrast, modulations induced in radiation-induced delay in G2 seem to alter cell survival in a way consonant to its action as a checkpoint. Thus, treatment of cells with caffeine,

a methylxanthine known to reduce the duration of radiation-induced arrest in G2, causes a significant radiosensitization [10, 11]. Although recent evidence directly implicates repair inhibition in the effect of caffeine on cell radiosensitivity to killing [12], checkpoint abrogation remains a strong candidate for the observed effects. Also treatment of cells with staurosporine, a non-specific kinase inhibitor [13], causes radiosensitization [14] and a reduction in G2-arrest [15, 16].

Furthermore, genetic manipulations causing an increase in cell radioresistance to killing are occasionally associated with an increase in G2-arrest. Cell lines generated by transfection of primary rat embryo fibroblasts with the oncogenes *H-ras* and *v-myc* are radioresistant compared to their normal counterparts, and show increased delay in G2 [17]. Also, human diploid fibroblasts transfected with SV40 large T antigen are more radioresistant than the parental cell lines and show an increased radiation-induced delay in G2 [18]. Finally, a correlation between G2 delay and cellular radiosensitivity was observed in a survey of several human tumor cell lines [19]. These observations suggest that radiation-induced delay in G2 contributes to intrinsic cell radiosensitivity and that its modulation offers means for altering the expression of potentially lethal damage.

Although several compounds have been described that reduce radiation-induced delay in G2 (e.g. caffeine, staurosporine, etc.), no compounds are available to date that specifically increase the duration of G2 arrest. Yet, such compounds may be of particular interest as they may enhance the ability of cells to carry out repair, and thus reduce radiation killing and mutation induction. In fact, such compounds may act

as radiation protectors. They will differ, of course, from radioprotectors presently available in that they will act via a modulation of repair pathways, and not via scavenging of radiation-induced radicals. Thus, they may define a new class of radioprotectors, and may be beneficial to individuals even when administered after exposure to ionizing radiation, because the repair processes they will modulate require hours for completion whereas radicals decay within msec. Here we provide evidence for the existence of such a factor, which we term G2-arrest modulating activity (GAMA).

2. Material and Methods

Two cell lines, A1 and T101-4, were primarily used for experiments. Cells were kindly provided by Dr. Arnold Levine, Princeton University, New Jersey. A1-5 is a subclone isolated by limiting dilution of A1 cells. A1 cells were generated by transfecting primary rat embryo fibroblasts with the murine p53^{val135} mutant (alanine to valine change at amino acid 135) together with an activated *ras* oncogene (EJ6.6) [20]. The p53^{val135} mutant is temperature sensitive for a conformational change detected by the binding of a monoclonal antibody, PAb246, which recognizes the wild type protein, and the great majority of p53^{val135} at 32.5°C. Cells grow normally when incubated at 37°C. T101-4 cells were generated from primary rat embryo fibroblasts after co-transfection with the KH215 mutant of murine p53 and activated *ras* (pEJ6.6) [21]. Cells are routinely maintained in DMEM supplemented with 10% fetal calf serum. All incubations were at 37°C in an atmosphere of 5% CO₂ and 95% air. Radiation exposures were carried out using an X-ray machine operating at 250 or 320 kV, 10 – 15 mA, with a 2-mm aluminum filtration. The effective photon energy was 70 - 90 keV. Radio-sensitivity to killing was determined by measuring loss of colony-forming ability. Induction and repair of DNA Double Strand Breaks (DSB) was measured by pulsed-field gel electrophoresis as described earlier [22]. To measure arrest in G2, 2×10^5 cells were plated in 60 mm dishes with 3 ml of growth medium. Approximately 30 h later, cultures were exposed to various doses of X-rays and returned to 37°C. At different times thereafter, cells were trypsinized and fixed in ice-cold 70% ethanol. Before analysis cells were washed with phosphate buffered saline and resuspended in a solution containing 0.1 M Tris.HCl pH 7.5, 0.1 M NaCl, 5 mM MgCl₂, 0.05% Triton X-100, and 2 µg/ml DAPI. The distribution of cells throughout the cell cycle was determined in a flow cytometer (Coulter Epics Elite) equipped with cell cycle analysis software.

