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RESPONSE OF HUMAN MYOBLASTS TO THERAPEUTIC DOSES OF X-IRRADIATION

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Abstract— **Objectives: The aim of this study was to investigate the response of human skeletal muscle myoblasts to therapeutic doses of X-irradiation.**

Methods: Myoblasts was established via skeletal muscle biopsies which irradiated in vitro by x-ray at doses 3, 5 and 7 Gy. Two effects of radiation were measured (acute effect after 24h and long term effect after 72h). Acute effect was monitored by interleukin-6 release, while long term effect was monitored by measuring the proliferation capacity and cell death.

Results: When comparing the non-irradiated control and cells processed with inhibitor of cell proliferation Ara C, myoblast proliferation reduced in 72h post-radiation, this change was significant with increasing doses. Post-irradiation myoblast survival determined by measurement of released LDH enzyme activity showed increased activity after exposure to x-rays. The acute reaction of myoblasts to lower doses of irradiation (3 and 5 Gy.) was reduced secretion of constitutive interleukin-6.

Conclusion: The current study approved that there was positive response of human skeletal muscle myoblasts to therapeutic doses of X-irradiation..

Index terms- **Myoblasts, Ionizing Radiation, Lactate Dehydrogenase, Necrosis, Interleukin-6, Proliferation.**

I. INTRODUCTION

Skeletal muscle tissue is repaired and maintained by activation of a specific type of adult stem cells called satellite cells. These dormant cells are activated to generate key cells responsible for muscle regeneration and differentiation [1]. An anti-inflammatory myokine interleukin 6 (IL-6) is secreted from activated myoblasts after trauma, especially burns or other tissue damage obtained by any physical stress. IL-6 plays an important role in promoting myoblast proliferation and differentiation during muscle regeneration [2,3,4,5]. Several studies have investigated ionizing radiation effects on skeletal muscle, demonstrating that skeletal muscle damage remains for many years after irradiation. Adult skeletal muscle is considered to be radio-resistant, unless higher doses of radiation are applied [6,7,8,9,10,11]. Direct effects of radiation inhibit muscle regeneration by damaging satellite cells, which can lead to mitotic failure and cell death [12]. Impaired muscle regeneration following irradiation may thus be due to a reduced number of activated satellite cells needed for reconstruction and repair of damaged muscle fibre. The lack of adequate muscle regeneration may also be due to impaired cytokine signalling pathway and, finally differentiation [13]. This brings to light that skeletal muscle is radiosensitive during childhood growth and development that is why radiotherapy in paediatric patients may induce muscular atrophy, a fact that is attributed to the large number of radiosensitive satellite cells during a child growth period [14]. The present study is a trial to understand the mechanism of radiation induced muscle atrophy by evaluating the radiation dose response of human muscle stem cells, primary mononucleated myoblasts, which are key cells involved in the development of adult muscle fibre and in the process of muscle regeneration. Two types of radiation effects on human myoblasts propagated in vitro were studied: acute effects (determined 24h post-irradiation) were followed by monitoring IL-6 secretion; long term effects (evaluated 72h post irradiation) were followed by assessing proliferation capacity and cell death.

II. MATERIALS AND METHODS

A. Cell culture

Human muscle tissue that usually disposed during orthopedic surgeries on donor subjects without diagnosing the muscular disease was cleaned and trypsinized. The released muscle satellite cells were cultured in advanced Minimum Essential Medium (aMEM) (Life technologies, Grand Island, USA) supplemented with 10% fetal calf serum (FCS) (Life technologies) at saturated humidity in mixture of 5% CO_2

enriched air at 37°C. Myoblast colonies identified by morphological characteristics and devoid of fibroblast contamination were trypsinized and further expanded. Cells were plated in six–well dishes and grown for 72h in aMEM compensated with 10% FCS prior to the experiments [6]. All the subjects were informed about the procedure of the study and written consent was obtained to participate in the study.

B. Exposure of Myoblasts to X-irradiation

Cells ($1x10^6$ cells/ml aMEM) were grown in six-well plates and irradiated with a dose rate of (3–7 Gy). An X-ray therapy unit DARPAC 150 MC (Raytec Inc., UK), run at 220kV, 10mA. Cu and Al filters with thicknesses 0.55mm and 1.8 mm respectively were used during irradiation.

