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Original scientific paper

THE RADIOPROTECTIVE EFFICACY OF THE RAT ACUTE-PHASE PROTEIN ALPHA2-MACROGLOBULIN ON BONE MARROW CELLS

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The rat acute phase protein α_2-macroglobulin (α2M) plays an important role in the restoration of disrupted homeostasis by inhibiting different types of non-specific proteases and facilitating the transport of cytokines, growth factors and hormones. Previously, we observed that administration of α2M to experimental animals prior to the infliction of life-threatening trauma in the form of scalding or total-body irradiation,

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significantly improved their survival rates. The aim of the present work was to evalute the radioprotective effect on blood cells of α2M that, when administered 30 min before irradiation with 6.7 Gy ($LD_{50/30}$), provides 100% survival of experimental animals where in unprotected irradiated rats the said dose results in 50% lethality. We observed that rats pretreated with α2M, after an initial decline, exhibited complete recovery of the leukocyte count due to the preservation of bone marrow cells, observed as a stable mitotic index. In untreated irradiated rats the decrease of the mitotic index reflected the significant destruction of bone marrow cells that resulted in a protracted decline in the leukocyte count. We conclude that the radioprotection provided by α2M was in part mediated through cytoprotection of new blood cells produced in the bone marrow.

Key words: α_2-macroglobulin, total body irradiation, mitotic index, bone marrow

INTRODUCTION

The deleterious effects of ionizing radiation are mediated through direct deposition of energy to biological molecules and indirectly through generation of highly reactive free radicals (NAIR *et al.,* 2001). Exposure to ionizing radiation induces the production of reactive oxygen species (ROS) which include superoxide, hydroxyl radicals, singlet oxygen, and hydrogen peroxide. Free radicals react with DNA, RNA, proteins, and membranes, resulting in cell dysfunction and death. Radiation sickness, also referred to as the acute radiation syndrome (ARS), is a serious illness that occurs when the entire body (or most of it) receives a high dose of radiation over a short period of time (WASELENKO *et al.,* 2004). There are three types of ARS syndromes: the bone marrow or hematological syndrome, gastrointestinal and the cardiovascular/central nervous system syndromes. The bone marrow syndrome is characterized by anorexia, fever and a decrease in all blood cell types, the gastrointestinal syndrome includes diarrhea, fever, dehydration and electrolyte imbalance and the central nervous system syndrome is characterized by damage to cells that do not reproduce, such as nerve cells (WASELENKO *et al.,* 2004).

Since ionizing radiation causes cell dysfunction and mortality, extensive research is devoted to the development of effective radioprotective compounds. (GANDHI and NAIR, 2004) that would diminish radiation injury in living organisms (NAIR *et al*., 2001). Radioprotective compounds are very important for the prevention of complications after radiotherapy of cancer patients and in treating patients after accidental radiation exposure. At a present, a number of synthetic radioprotective compounds are used in radiotherapy (GRDINA *et al.*, 2002). Aside from these compounds, certain adaptogens – natural nontoxic products that produce a nonspecific response in the body that increases resistance and restores homeostasis – stimulate radioresistance and are also considered as radioprotectors (LIVESEY and REED, 1987; NAIR *et al*., 2001). Previously, we observed that the administration of

the rat acute phase (AP) protein α_2-macroglobulin (α_2M) improves the survival of experimental animals, both after a lethal scalding (ŠEVALJEVIĆ *et al*., 1994) and total-body irradiation (ŠEVALJEVIĆ *et al.,* 2003). In view of the important role of α_2M in the restoration of homeostasis during the AP response (MIHAILOVIĆ et al., 2007; USKOKOVIĆ *et al*., 2007), we concluded that in the rat α_2M behaved as an adaptogen with a radioprotective function. α_2M is a tetrameric, disulfide-rich plasma glycoprotein (SOTTRUP-JENSEN, 1989). Some of its functions, such as inhibition of different types of non-specific proteases and transport of cytokines, growth factors and hormones (BORTH, 1992) are well established, however, the molecular mechanisms that underlie the significant improvement of survival after irradiation exposure by exogenously administered α_2M remain largely unknown.

