Abdominal Radiation Initiates Apoptotic Mechanism in Rat Femur Bone Marrow Cells in vivo that is Reversed by IGF-1 Administration

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Apoptosis/Haematopoetic cells/Low dose radiation/IGF-1.

Purpose: Radiation induces apoptosis as a result of damage to cellular DNA and RNA. The aim of our work was to study the effect of radiation on rat bone marrow cells (as a neighboring tissue) in the context of a model of experimental radiation enteritis in rats. The effect of systematic administration in irradiated animals of r-IGF-1 and GH was also studied. Materials and methods: Wistar type, normal rats, were divided in 4 groups. One control group and the other 3 groups were irradiated in the abdomen. The measured scattered irradiation in the femur ranged from 16.5 to 47.3 cGy. In 2 groups of irradiated animals, rIGF-1 (0.1 μg/g of body weight twice/d) and rGH (0.25 μg/g of body weight /d) were administered. Bone marrow cells were harvested from both femurs. DNA and RNA were analyzed in specific gels. The m-RNA was hybridized for c-fos proto-oncogene expression. Results: The calculated low dose of radiation that affected the femurs of the animals induced reduction in bone marrow cell numbers and endonuclease activation manifested by subsequent fragmentation of DNA and RNA. This phenomenon was reversed by rGH and rIGF-1 administration. The c-fos proto-oncogene expression was upregulated by irradiation. Conclusion: These observations indicate that scattered low dose radiation is capable of initiating apoptosis in rat bone marrow cells and rGH and rIGF-1 administration reverse this process.

INTRODUCTION

Genotoxic agents like antineoplastic drugs, UV light and ionizing radiation induce apoptosis in hematopoetic cells. Radiation affects not only the target tissue but neighboring tissues as well, by scatter of radiation or by products of damaged cells or cytokines released by the irradiated cells, phenomena that are extensively reviewed by Mothersill and Seymour and others.

There is accumulating evidence that DNA is the principal target of the biological effect of radiation. This effect begins with a break in one or both strands of the DNA molecule leading to activation of apoptotic machinery of the irradiated cell.

A hallmark of apoptosis is the DNA and RNA fragmentation due to endonuclease activation giving the characteristic picture of “laddering” in DNA and RNA analysis in agarose gels. Continuous signaling by growth factors, hormones, cytokines, cell-cell contact and cell matrix interactions are necessary for hematopoetic cells to refrain from undergoing apoptosis, by activating mechanisms of DNA repair, keeping them alive after radiation or other genotoxic agents. Insulin –like Growth Factor (IGF-1) and Growth Hormone (GH) are known to support growth and to prevent apoptosis in many cell types. GH and IGF-1 indirectly control the volume of bone marrow and the production of hematopoetic cells. Although IGF-1 is regulated by GH dependent pathways, it is well documented that the stromal cells and the hematopoetic stem cells (CD34+) produce and secrete IGF-1 with an autocrine and paracrine effect on growth, differentiation and apoptosis of hematopoetic cells.

Our aim was to study whether the low dose radiation (scattered to neighboring femur area) could initiate apoptotic phenomena on bone marrow cells taken from the femurs of animals, in an in vivo model of normal rats irradiated in the abdomen, and in the context of another protocol that involved radiation induced enteritis. The parallel aim in this study was to observe whether IGF-1 and GH administration
could anticipate the radiation induced DNA and RNA damage, in bone marrow cells.

**MATERIALS AND METHODS**

**Experimental animals**
Male albino Wistar rats weighing 200–250 g were used in this study. The animals were housed in stainless-steel cages (3 rats per cage), under controlled temperature (23°C), humidity conditions and 12 hour dark/light cycle. They were allowed three days to become acclimatized to the animal care facility prior to the study and had free access to standard rat chow and water. The rats were divided randomly in four experimental groups of 4 or 5 animals according to the treatment they received. Group I (controls) received no radiation and no treatment, Group II (radiation only), Group III (radiation plus GH treatment) Group IV (radiation plus IGF-1 treatment).

Animals were injected for three days with the hormones and on the 4th day were sacrificed. All experiments were carried out according to the rules of the Ethical Committee of the Patras University Hospital, Rio, Greece.

