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Pro-angiogenic effects of X-rays on murine endothelial cells

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Abstract Recently, significant attention has been paid to the possibility of thwarting cancer progression by inhibition of neoangiogenesis (formation of new blood vessels) in growing tumors. Although general mechanisms of angiogenesis have been elucidated, virtually nothing is known about the effects of low doses of ionizing radiation on pro-angiogenic properties of endothelial cells. In the present study, we evaluated the effects of a low (0.2 Gy), intermediate (1 Gy), and high (4 Gy) doses of X-rays on a few angiogenesis-related parameters of isolated murine endothelial cells. We show here that 24 to 48 hours after irradiation with 0.2 Gy the cell proliferation was inhibited to a similar extent as after the exposure to 1 Gy. Also, adhesion of the 0.2 Gy-irradiated cells to both gelatin and Matrigel® was inhibited 24 hours post-exposure, whereas irradiation with 1 or 4 Gy resulted in the increased adhesion of the cells to these substrata. Similar effects were observed during the "wound" migration assay. Finally, 24 hours after exposure of the cells to 0.2 Gy of X-rays, the surface expression of the β_3 integrin subunit was down-regulated, whereas irradiations with 1 and 4 Gy of X-rays resulted in the significantly elevated expression of this subunit. These results indicate that proliferating endothelial cells are sensitive *in vitro* to relatively low doses of ionizing radiation and that the effects of a low dose are opposite to those observed after irradiations with 1 and 4 Gy of X-rays.

Key words angiogenesis • ionizing radiation • cell adhesion • integrins

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Introduction

A promising strategy of therapy of solid tumors rely on interference with the development of new blood vessels (neoangiogenesis) in a growing neoplastic tissue [5]. Neoangiogenesis results from proliferation and migration of endothelial cells which bud-off from the walls of the vessels in the surrounding healthy tissues. These two features of endothelial cells are stimulated by the tumor-derived pro-angiogenic factors as well as by interactions of these cells with the tumor stroma, especially via the integrin receptors [13].

So far, the effect of ionizing radiation on neoangiogenesis has not been examined in details. A few reports have indicated that irradiation with X-rays can modulate formation of new blood vessels *in vitro* and *in vivo* suggesting that the mechanism involved depends on a release of growth factors and/or on interactions with extracellular matrix (ECM) [9, 12]. Notably, more than additive effect of radiotherapy and anti-angiogenic factors or inhibitors of the cell-to-cell or cell-to-ECM interactions has been described [14].

The aim of the present study was to quantitatively and qualitatively evaluate the effect of a low (0.2 Gy), intermediate (1 Gy), and high (4 Gy) doses of X-rays on the proliferative, adhesive, and invasive potentials of

isolated endothelial cells as well as on expression of the β_3 integrin subunit on their cellular membrane, that is the cell parameters potentially associated with the neoangiogenic process.

Materials and methods

Endothelial cells' isolation and culture

Endothelial cells (EC) were obtained using the lungs of C57Bl/6 mice as described elsewhere [4]. The isolated cells were cultured in flasks precoated with 1% gelatin in the DMEM medium supplemented with 20% fetal calf serum (FCS), 1% nonessential amino acids (NAA), heparin, and 50 µl/ml EC growth factor. For the experiments, cells from passages 6 to 12 were used.

Exposure to X-rays

The cells were irradiated *in vitro* at room temperature with a 240 kV X-ray generator (Isovolt 320/10, Seifert, Germany) at 0.6 Gy/min dose rate. As controls, sham-irradiated (the generator in a turn-off mode) cells were used.

Proliferation assay

Endothelial cells plated at different cell numbers onto the gelatin-coated flasks were irradiated 24 hours later and incubated in the medium for up to 120 hrs. Everyday during the incubation the cells were counted.

Adhesion assay

Adhesion of the cells to Matrigel® or gelatine was determined as described elsewhere [1]. Briefly, 24 and 48 hrs post-irradiation, the cells were seeded onto the 96-well plates pre-coated with either of the two substances. After 45 min, non-adherent cells were removed and the remaining cells were fixed with 70% ethanol and stained with 1% methylene blue. The rate of adhesion was determined using a spectrophotometer (Spectra max® 190, Molecular Devices, Germany), from the absorbance at 630 nm of the liquid solution obtained after washing stained cells with distilled water followed by the addition of 100 µl of a 0.1 M HCl solution to each well.