In the present work we examined further the effects of α_2M in radioprotection on the survival rate, the leukocyte count in peripheral blood and on the mitotic index of bone marrow as the most radiosensitive tissue, during a four week follow-up period in rats with exogenously applied α_2M.

MATERIALS AND METHODS

Animals

Experiments were performed on 2.5 month-old adult male albino rats weighting 220-250 g of Wistar strain. All the animal procedures were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85/23, revised in 1986).

Experimental protocol

Rats were whole body X irradiated using a Philips linear accelerator SL 75-80, 8 MeV with 6.7 Gy ($LD_{50/30}$) at a rate of 3.4 Gy/min. Ten animals were placed in a Plexiglas container divided into 10 chambers. The container was covered with a 1 cm-thick Plexiglas sheet. The animals were exposed to radiation at 100 cm distance from the source. α_2M was administered i.p. (4.5 mg in a saline solution) 30 min before irradiation. Rats from the unprotected control group were injected with the same volume of saline solution 30 min prior to irradiation with 6.7 Gy. The control rats proper (referred to as the "intact control"), were injected with the same volume of saline solution. The three experimental groups each contained 30-50 rats.

The number of surviving rats were recorded for 4 weeks after irradiation, the standard follow-up period for evaluating biological parameters during the ARS. Peripheral leukocytes were measured with Hemocount ERP-9 (Ortho Diagnostic System, Germany).

Isolation of α_2M

α_2M was isolated from the sera of rats during the AP response when the concentration of α_2M undergoes a dramatic increase. The AP response was induced

by an i.p. administration of turpentine oil (200 µl/rat). The sera were collected 24 h after injection, pooled and sequentially fractionated with dextran sulphate, DEAE cellulose chromatography and by gel filtration as described by OKUBO *et al.* (1981). The purity of the isolated α_2M was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Preparations that generated a single protein band after electrophoresis were further processed as described above for subsequent injection into rats.

Metaphase chromosome analysis

Animals were injected intraperitoneally with colchicine solution (1µg/1g) 1 h before sacrifice. Both femurs were removed and the ends of each femur were cut off. Bone marrow cavity were washed out, with 9 ml pre-warmed (37˚C) hypotonic solution (0.075M KCl) and obtained cells were resuspended and incubated at 37^{o}C for 25 minutes, then spin at 1500 rpm for 7 min. Supernatant was removed and the pellet was resuspended in freshly prepared 3:1 fixative (methanol [100%]:glacial acetic acid). This step was repeated two times. Total time of the fixation was 30 minutes. At the last step cells were resuspended in a small amount of fixative (about 0.5ml). Slides were made using flame dried method and stained 15 min. in 10% Giemsa solution in Sorensen's phosphate buffer.

Mitotic index

In order to analyse cytotoxic activity of irradiation and radioprotective activity of α_2M mitotic index was calculated in bone marrow cells as the percentage of dividing cells of the total number of counted cells.

Statistical method

The data were expresed as means and standard errors from four separate experiments. The χ^2 test was used to compare obtained differences in the values of mitotic indexes among experimental and control groups (Statistics ver. 5).

RESULTS

The radioprotective efficacy of α_2M was examined by comparing the survival times of rats that received α_2M 30 min before irradiation with 6.7 Gy and unprotected irradiated rats (Figure 1). The administration of α_2M effectively raised its plasma concentration about 15 fold from the basal value. The survival rates were recorded for 28 days. Rats from both groups survived the 1st post-irradiation week, however, at the end of the 2nd week, the number of surviving rats in the irradiated control group decreased to about 50%, more or less the number of animals that remained until the end of the follow-up period. In contrast, the pretreatment with α_2M provided 100% protection.

Peripheral blood leukocytes are the most sensitive target for irradiation. Their number falls rapidly in proportion to the dose of exposure. The effectiveness of

α_2M administration on the ensuing leukocyte replenishment was assessed by measuring the number of peripheral blood leukocytes.

