Final Report

Factors Modulating Radiation-Induced G2 Delays

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Introduction

Rationale and background: The orderly progression of cells through the cycle is maintained by control mechanisms operating through the activation of checkpoints at defined positions. It is generally believed that at these checkpoints, feedback controls operate detecting a failure to complete replication, repair, or spindle assembly and arresting further progression of cells through the cycle until proper completion of these events. Delays have been described in the progression through the cell cycle after exposure of mammalian cells to ionizing radiation, corresponding to the activation of specific checkpoints in G1, S, and G2. These delays in cell cycle progression are thought to be exploited by the cell to perform repair, and as a consequence to reduce the adverse effects of radiation, such as cell killing and induction of mutations. Based on this definition 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 compares 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 aiming at the modulation of cell radiosensitivity to killing.

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 the G2 arrest. Yet, such compounds may be of particular interest as they may enhance the ability of cells to carry out repair, which may reduce cell killing and mutation induction. In fact, such compounds may protect cells against the adverse effects of ionizing radiation. They will differ, of course, from radioprotectors presently available as they will act via a modulation of repair pathways rather than via scavenging of radiation-induced radicals. Such hypothetical compounds may define a new class of radioprotectors, and may be beneficial to individuals even when administered after exposure to ionizing radiation, because repair processes require hours for completion whereas radicals decay within msec.

Project Aims: The proposal was initiated by the fortuitous observation that mammalian cells produce factors with G2-arrest modulating activity (GAMA), and had the goal of characterizing and defining the effects of these factors in mammalian cells. The hypothesis was that GAMA will act as radioprotector by promoting repair in irradiated cells, and could therefore find application in the radiation protection of humans engaged in the exploration of space. The Aims were to demonstrate, in principle, the production and excretion of GAMA by mammalian cells; to standardize production conditions and begin with the characterization of the biological properties of GAMA. We wished to examine the secretion of similar activities by other cell lines, as well as the action of GAMA on isogenic and non-isogenic cell lines. We also wished to obtained information on apparent size and other physicochemical properties of GAMA and inquire whether GAMA was a peptide. Further we wished to characterize the mode of GAMA production including the possible characterization of the gene encoding for the peptide and study the presence of receptors for the peptide. Finally we wished to study the effects of GAMA on cell survival and mutation induction.

isogenic - of a group of individuals (cells) genetically alike with respect to specified gene pairs

Relevance to CPR: The results of this research have the potential of providing information useful for the development of countermeasures to mitigate acute and late effects of radiation. GAMA and substances with similar properties could be developed to biological agents useful against several effects of radiation including early or acute effects, cancer development and damage to the central nervous system.

Materials and Methods

Two cell lines, A1-5 and T101-4 were used for the experiments designed to demonstrate the production of GAMA and its fundamental biological properties. 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. 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. At 37°C both mutant and wild type p53 conformational forms co-exist in the cells, while at 39.5°C the majority of the $p53^{val135}$ is in a mutant conformation not recognized by the PAb246 antibody. Cells grow normally when incubated at 37°C. However, when transferred to 32.5°C cells stop replication and arrest in G1 in agreement with a wild type function of $p53^{val135}$. Cells were kindly provided by Dr. Arnold Levine, Princeton University, NJ, and were further subcloned for this project to generate a line displaying a single G1 peak in flow cytometry measurements. This step was necessary for the studies described here as extensive flow cytometry measurements were required. T101-4 cells were used as a control to A1 cells and were generated from primary rat embryo fibroblasts after co-transfection with the KH215 mutant of murine p53 (in frame insertion between amino acids 215 and 216 that mutates VP to PSLA and eliminates the ability of the protein to bind SV40 large T antigen, or the wild type specific antibody PAb246) and activated *ras*. Both A1-5 and T101-4 cells display a transformed phenotype when grown at 37°C. Initially these cell lines were selected on the basis of their p53 status and were introduced in the laboratory for an unrelated project studying the effects of p53 on radiation response. Experiments for that project, which was then discontinued, generated the first observations from which the present project originated.