**Radiation**
On day 1 the rats were anesthetized with ether and were placed in the supine position. The radiation field measured 5x5cm and was centered on the abdomen at the midpoint of the distance from the xyphoid to the pubis. Radiotherapy was delivered by a linear accelerator (Philips SL-75) with 6MV photons (X-rays) with an average dose rate of 5.0 Gy per min. The rats received a single dose of 11 Gy to the abdomen, at a focus –skin-distance of 100 cm. Scattered irradiation to the proximal, middle and distal points of the femur was calculated directly using an ionization chamber in a plexiglass phantom, (Fig. 1). The length of the femur was calculated to be approximately 35 mm.

**Hormone treatment**
Recombinant human GH (specific activity 1 mg = 3IU) and recombinant human IGF-1 (a gift from Pharmacia-Upjohn) were injected in the following doses: a) GH: 0.25 μg/g b.w. subcutaneously once daily, on days 1–3 b) IGF-1: 0.1 μg/g b.w., s.c. twice daily on days 1–3. The first injection was given immediately after radiation.

**Specimens and Methods**
On day 4 all animals were sacrificed under ether anesthesia, by blood withdrawal from the aorta. The bone marrow cells from both femurs of each animal were flushed with a syringe containing PBS into a sterile tube with 5 ml RPMI and 0.1% EDTA. The cell-suspension from each experimental animal was centrifuged and washed twice with RPMI. From each experimental group of animals, cells were collected, counted and viability was assessed by trypan blue staining. We obtained bone marrow cells from 4 radiation experiments with the same procedure.

**Isolation of cells**
The crude bone marrow cell suspension from each group of experimental animals was layered in Ficoll solution (Biochrom AG) and after centrifugation (1800 rpm for 30 min) the interphase cell layer was taken and washed once with RPMI plus 2% FCS and twice with PBS.

**RNA and DNA isolation/analysis**
RNA was isolated with the single step method of RNA isolation by acid guanidinium –phenol –chloroform extraction. DNA was isolated with the method described in Molecular Clonig laboratory manual by J. Samrook, E. Fritsch, and T. Maniatis.

DNA samples (1 μg/μl, 5 μl loaded in each well) and RNA samples (3 μg/μl, 5 μl loaded in each well) were analyzed by the corresponding Southern (1.8% agarose gel) and Northern (1.6% agarose gel in 1X TBE-Tris borate/EDTA buffer) procedure and the degradation bands were visualized by ethidium bromide staining. RNA analysis gels were transferred to nylon membrane for hybridization.

**Hybridization**
15 μg of total cellular RNA/ sample, on a nylon mem-

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Fig. 1. Simulation film of a rat in treatment position. C: center of the field, A: proximal, B: distal.
brane, were prehybridized and hybridized at 60°C in 0.5 M sodium phosphate buffer (pH 7.2) containing 7% sodium dodecyl sulfate, 1 mM EDTA, 0.1 mg of sheared and denatured herring sperm DNA per ml. The membranes were washed twice in 2XSSC (1XSSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and then three times at high stringency in 0.2 XSSC at 50°C for 30 min. The probe labeled with $^{32}$P-CTP (NEN) with a random-prime kit (Boeringer, Germany), was the mouse c-fos cDNA 1970 bp, into BamH1 restriction site of the pGEM3 plasmid. After washings the nylon membrane was dried and exposed to Kodak XAR-5 film. Three membranes were hybridized with c-fos and optical density of the bands was quantified by densitometric analysis on scanned films with Scion Image Software (Scion Corporation Maryland, USA).

For statistical analysis we used the SPSS programme.

RESULTS

Radiation

Based on the direct measurements the peripheral dose to the femur was 47.3 cGy (0.473Gy) and 16.5 cGy (0.165Gy), to the proximal and distal point, respectively (Fig. 1).

Bone marrow cells

The mean of cell numbers in 4 radiation experiments, from the 4 experimental groups was: Group I (control) = 59300 cells/dl, Group II (radiation) = 34400 c/dl, Group III (GH treated) = 41100 c/dl, and Group IV (IGF1 treated) = 38800 c/dl. The yield of cells in each experimental group and between groups was different due to different size/weight of animals (high standard deviation in absolute numbers of cells in each group) (Fig. 2, panel A). There was no statistical difference between control and irradiated bone marrow cells (p > 0.05) and no difference between irradiated and GH or IGF-1 treated animals (Fig. 2, panel A).