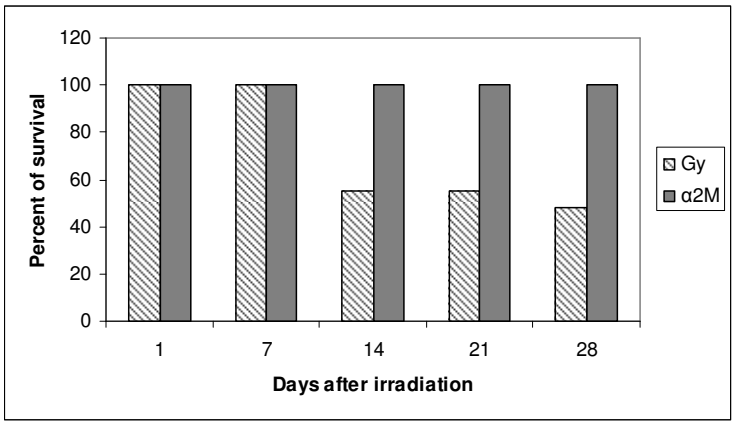

Figure 1. The effects of alpha$_2$-macroglobulin on the survival of rats exposed to total body irradiation. Adult rats were exposed to whole body irradiation with 6.7 Gy ($LD_{50/30}$). 30 min before radiation exposure the rats either received an i.p. injection of 4.5 mg alpha$_2$-macroglobulin in saline (α_2M) or only saline (Gy). Each group consisted of 30-50 rats. The values represent means ± S.E.M. from four separate experiments.

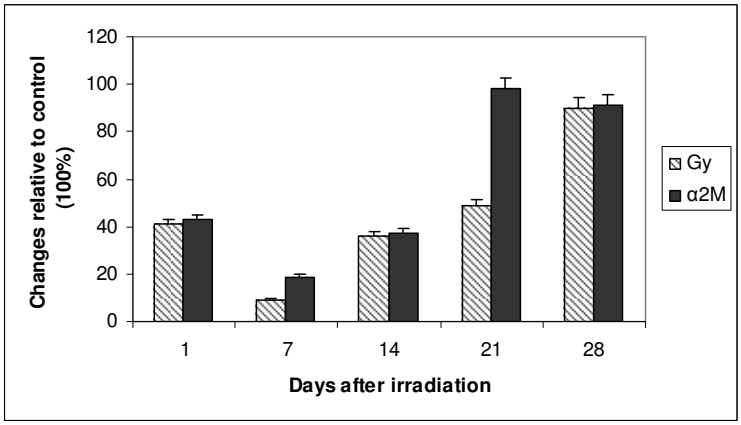

Figure 2. The effects of alpha$_2$-macroglobulin on leukocyte counts after total body irradiation. Adult male were exposed to whole body irradiation with 6.7 Gy ($LD_{50/30}$). 30 min before radiation exposure the rats either received an i.p. injection of 4.5 mg alpha$_2$-macroglobulin in saline (α_2M) or only saline (Gy). Each group consisted of 30-50 rats. The number of leukocytes is expressed relative to the control (100%). The values represent means ± S.E.M. from four separate experiments.

After exposure to 6.7 Gy, in both experimental groups the leukocyte count decreased to about 40% of the initial value by the 1st day (Figure 2), and by the end of the 1st week the leukocyte count was at its lowest. In the irradiated control group the leukocyte count fell to just below 10% of the initial value whereas in α_2M-pretreated groups the leukocyte count decreased to about 20% of the control value. During the 2nd and 3rd weeks a gradual recovery of the number of leukocytes was observed in all groups, and at the end of the follow-up period the number of leukocytes rose to about 90% of the control level in both the surviving control irradiated and α_2M-pretreated groups. Clearly, α_2M provided increased leukocyte protection and allowed for improved leukocyte recovery.

The bone marrow contains hematopoietic stem cell that give rise to the three classes of blood cells found in the circulation: leukocytes, erythrocytes and thrombocytes. The number of differentiated cells in the peripheral blood is proportional to the level of bone marrow protection, representing a very important parameter of survival after irradiation. Bone marrow cells were examined for mitotic activity by scoring the number of cells in mitosis as described in the Materials and Methods. The mitotic index is presented on Figure 3.