A1-5 and T101-4 cells were grown in DMEM supplemented with 10% fetal calf serum under standard conditions. Cells were grown in large volumes as appropriate for preparing adequate material for testing the presence and the effects of GAMA. Particular emphasis was place in the conditions of production, as cell concentration was found to be important in the generation of conditioned medium with good GAMA activity, as well as for the cultures used in the experiments (set to 10^5 cells per 60mm dish for 30h). We adopted conditions where C-Med was prepared by growing 10^6 cells for 4 days in 20 ml complete growth medium (100mm dishes). The distribution of cells in the various phases of the cell cycle was monitored by flow cytometry. Standard protocols were also used to assay for colony forming ability. Other cell lines were also introduced into this project as appropriate to test the effects of GAMA, as well as their ability to produce GAMA. They were grown under standard conditions. For irradiations, a Siemens therapeutic X-ray machine operated at 250 kV, 15 mA with a 2 mm Al filter was used. The dose rate at the irradiation distance (50 cm) was 2 Gy/min and the effective photon energy about 70

keV. Dosimetry was performed regularly with a Victoreen dosimeter. Experiments were also carried out using the BNL-facility.

To investigate the size of GAMA, AMICON filters with pore size retaining molecules larger than 10kD, 5kD and 1kD were used. This procedure was carried out using an AMICON filtering unit. To test whether GAMA is a small peptide we immobilized trypsin on beads and treated aliquots of C-Med for different periods of time. First attempts for the purification of GAMA used a Biocad station (PE Biosystems).

For Western blot analysis, aliquots of cell lysates were electrophoresed on SDS-PAGE, blotted onto Immobilon-P membranes for one hour using standard procedures. In some experiments transfer was tested by staining the membrane with 0.5% Ponceau S in 1% acetic acid. The membrane was destained in 5% acetic acid, rinsed with deionized water, and nonspecific binding sites blocked by incubation of membrane in 5 % bovine serum albumin (BSA) in Tris-buffered saline (TBS). The blot was washed and then incubated for 2 hours at RT with an appropriate dilution of the primary antibody. The membrane was washed and incubated for one to two hours with an appropriate dilution of alkaline phosphatase-conjugated secondary antibody. The blot was washed and incubated with the substrate solution. The ECL detection system of Amersham was used. Detection was carried out using the Storm (Molecular Dynamics).

Induction and rejoining of radiation-induced DNA DSBs was measured using asymmetric field inversion gel electrophoresis. This technology is standard in our laboratories.

Results

A1-5 cells are extremely radioresistant

One of the first observations in this project was that A1-5 cells were much more radioresistant than the control T101-4 cells. The difference in radiosensitivity was among the largest we and others have observed with cell lines generated after transfection of primary rat embryo fibroblasts with oncogenes and/or anti-oncogenes of different families. Therefore, we initiated experiments to investigate the mechanism(s) underlying this phenomenon. Since radiation-induced DNA double strand breaks (DSB) are thought to be severe lesions which if unrepaired or misrepaired will lead to cell death, we examined their induction and repair in irradiated exponentially growing A1-5 and T101-4 cells. For the experiments, we used asymmetric field inversion gel electrophoresis (AFIGE), a pulsed field gel electrophoresis assay. There was no difference in the dose response curves measured with A1-5 or T101-4 cells, suggesting similar yields of radiation-induced DNA DSBs. Rejoining of DNA DSBs was also similar in the two cell lines suggesting a similar capacity in repairing this type of lesion.