C. Measurement of the proliferation capacity

The proliferation capacity was determined by Bromodeoxyuridine (BrdU) Cell proliferation Assay, (Calbiochem, Merk, Darmstadt, Germany). Cells were cultured in 96-well plates (1500 cells per well in 100µl MEM), the next day they were irradiated and the proliferation test was performed 72h later. 10µM arabunifuranosyl cytidine (Ara C), an inhibitor of DNA synthesis, was added to the cells to produce the negative proliferation control. BrdU was allowed to incubate with cells for 18 h. BrdU labeled cells were visualized by Anti-BrdU Antibody diluted 1:100 (supplied with the kit). The diluted Peroxidase Goat Anti-Mouse IgG HRP Conjugate was filtered through 0.2 micron filter according to instructions. Fifteen minutes after adding the Substrate Solution absorbance was measured at 450 nm and 540 nm (Victor 3 plate reader from PerkinElmer, Shelton, CT, USA).

D. Assessment of cell death

Cells were plated in white 24-well plates (10^5 cells per well in 500µl MEM) (Visiplate-24 TC, PerkinElmer, Shelton, CT, USA). After irradiation, the cells were treated for caspase detection and the culture media were collected and used to assess the release of lactate dehydrogenase (LDH, EC 1.1.1.27). Apoptotic initiator caspase 9 (LEHD-ase) and executor caspase 3/7 (DEVD-ase) activity were measured using CaspaseGlo 9 Assay and CaspaseGlo 3/7 Assay Kits from Promega (Madison, WI, USA). The level of necrotic cell death after cell irradiation was determined by measuring the activity of LDH in the cell culture media using a Cytotoxicity Detection Kit PLUS (Roche Diagnostics GmbH, Mannheim, Germany). Luminescence and absorbance (490nm) were measured on a Victor 3 plate reader (PerkinElmer, Shelton, CT, USA) immediately after irradiation, 36h, and 72h later.

E. Measurements of IL-6 secretion

Levels of secreted IL-6 in the culture supernatant collected from the cultures 24-h after exposure to irradiation were measured using an Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit (Endogen, Rockford, USA), according to manufacturer's instructions and as described previously **[16]**. IL-6 levels were calculated from concentrations of IL-6 measured in supernatant in each well.

F. Statistical analysis

Results are presented as mean ± standard error S.E. The differences among experimental groups were calculated using one-way ANOVA for intragroup comparisons. SPSS 17.0 for (SPSS, Chicago, IL, USA) was used for data analysis.

III. RESULTS

A. Cell proliferation

X-ray doses of the therapeutic range used in the experiments effectively prevented human myoblast proliferation. Irradiation at all doses statistically significantly inhibited myoblast proliferation to the same level (~ 0.3 of the untreated control) as the blocker of DNA synthesis Ara C.

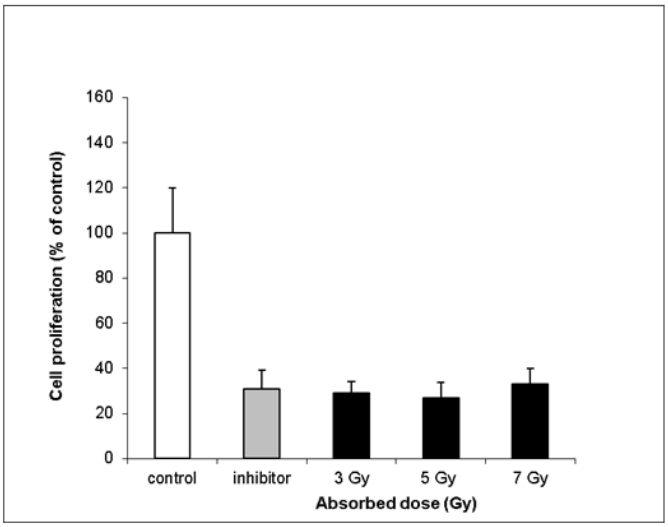

Fig. 1 ; Inhibition skeletal myoblasts proliferation capacity as a function of absorbed dose measured 72h post-irradiation. Columns and bars represent means ± SD (n=9). Means are expressed as % of control of absorbance measurement at dual wavelengths of 450-540 nm. The control was used as the predetermined reference measurements. Statistically significant differences ($p< 0.001$). Control-non irradiated myoblast; inhibitor-myoblasts treated with 10µM AraC, an inhibitor of cell proliferation; 3 Gy- myoblasts irradiated with 3 Gy; 5 Gy-myoblasts irradiated with 5 Gy; 7 Gy- myoblasts irradiated with 7 Gy.