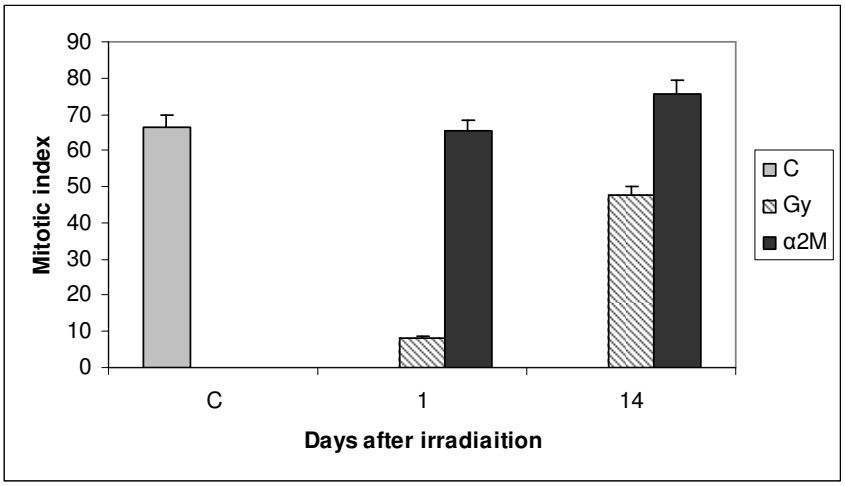

Figure 3. The effect of alpha$_2$-macroglobulin on the mitotic index of bone marrow cells after total body irradiation. Adult male rats were exposed to whole body irradiation with 6.7 Gy ($LD_{50/30}$). 30 min before radiation exposure the rats either received an i.p. injection of 4.5 mg alpha$_2$-macroglobulin in saline (α_2M) or only saline (Gy); C-intact control. Each group consisted of 30-50 rats. The values represent means ± S.E.M. from four separate experiments.

On the 1st day after irradaition the mitotic index in unprotected animals was only 12% of the value observed for intact control (X^2=149.19, p<0.001). At the end of the 2nd postirradaition week, the mitotic index increased to 70% of the control level, but it was still significantly different from the control (X^2=10.08, p<0.001). In α_2M-pretreated animals the mitotic index did not change on 1st post-irradiation day in comparison to control group (X^2=0.02, p=0.880). At the end of the 2nd postirradaition week, the mitotic index in this experimental group was even slightly above the control level, but statisticaly unsignificant (X^2=1.99, p=0.159).

DISCUSSION

α_2M is a major AP protein in rats (BAUMANN and GAULDIE, 1994). During the AP response, a systemic reaction of organism against a variety of stress stimuli ranging from inflammation to tissue injury (BAUMANN and GAULDIE, 1994), the serum concentration of α_2M increases several fold. Evidence has been provided which implicates the α-globulin fraction (19S) (containing α_2M) in the recovery of mice from radiation damage. The α-globulin fraction has been shown to enhance the regeneration of haematopoietic cells (HANNA *et al*., 1976) and lymphopoietic cells (BERENBLUM *et al.,* 1968) in X-irradiated mice. Cytological studies of the haematopoietic recovery indicated that the injection of α-globulin fraction into irradiated mice enhanced the recovery, both in time and magnitude, of the granuloid, lymphocyte and lymphocyte-like elements of the bone marrow, as well as of leucocytes in the peripheral blood. The bone marrow, together with lymphoid tissue, the gastrointestinal epithelium, gonads and embryonic tissues, is highly radiosensitive. In the present study, rats from the unprotecdted irradiated group displayed the highest mortality rate within two weeks of X-irradiation. Up to the end of the 2nd week after irradiation, animals from this group exhibited decreased leukocyte counts, whereas the pretreatment with α_2M-pretreatment provided full survival and restoration of leukocyte numbers by the end of the follow-up period.

In untreated irradiated animals, the number of leukocytes decreased rapidly after irradiation exposure whereas in the α_2M-pretreated animals during the followed up period it increased. Compared to the intact control, the mitotic index in the bone marrow of α_2M-pretreated animals remained almost unchanged during the critical two week period after irradiation. The lowest mitotic index in unprotected animals was observed on the 1st post-irradiation day. This dramatic decrease of the mitotic index on the 1st post-irradiation day in the unprotected group was probably responsible for the high level (50%) of mortality. The recovery of the mitotic index observed at the end of the 2nd week in unprotected irradiated animals is a reflection of the physiological state of rats possessing increased chances of surviving the ARS. Recovery of bone marrow cells after irradiation is possible if at least 10% of the cells survive irradiation exposure (STROBER, 1984). In contrast to the unprotected irradiated animals that exhibited a high (50%) mortality and incomplete recovery of the bone marrow during the follow-up period, the α_2M-pretreated animals displayed

100% survival that was accompanied by a high mitotic index as a result of α_2M-afforded protection on the bone marrow.