A1-5 cells display an unusually long G2 arrest

The above results suggested that differences in the induction and/or rejoining of DNA DSBs cannot be invoked to explain the increased radioresistance of A1-5 cells. It was possible, however, that A1-5 cells were more radioresistant, as compared to T101-4 cells, because of a lower incidence of misrepair of radiation-induced lesions - possibly DNA DSBs. Because a

reduction in the incidence of misrepair may be associated with alterations in the kinetics of cell cycle progression after irradiation, we compared radiation-induced delays between the two cell lines. Progression from G1 into S after exposure to X-rays was similar in A1-5 and T101-4 cells and could not, therefore, explain the increased radioresistance of the former cells. We observed a moderate increase of radiation-induced inhibition of DNA replication in A1-5 cells, as compared to T101-4. When we examined, however, radiation-induced delay in G2, we found dramatic differences between A1-5 and T101-4 cells. T101-4 cells showed a modest accumulation of cells in G2 that increased with increasing radiation dose. On the contrary, A1-5 cells experienced a much longer delay in G2 with more than 70% of cells accumulated in G2 after exposure to 6Gy. Cell division did not take place for up to 30h after irradiation. This response was the strongest we had ever observed in a repair proficient cell line and marked the beginning of the research in this project. To exclude that the response was not the result of a lethal accumulation of cells in G2 we tested the effect of caffeine. Caffeine is known to sensitize cells to radiation, and to reduce the duration of G2 arrest. We argued that if the accumulation of A1-5 cells in G2 was a reflection of a genuine G2 arrest, caffeine should reduce its duration. If, on the other hand, the observed accumulation in G2 stemmed from non-specific, cell-line-related artifacts, caffeine should only have a minimal effect. The results obtained indicated that 2 mM caffeine caused a substantial reduction in the accumulation of A1-5 cells in G2, suggesting that it reflects a genuine G2 arrest. We observed a significant reduction in the duration of G2 arrest in T101-4 cells as well, but it was modest when compared to the reduction achieved in A1-5 cells. We also tested staurosporine, a non specific inhibitor of protein kinases known to also affect cell radiosensitivity and G2 arrest, and obtained similar results. The above findings provided strong support for a genuine long G2 arrest in A1-5 cells.

A low molecular weight factor modulates G2 arrest in A1-5 cells

A series of observations from a large number of different experiments led us to suspect that G2 arrest was modulated in A1-5 cells by a factor produced by the cells and excreted into the growth medium. Two types of experiments provided support for this mechanism. First, the duration of the G2 arrest was shorter when cells were grown at a lower density before irradiation and follow up for cell cycle progression. Second, we observed that medium change a few hours before irradiation significantly reduced the duration of the G2 arrest. We argued therefore that if GAMA is produced by the cells and excreted into the growth medium, change of medium at some point after irradiation should also reduce the duration of the G2 arrest. Indeed, radiation exposure caused an accumulation of cells in G2 that persisted for over 40h, whereas cells supplied with fresh growth medium at the peak of G2 accumulation, started dividing a few hours later. These observations suggested the presence of GAMA in the conditioned medium of non-irradiated cells, and indicated that the G2 arrest can be reverted if cells are transferred to fresh medium, which is equivalent to removing GAMA. We established that GAMA was present in the conditioned medium (C-Med) of cultures grown for 4 days starting at a concentration of 10^6 cells per dish (20 ml), and produced C-Med (and therefore non-purified GAMA) in this way for all subsequent experiments. We further established that the concentration of GAMA in cultures of A1-5 cells grown for 30h starting from 10^5 cells per 60 mm dish was not high enough to modulate G2 arrest, unlike what was observed with cells grown for 30h starting from 2.5×10^5 cells per dish.