3. Results

Figure 1 shows dose response curves for cell killing in A1-5 and T101-4 cells. It is evident that A1-5

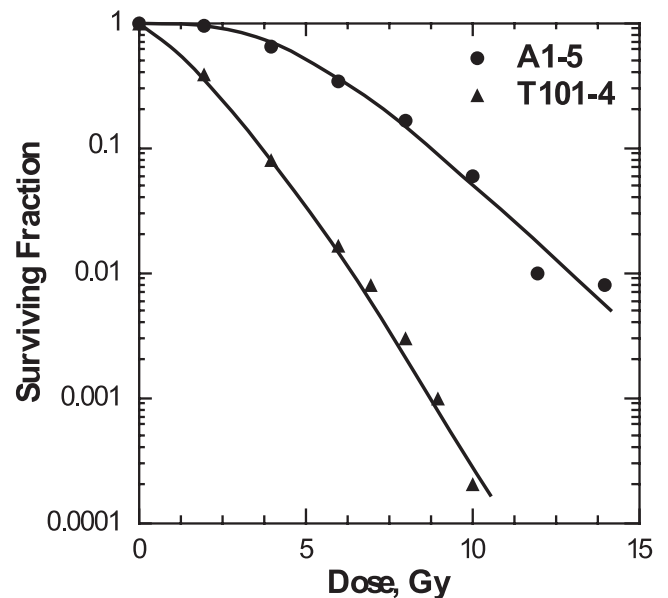


Fig. 1 – Survival curves of exponentially growing A1-5 and T101-4 cells as determined using the clonogenic assay. Cells were processed immediately after irradiation.

cells are significantly more radioresistant, as compared to T101-4 cells. The difference in radiosensitivity is among the largest observed with cell lines generated after transfection of primary rat embryo fibroblasts with oncogenes and/or anti-oncogenes of different families [18, 23-25]. Therefore, we initiated experiments to investigate the mechanism(s) underlying this phenomenon.

Since radiation-induced DNA DSBs are thought to be severe lesions which if unrepaired or misrepaired will lead to cell death, we examined their induction and rejoining in irradiated exponentially growing A1-5 and T101-4 cells using pulsed-field gel electrophoresis. There was no difference between the two cell lines either in the yield or the kinetics of DNA DSB rejoining.

As differences in the induction and/or rejoining of DNA DSBs could not be invoked to explain the increased radioresistance of A1-5 cells, we examined whether enhancements in checkpoint response, as it was also previously found for other transformed rat cell lines [25], underlie this phenomenon. Flow cytometry indicated dramatic differences in the response between A1-5 and T101-4 cells. As the results in Figure 2A indicate, T101-4 cells showed a modest accumulation of cells in G2 after exposure to 6 Gy leading to a maximum accumulation of approximately 35% at 4-6 h. Cells overcame the arrest induced by this dose and divided after approximately 10h. On the contrary, A1-5 cells, Figure 2B, experienced a much longer delay in G2 that led to the accumulation of over 70% cells in G2 after the same dose of X-rays. Cells were unable to overcome the delay during the 34 h of observation. This response was the strongest we had ever observed in a repair proficient cell line and suggested a