Expressed in per cent to non irradiated-control cells, loss of cells due to radiation was calculated to be 41% to the control, being statistically significant (p = 0.02). Non-significant gain of cells due to GH or IGF1 treatment of irradiated animals for 3 days was observed 19% (p = 0.055) and 12% (p > 0.05) respectively (Fig. 2, Panel B). Mean cell viability in the 4 experimental groups was found: 97% control, 58% irradiated, 71% GH treated and 74% IGF1 treated groups.

The interphase layer of bone marrow cells after Ficoll gradient centrifugation consisted mainly of lymphocytes, mononuclear cells and haematopoietic progenitors cells (CD34+ cells = 1–2%). In a Giemsa staining of the smears from bone marrow cells, clusters of apoptotic bodies were noticed in irradiated cells by light microscopy (data not shown).

DNA fragmentation

The scattered low dose radiation calculated approximately 16.5–47.3 cGy/ femur area, induced DNA fragmentation.
characteristic of endonuclease activation (DNA laddering, fragments of different molecular weights) of rat bone marrow mononuclear cells, a phenomenon that was reversed almost totally by the parenteral systematic administration of rGH and rIGF-1 for 3 days, to the experimental animals (Fig. 3).

**RNA cleavage of bone marrow cells**

Specific 28s rRNA cleavage, accompanying the DNA internucleosomal degradation, was observed in total RNA analysis in a denaturing gel. As shown in Fig. 4 there are 3 bands in 28s region and 2 bands in 18s region of ribosomal RNA. The 28s band density was diminished by the radiation induced activation of RNAse and the cleavage in D2 and D8 domains of the ribosome was visualized. 18s rRNA band appeared to be cleaved in two regions (D6, D8 domains) as well.

The RNAse activation and the specific fragmentation of 28S and18S rRNA in certain regions, after radiation exposure of bone marrow cells was stably found in all experiments and seemed to be reversed by the IGF-1 administration but not by GH administration (Fig. 4)

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**Fig. 3.** Total DNA extracted from hematopoietic cells analyzed in 1.8% agarose gel electrophoresis (5 μg/well). Lane 1: DNA from control group of animals, lane 2: DNA from irradiated animals, lane 3: DNA from irradiated animals treated 3 days with Growth Hormone, lane 4: DNA from irradiated animals treated 3 days with Insulin like Growth Factor-1.

**Fig. 4.** Total RNA extracted from hematopoietic cells analyzed in 1.6% agarose gel electrophoresis (15 μg/well). Lane 1: RNA isolated from the HL-60 cell line, as a marker, lane 2: control group of animals, lane 3: radiation group, lane 4: radiation plus Growth Hormone treated group, lane 5: radiation plus Insulin like Growth Factor-1 treated group.

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**Fig. 5.** Panel A: The RNA (15 μg/well) analyzed with gel electrophoresis was transferred to nylon membrane and hybridized with the c-fos probe as described. 1: RNA isolated from the HL-60 cell line served as marker, 2: control group of experimental animals, 3: radiation group, 4: radiation plus Growth Hormone treated group, 5: radiation plus Insulin-like Growth Factor-1 treated group. Panel B: Optical density of c-fos signal in the hybridized membranes (mean of three experiments). Statistical significant difference in the signal between non-irradiated and irradiated cells (bars 2 and 3) and reversal to proximal levels of non-irradiated cells with GH and IGF-1 treatment.

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C-fos expression

The membranes from the transferred gel that RNA-degradation was visualized, (Fig. 4) were hybridized with a mouse c-fos cDNA probe known to have about 80% homology to rat cDNA. As shown in Fig. 5, c-fos expression was highly upregulated by radiation of cells (Fig. 5, panel A).

In optical density measurement, these findings were confirmed, where c-fos expression was significantly upregulated (p = 0.03) in irradiated cells compared to the non-irradiated and returned to normal levels of expression with the GH and IGF-1 treatment, (Fig. 5, Panel B).