To obtain information on the molecular weight (MW) of GAMA, we passed C-Med through an Amicon filter with a nominal MW cutoff limit of 10 kD. We tested the effect of ultrafiltrate and retentate on radiation-induced G2 arrest in A1-5 cells. Cells incubated in fresh medium supplemented with retentate displayed kinetics of accumulation in G2 and subsequent division practically identical to those of cells transferred to fresh medium. On the other hand, cells transferred to ultrafiltrate displayed an accumulation and retention in G2 similar to that of cells kept in C-Med. Similar results were obtained when C-Med was passed through a filter with a nominal molecular weight cutoff limit of 3 kD. These observations suggested that GAMA passes the 10 kD and 3 kD filters and is present in the ultrafiltrate. Therefore, it should have a molecular weight of less than ~3 kD. Reduced effect was observed on the other hand with material obtained after passing through a filter with a 1 kD molecular weight cutoff suggesting partial retention. Based on these results we estimated that GAMA has a molecular weight in the range of 1 kD. Dialysis experiments also produced similar results and confirmed that GAMA is a low molecular weight factor. To test the stability of GAMA we exposed C-Med to 56°C for 1h and tested its ability to arrest irradiated A1-5 cells in G2. The results showed that incubation at 56°C for 1h did not alter the ability of C-Med to arrest cells in G2, suggesting that GAMA is stable to heat. Finally, we inquired whether GAMA is a peptide. We argued that if GAMA were a peptide it should be sensitive to proteases. Therefore, we first treated a 10 kD ultrafiltrate for 2h at 37°C with 0.25% trypsin and tested its effect in arresting irradiated cells in G2 after trypsin neutralization with serum. The results showed a shorter G2 delay after trypsin treatment suggesting that GAMA is a peptide. We also confirmed this observation using a different protease with less specificity than trypsin, proteinase K. We argued that a less specific protease should have a more dramatic effect on GAMA activity. To avoid extensive contamination of the ultrafiltrate with the protease, we selected a form of Pro-K immobilized on agarose beads. We treated overnight at 37°C ultrafiltrate or fresh medium without serum with Pro-K-crosslinked agarose beads, and tested the effect of the treatment on the ability of the ultrafiltrate to induce G2 arrest in irradiated cells. Ultrafiltrate incubated in the presence of Pro-K showed a dramatically reduced ability to arrest cells in G2.

GAMA may be produced by a variety of cell lines but requires cognate receptors to be effective

To test whether production of GAMA is a specific property of A1-5 cells, or whether it can be produced by other types of cells as well, we prepared C-Med, from a number of cell lines and tested its effect on G2 arrest in A1-5 cells. C-Med from CHO cells did not retain cells in G2 and allowed division with kinetics similar to the control. On the other hand, C-Med from *ts*BN2 cells, a mutant of BHK cells with a temperature sensitive defect in chromosome condensation, from T101-4 cells, from wild type BHK cells and from V79 cells showed a significant ability to retain irradiated A1-5 cells in G2, suggesting that GAMA is produced by a variety of cell lines.

We also inquired whether GAMA produced by a variety of cells can modulate the G2 arrest in cell lines other than A1-5. C-Med from A1-5, V79 and BHK cells was unable to arrest irradiated T101-4 cells in G2. We obtained similar results when testing irradiated CHO and BHK cells. An intermediate response was found with *xrs-5* cells, a mutant of CHO with a defect in the 80 kD subunit of the *Ku* autoantigen that causes a defect in the rejoining of DNA DSB. We interpret these results as indicating that GAMA acts via a receptor which must also be present for GAMA to exert its G2 arrest modulating activity.

Experiments using high LET particles at BNL

Because it is important for the NASA programs that our findings hold for high LET radiations as well, we extended our investigations to include experiments with heavy ions. We participated in BNL-5 and carried out one experiments designed to investigate the effect of GAMA on A1-5 cells exposed to 1 GeV Fe. We also participated in BNL-6 with a similar experiment. These experiments used exponentially growing A1-5 and T-101 cells exposed to various doses of 1 GeV/a Fe ions and growth was followed for 36 h by cell counting. In addition, cells were collected and fixed in ethanol to study progression through the cycle by flow cytometry. The role of caffeine and staurosporine, agents known to abrogate G2-arrest, was also investigated. A combination of equipment difficulties and the relocation of the PI to Europe have delayed analysis of these experiments; this analysis is expected to be complete in the next six months. However, preliminary analysis of available data suggests that after exposure to high LET radiation effects similar to those after exposure to low LET radiation are observed.