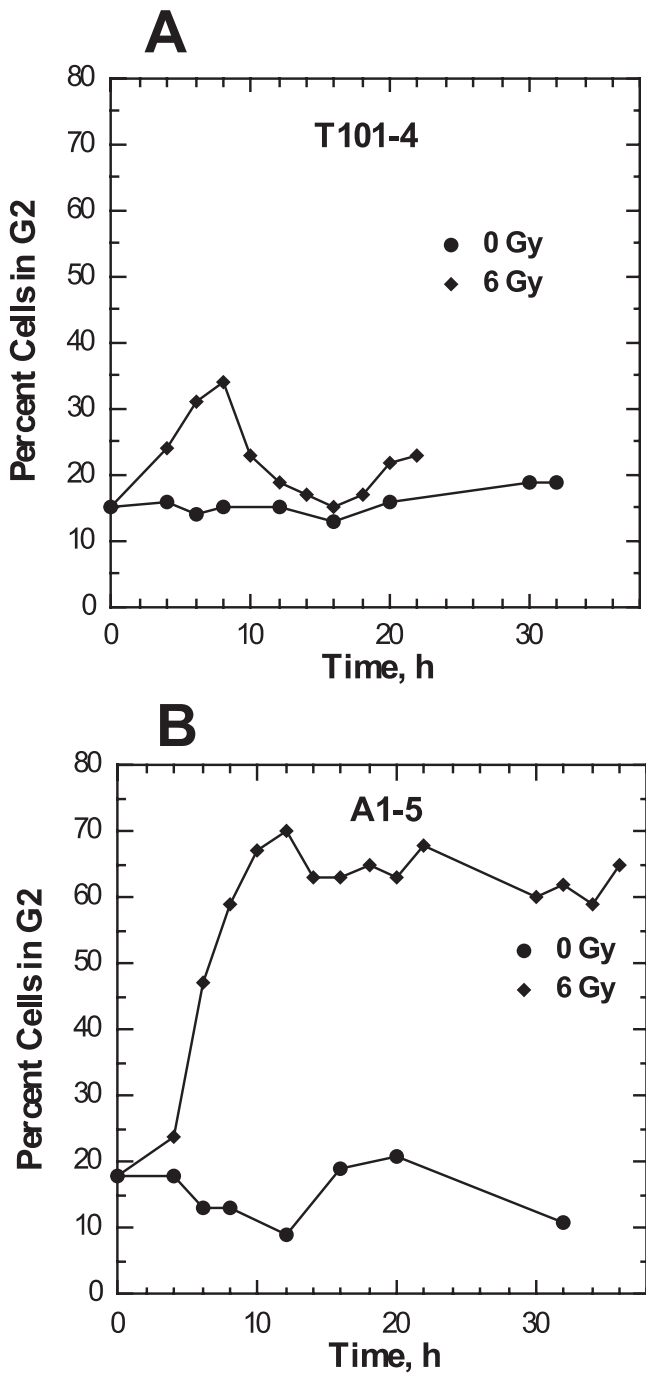


Fig. 2 – Panel A: Exponentially growing T101-4 cells were exposed to 6 Gy or left unirradiated and then returned to 37 °C without any further manipulation. At various times after irradiation, cells were trypsinized and stained with DAPI. The distribution of cells through the cycle was measured by flow cytometry and the fraction of cells in G1, S, and G2+M estimated for each time point. Shown is the fraction of cells in G2+M phase as a function of time after irradiation. Panel B: Results similar to those shown in Panel A but for A1-5 cells.

correlation between checkpoint activation and radioresistance to killing. The enhanced G2 arrest of A1-5 cells could be abrogated by treatment with caffeine or staurosporine indicating that it is the result of activation of kinases normally involved in this type of response.

Further experiments provided evidence that the G2-arrest of irradiated A1-5 cells is sustained by a low molecular weight factor produced by the cells and excreted into the growth medium. In the experiment shown in Figure 3, A1-5 cells were exposed to 6 Gy X-rays and allowed to accumulate in G2 for 12h. At this time, one set of dishes was trypsinized and replated in fresh growth medium plus 10% fetal calf serum, one set of dishes was washed with PBS and transferred to fresh growth medium plus 10% FCS, while one set of dishes was left undisturbed as a control. The results show that radiation exposure caused an accumulation of cells in G2 that persisted for the entire period of follow-up (40h) in cultures in which growth medium was not changed. However, cells supplied with fresh growth medium at the peak of G2 accumulation, started dividing a few hours later. We did not observe differences in the kinetics of division between cells transferred to fresh growth medium after trypsinization as compared to those transferred to fresh growth medium without trypsinization. These observations suggest the presence of GAMA in the conditioned medium, and indicate that the G2 arrest can be partly reverted if cells are transferred to fresh medium – which is equivalent to removing GAMA.