B. Assessment of cell death

The influence of x-irradiation on cell death was studied at room temperature ~21ºC to detect whether the cell death mechanism was either by necrosis or apoptosis. Myoblasts irradiation did not cause necrotic cell death, measured by LDH activity, up to 36 h after cell manipulation with all tested doses (Figure 2A). However, a statistically significant increase in necrotic cell death occurred 72 h after irradiation with 3 Gy. LDH activity in the media at this time point increased to 42 ± 9%. Furthermore, a similar pattern was seen after irradiation with higher doses, although the increase in LDH activity was not statistically significant compared to control untreated cells. Apoptosis of cells was measured by activation of caspases 3, 7 and 9.

Irradiation of myoblasts did not result in either increased activity of initiator caspase 9 (data not shown) or activation of execution caspases 3 and 7 (Figure 2 B).

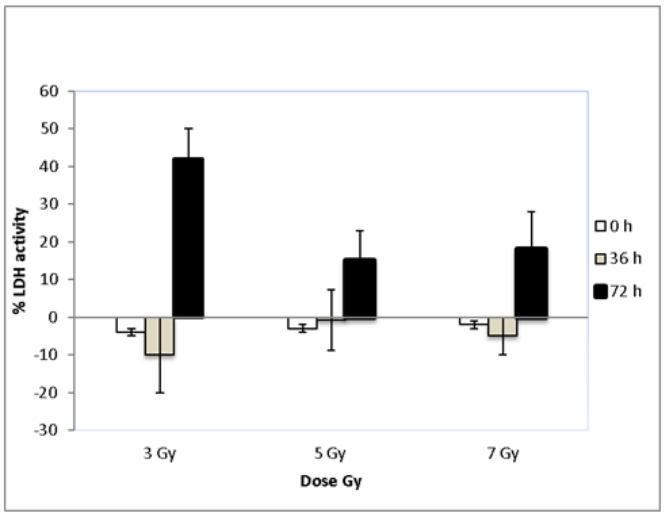

Fig. 2A ; The influence of selected therapeutic doses of x-rays (3 Gy, 5 Gy, 7 Gy) on cell death immediately after irradiation (0 h after IR), 36h after irradiation (36h after IR) and 72h after irradiation (72h after IR). Columns and bars represent means ± S.E. (n=6). (A) The assessment of myoblasts membrane integrity at different time points after irradiation is shown as the activity of lactate dehydrogenase LDH released in the medium. Statistically significant differences ($p < 0.001$).

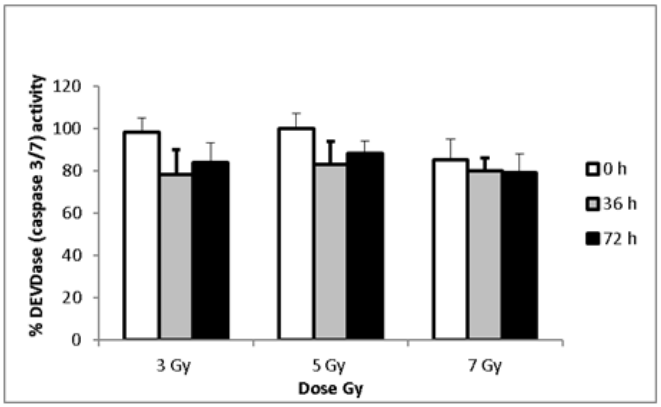

Fig. 2B; The influence of selected therapeutic doses of x-rays (3 Gy, 5 Gy, 7 Gy) on cell death immediately after irradiation (0 h after IR), 36h after irradiation (36h after IR) and72 h after irradiation (72h after IR). Columns and bars represent means ± S.E. (n=6). (B) The cleavage activity of the amino acid sequence DEV (recognized and cleaved by apoptotic caspase 3 and 7) is shown at different time points after irradiation. There were no statistically significant differences among groups.

C. Effects of irradiation on the secretion of IL-6

Levels of IL-6 were measured in supernatants of cultures 24h after exposure to different doses of irradiation. In control cultures that were kept in the same conditions as other cultures and were not exposed to irradiation, the constitutive level of IL- 6 was 12,988 pg/ml (n=18). Exposure of myoblasts to irradiation resulted in a decreased level of IL-6 secretion in a dose dependent manner, being most pronounced in myoblasts irradiated with 3 Gy in comparison with myoblast irradiated with 5 Gy ($p<0.05$) or with 7 Gy ($p<0.05$). Irradiation of cells with 5 Gy also resulted in a statistically significant decrease of IL-6 secretion, while irradiation of myoblasts with the highest tested dose (7 Gy) did not result in decreased secretion of IL-6 (Figure 3).