One aspect of the radioprotective efficacy of α_2M could be attributed to its unique ability to inhibit all classes of proteases in circulation (SOTTRUP-JENSEN, 1989, BORTH, 1992). Irradiation induces primary oxidative damage of biomolecules, including lipids, proteins, and DNA (NAIR *et al*., 2001). Damage to cellular proteins by oxygen free-radicals formed as a result of action of exogenous factors (radiation, oxidants etc.) underlies the pathogenesis of many diseases (CARNEY and CARNEY, 1994). Thus, oxidation of amino acid residues in the active centers of enzymes could induce considerable alterations of their catalytic properties and, as a consequence, impair cellular regulatory processes. Ionizing radiation also stimulates proteolysis by proteases. (BARTOSZ et al., 1992). Interaction between α_2M and proteases in the plasma and extracellular fluids involve a unique trapping mechanism by which proteases are incorporated covalently into the α_2M molecule, diminishing their proteolytic effect during irradiation.

The proliferation and differentiation of cells of the hematopoietic system depends directly on cytokine growth and differentiation factors produced by the host. Cytokines induce the growth and differentiation of many cells of connective tissue and immune, central nervous, vascular and endocrine systems (GUTTERMAN, 1994). The multiplicity of important functions that are induced by the cytokines indicates that these agents play essential roles in defense reactions against infections, in the recovery from injury and in mediating the radioprotective effect of immunomodulators. In addition to the importance of α_2M in maintaining the proteinase-proteinase inhibitor equilibrium during inflammation and its antiapoptotic effect (BORTH, 1992), α_2M also binds different acute inflammatory mediators of which the most important are the cytokines (TNF-α, IL-1β, IL-2, IL-6) and growth factors (β-NGF, PDGF-BB, TGF-β1, TGF-β2) that play essential roles in the regulation of cellular functions that bring about tissue repair and remodeling (JAMES, 1990) and participate in mechanism responsible for radioprotection (NETA *et al*., 1991; NETA *et al*., 1992). α_2M was identified as a major component of the α-globulin fraction in the serum that modulates cell growth (HANNA *et al*., 1976; BERENBLUM *et al.,* 1968; OLINESCU *et al*., 1980), and a direct link between growth factors and α_2M was established (MATSUDA *et al*., 1989).

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RADIOPROTEKTIVNA ZAŠTITA AKUTNO FAZNOG PROTEINA A2-MAKROGLOBULINA NA ĆELIJE KOSNE SRŽI PACOVA

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I z v o d

Pacovski alfa2-makroglobulin (α2M) ima važnu ulogu u uspostavljanju narušene homeostaze inhibicijom različitih tipova nespecifičnih proteaza i olakšavajući transport citokina, hormona rasta i hormona. Naša ranija istraživanja su pokazala da administracija α2M eksperimentalnim životinjama u traumama tipa opekotine ili ozračivanja celog organizma značajno povećava njihovu stopu preživljavanja. Cilj ove studije je bio izučavanje radioprotektivne uloge α2M na ćelije kosne srži. α2M je apliciran 30 minuta pre ozračivanja pacova dozom od 6.7 Gy ($LD_{50/30}$) X-zraka i omogućio je 100% preživljavanje pacova za razliku od ozračenih pacova bez tretmana kod kojih je smrtnost bila 50%. Rezultati su pokazali da tretiranje životinja sa α2M, nakon inicijalnog pada, omogućavaju potpuni oporavak broja leukocita kao posledica očuvanja ćelija kosne srži, što se uočava preko stabilnog mitotskog indeksa. Kod ozračenih pacova bez tretmana signifikantno smanjenje mitotskog indeksa, kao posledica narušavanja ćelija kosne srži, rezultuje i u prolongiranom padu broja leukocita. Na osnovu ovih rezultata može se zaključiti da se radioprotektivna uloga α2M delom odvija putem citoprotekcije novih krvnih ćelija u kosnoj srži.

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