In a set of experiments that will be presented in greater detail elsewhere we established that GAMA was present in the conditioned medium (C-Med) of cultures grown for several days, and that GAMA is heat stable and has a molecular weight in the range

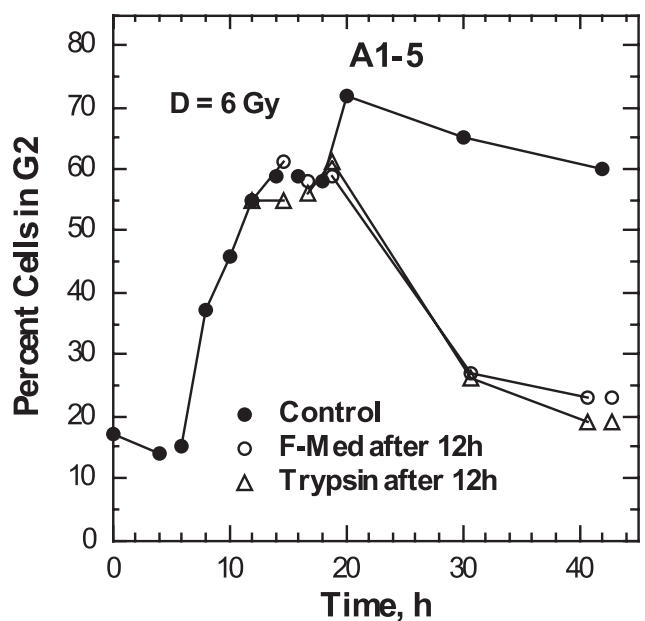


Fig. 3 – A1-5 cells were exposed to 6 Gy and were returned to 37°C for 12 h. At this point one set of dishes was trypsinized and transferred to fresh growth medium (open triangles), a second set of dishes was transferred to fresh growth medium without trypsinization (open circles), and a third set of dishes was left undisturbed and used as a control (closed circles). The distribution of cells through the cycle was measured at various times after irradiation.

of 1 kD. Most importantly, we observed that GAMA is sensitive to proteases and should therefore be a peptide. We examined the ability of GAMA to modulate the response of cells exposed to heavy ion radiation, similar to that encountered in outer space. Exponentially growing A1-5 and T-101 cells were exposed to various doses of 1 GeV/a Fe ions at the Brookhaven National Laboratory and cell cycle progression was followed for 36 h by flow cytometry. While the final analysis has not been completed yet, preliminary results confirm a prolonged G2-delay in A1-5 cells and a modulation of this response by media containing GAMA. The results suggest that low LET responses to GAMA are preserved after exposure to high LET radiation and broaden the potential utility of this compound as a radioprotector.

4. Discussion

The results presented above identify a cell line with extreme radioresistance and point to an enhancement in checkpoint response, particularly the one activated in G2, as the cause of the phenomenon. Because other cell clones generated by transfection with the same mutants of *ras* and *p53* used to generate A1-5 cells show radioresistance to killing similar to that of T101-4 cells, it can be concluded that the phenotype of A1-5 cells is not a direct consequence of the particular mutant of *p53* used for transformation.

It is particularly interesting that the maintenance of the G2 arrest in A1-5 cells relies on a low molecular weight, heat-stable factor. This is, to our knowledge, the first experimental demonstration of the existence of a molecule with such properties. The fact that it is produced by a very radioresistant cell line also suggests a correlation between its presence, its effect on G2 arrest, and the observed radioresistance to killing. These results suggest that cells may be endowed with a variety of systems able to modulate their response to radiation and other DNA damage inducing agents. The mechanisms are just starting to be recognized and studied. However, their potential importance in the development of strategies for the radiation protection of humans during space travel cannot be underestimated.

It will be particularly important to study the effect of GAMA on cell lethality and mutation induction following radiation exposure and inquire on its use as a radioprotector. We speculate that characterization of GAMA will define a novel group of compounds which modulate G2 arrest and thus, probably also radiosensitivity and mutagenicity. Such compounds may be useful radioprotectors, acting via modulation of repair pathways, as opposed to the radical scavenging properties of classical radioprotectors. They may be beneficial to individuals even when administered after exposure to ionizing radiation, because the repair processes they modulate require hours for completion whereas radicals decay

within msec. Finally, our findings provide evidence for an autocrine or paracrine regulation of cell cycle progression in irradiated cells that is worth further investigations.

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