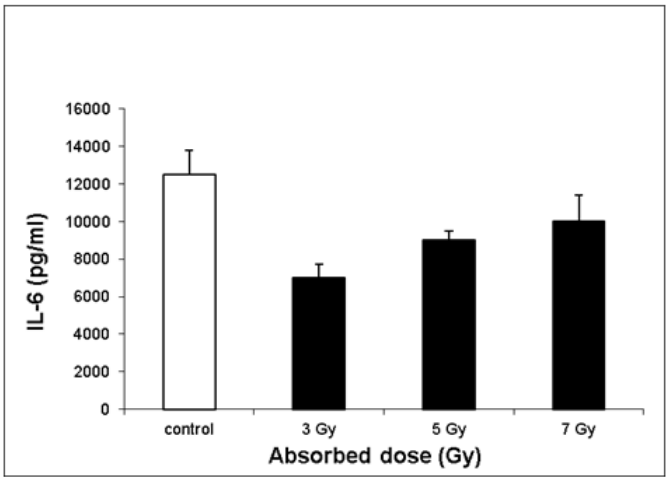

Fig. 3; IL-6 secretion level was estimated by ELISA in control myoblast cultures and compared with level of IL-6 secretion in myoblast culture 24 h after exposure to 3 Gy, 5 Gy and 7 Gy dose of irradiation. Data are means ± S.E. (n = 18 in each independent experiment). * $p < 0.05$ denotes difference in level of IL-6 in exposed cultures vs. respective level of control cultures.

IV. Discussion

Previous studies revealed that ionizing radiation is capable to prohibit mitosis of the primary stem cells in adult skeletal muscle which is called satellite cells. Satellite cells play the major role in muscle growth and hypertrophy after birth. Ionizing radiation breaks DNA strands of the cells and in this way inhibiting muscle regeneration [12]. Human muscle precursor myoblasts derived from satellite cells are responsible for muscle growth and regeneration [15-18]. Ionizing radiation impacts muscle cells during development by reducing their activation, proliferation and differentiation [19]. It also prohibits muscle growth during development. The results of this study showed a dose-dependent effect on human myoblast proliferation and regeneration capacity due to x-irradiation. These results suggest the appearance of a significant change in myoblast proliferation and regeneration capability. The myoblast stage of developing skeletal muscle is sensitive to ionizing radiation, and it is thus essential to give particular attention to radiotherapy during childhood. Previous investigations [6-11] suggest that adult skeletal muscle is radio-resistant unless higher doses of radiation are applied. However, higher doses of radiation can cause myopathies and, in most cases, induced muscle atrophy. Skeletal muscle in cancer patients is often exposed to ionizing radiation during radiotherapeutic treatment. One of the most probable mechanisms contributing to post radiation myopathy and muscle atrophy is the inability of muscle to regenerate. Muscle

regeneration is a key process, responsible for maintaining the integrity of the muscle mass and function throughout life and particularly after muscle injury [20,21]. It has been demonstrated that autocrine secretion of IL-6 plays an important role in the regulation of myoblast proliferation and differentiation [22]. There was observed that an acute decrease in IL-6 secretion, which was more prominent when myoblasts were exposed to lower doses of irradiation. The less prominent decrease of IL-6 secretion in myoblasts exposed to higher doses of irradiation might be the result of IL-6 release from damaged cells, although we could not detect significantly increased levels of necrotic cell markers. In some in vivo and in vitro studies, it was shown that radiation increases cytokine IL-1 and IL-6 levels [21,22,23]; higher levels of cytokines might be due to their release from cells other than muscle. Similarly, a reduction in constitutive IL-6 secretion was also observed in primary myoblast culture after exposure to high doses of glucorticoids or to environmental stress or organophosphates. My results and the results of previous studies show high sensitivity of human myoblast to various chemical and physical factors. IL-6 is an effective stimulator of myoblast proliferation and a diminished level of IL-6 secretion post irradiation might severely decrease autocrine and paracrine IL-6 activity.

V. Conclusion

In conclusion, my results show that myoblasts are sensitive to irradiation in terms of their proliferation capacity and the ability to secret IL-6. Since myoblast proliferation and differentiation are a key stage in muscle regeneration, this effect of irradiation needs to be taken into account, particularly in certain clinical conditions, especially in Children radiotherapy.

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