DISCUSSION

In an *in vivo* system of scattered radiation effect on animal’s femurs, an early observation was that low dose radiation (16.5–47. cGy) induced 40% loss of bone marrow cells which was partially corrected by GH and IGF1 treatment due to either proliferation or protection of cells from apoptosis[11,13] (Fig. 2 Panel A and B). The correction in cell numbers by GH or IGF-1 was not significant, probably of inadequate time of hormone administration as shown in previous experiments of protection in mice.[20]

Next observation was that low dose scattered radiation is capable of inducing activation of endonucleases resulting in DNA and RNA cleavage in certain sites of the molecule (DNA and RNA fragmentation), a biologic phenomenon that is known to be a hallmark of apoptosis in irradiated cells.[6–8] This phenomenon is reversed in DNA both with GH and IGF-1 presence; albeit in RNA, the cleavage is reversed only by the presence of IGF-1.

This specific cleavage of 28S and 18S sites of rRNA was located in D2, D8 and D6 regions respectively, and this type of selective cleavage of ribosomal RNA has been described in hematopoietic cells before, as a specific RNAse activation in cells undergoing apoptosis.[21]

Whether the induced DNA and RNA damage in the bone marrow cells of the femurs of experimental animals was a direct effect of the low dose scattered radiation as calculated by direct measurements, or an abscopal or bystander effect of radiation, remains a question. In our experiments radiation to bone marrow cells could be defined as “scattered” because the femurs were out of the radiation field (Fig. 1) and as an “abscopal” or bystander effect, because of the very low calculated dose of radiation.[22] Both mechanisms are referred in the literature either as biological effects of radiation on tissues being only 1 mm next to irradiation field,[5] or as a second mechanism due to secreted soluble factors (TNF-a and other factors or free radicals) from irradiated cells,[23,24] communicating the radiation response to non-irradiated cells (distant bystander effect).[25]

Another finding that support the apoptotic event in our experiments was the upregulation of c-fos mRNA expression. C-fos is a rapidly induced proto-oncogene when cells are exposed to genotoxic agents and particularly after DNA-damage due to irradiation.[26,27] In our data a high expression of c-fos was found in irradiated cells and it was down regulated by both GH and IGF-1. This high level of c-fos expression may be due, as well, to mRNA stabilization by the irradiation and the accumulation of c-fos mRNA, as described before.[28]

Previously, using the same experimental system that we studied apoptosis of bone marrow cells, (normal rats subjected to radiation enteritis) we have seen the protecting effect of GH and IGF-1 as a totally corrected intestinal epithelium.[29]

In the recent literature is stated that irradiation may induce cellular processes like apoptosis and genomic instability translated to single or double strand breaks of DNA and reduced DNA repair capacity in human haemopoietic cells, even at very low doses (5–10 cGy). A double strand break followed by DNA repair recombination could cause translocation and alter genetic integrity even in a single cell.[30–32]

These data, although preliminary, are indicative that low doses of scattered radiation initiate apoptosis of rat hematopoietic bone marrow cells, manifested by DNA and RNA intermolecular cleavage and high expression of the c-fos transcription factor. These phenomena were reversed by treatment with Growth hormone or IGF-1. The involved pathways of antiapoptotic effect of IGF-1 to irradiated hematopoietic cells[33] and the potential of the hematopoietic cells to recover their functional integrity,[34] have to be elucidated with further research *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

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REFERENCES

3. Mothersill, C. and Seymour, C. (2001) Radiation–induced apoptosis in hematopoietic bone marrow cells, (normal rats subjected to radiation enteritis) we have seen the protecting effect of GH and IGF-1 as a totally corrected intestinal epithelium.[29]
4. Mothersill, C. and Seymour, C. (2001) Radiation–induced apoptosis in hematopoietic bone marrow cells, (normal rats subjected to radiation enteritis) we have seen the protecting effect of GH and IGF-1 as a totally corrected intestinal epithelium.[29]
5. Mothersill, C. and Seymour, C. (2001) Radiation–induced apoptosis in hematopoietic bone marrow cells, (normal rats subjected to radiation enteritis) we have seen the protecting effect of GH and IGF-1 as a totally corrected intestinal epithelium.[29]
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