Discussion

The above observations allow the following conclusions:

1. Several cell lines grown in vitro produce factors with the potential of modulating radiation-induced cell cycle checkpoints.

2. Such factors can be low molecular weight stable peptides.

3. Response to these factors likely requires the presence of appropriate receptors in the cells, as more cells produce them than respond to them.

4. These factors are likely to be active both after exposure to low, as well as to high LET radiation.

These properties of GAMA are very interesting and allow practical exploitation. Characterization of a low molecular weight compound that prolongs G2-arrest and possibly protects cells from radiation-induced killing and mutation induction is very interesting for several reasons. First, it defines a new class of radioprotectors with direct application in radiation protection. Second, because the compound is synthesized by the cells it is expected to be of limited toxicity in the range of concentrations where it exerts a protective action. This may expedite its application in radiation protection during space exploration. Third, because GAMA is expected to act by somehow modulating repair, it could also be taken shortly after radiation exposure, as soon as the radiation monitors in a Space Craft, or the Space Station, register increased cosmic ray activity. These potential applications of GAMA warrant further work to characterize the spectrum of its biological activities and to proceed with its further characterization. For this purpose the following questions will need to be addressed:

1. What level of protection against cell killing and mutation induction do media containing GAMA provide? This test was initially planned within this project, but could not be completed.

2. What is the molecular nature of GAMA and how is it produced in the cells? Initial information was provided in this proposal but a lot more needs to be done. Characterization of low molecular weight compounds is an arduous time consuming task.

3. Is GAMA effective in vivo and is it without side effects? What effects of radiation can be mitigated by such compounds?

4. Can GAMA-like factors be synthesized in vitro?

The information provided by the results obtained in this proposal may ultimately be useful in several ways for the Critical Path Roadmap (CPR).

1. It indicates in principle the possibility of developing countermeasures to unwanted exposures to radiation on an entirely new concept – by promoting repair processes.

2. Such compounds may mitigate early or acute form radiation exposure (Risk No. 41).

3. They may mitigate the carcinogenic effects of radiation (Risk No. 38).

4. They may help mitigate damage to central nervous system from radiation exposure (Risk No. 39).

In addition to the potential application of these compounds as radioprotectors, study of their properties may provide new insights into the ways multicellular organisms regulate cell cycle progression after radiation exposure. The possible production by cells of low molecular weight compounds that act as extracellular signals to regulate radiation-induced G2 delay, and the possible existence of cognate receptors, suggest the operation of autocrine and possibly also of paracrine pathways in the regulation of checkpoints. If confirmed, this observation will open new areas of investigation and will add an additional level of complexity to the mechanism by which multicellular organisms and humans respond to ionizing radiation exposure. At the same time it may also offer new ways for modulating radiation response.

Other Information and Materials

Presentations

1. 12th Annual NASA Space Radiation Health Investigators' Workshop, Brookhaven, May 2001.

2. 11th Annual NASA Space Radiation Health Investigators' Workshop, Arona, Italy, May 2000.

3. 10th Annual NASA Space Radiation Health Investigators' Workshop, Brookhaven, May 1999.

4. 9th Annual NASA Space Radiation Health Investigators' Workshop, Loma Linda, June 1998.

Published Abstracts

No published abstracts beyond those associated with the above presentations.

Published Articles

1. N. Cheong, Z.-C. Zeng, Y. Wang, G. Iliakis. Evidence for factors modulating radiation-induced G2-delay: potential application as radioprotectors. Physica Medica-Vol XVII, Supplement 1, p 205-209. 2001

More articles will result from this work upon completion of the analysis of the last sets of experiments.

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