Migration assay

Cells were seeded onto the gelatin- or Matrigel®-coated plates and allowed to grow into confluence. Immediately after exposure of the confluent cultures to X-rays a nick in the cell layer was made with a sterile tip. Twenty-four or 48 hrs later the cells were stained with methylene blue and the total number of cells that migrated into the nick was counted.

Flow cytometry analysis of the β_3 integrin subunit

EC were seeded onto the gelatin coated flasks and irradiated after 24 hrs of growth. Two, 6, 24 and 48 h post-exposure the cells were collected, washed and stained with the FITC-conjugated anti-mouse β_3 monoclonal antibody (clone C 27, Becton-Dickinson) at room temperature in the dark. Surface expression of the integrin subunit was determined using a FACSCalibur flow cytometer (Becton-Dickinson) equipped with a CellQuest plus software.

Results and discussion

As shown in Fig. 1, during the first 24 hrs after exposure to the three doses of X-rays proliferation of the EC was unaltered. However, beginning from the 48th hour after the irradiation a significant inhibition of the proliferation was detected, most pronounced following the exposure to 4 Gy of X-rays. Interestingly, irradiation with both 0.2 Gy and 1 Gy resulted in a similar inhibition of the proliferation detected at 48 and 120 hours after exposure. Although the dose-dependent inhibition of proliferation of endothelial cells by X-rays was previously reported [7, 10], the approximate result of the exposures to 0.2 and 1.0 Gy is an original observation of the present study.

Twenty-four hours after the irradiation with 0.2 Gy of X-rays adhesion of the cells to Matrigel® and gelatine was significantly inhibited compared to the effect detected in the sham-irradiated cells (Fig. 2). So far, only Hildebrandt *et al.* [6] reported that endothelial cells irradiated with 0.3 to 0.6 Gy of X-rays less eagerly bind peripheral blood mononuclear cells than do their non-exposed counterparts. In contrast, in the present study, exposure to 1 or 4 Gy increased the number of cells adhering to both Matrigel® and gelatine, but the more pronounced effect was in cells seeded on the former substrate. These results corroborate the findings of Meineke *et al.* [8] who detected the dose-dependent

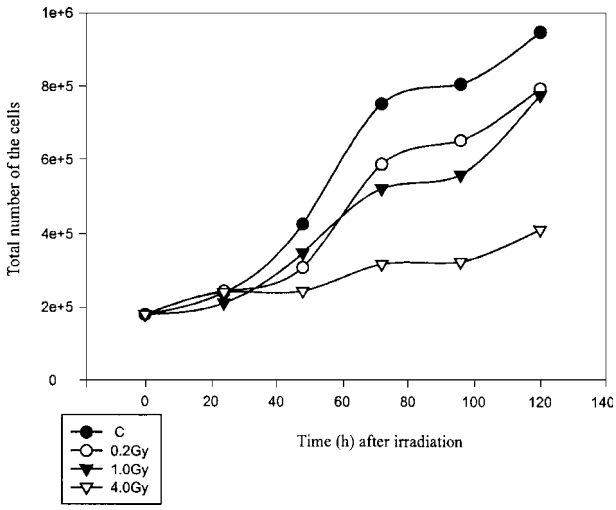

Fig. 1. Proliferation of murine endothelial cells after exposure to different doses of X-rays, expressed as total number of cells after the irradiation.

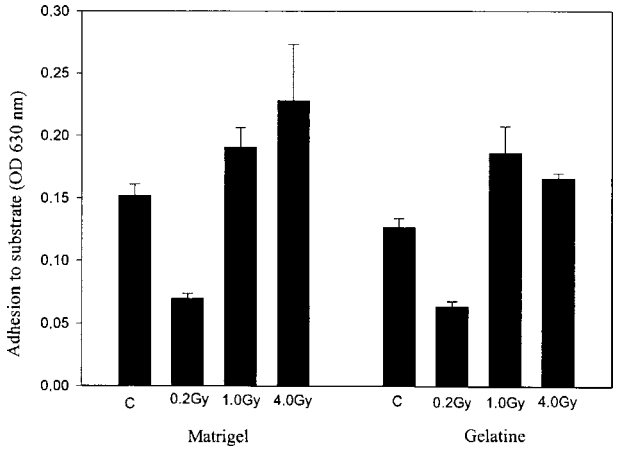

Fig. 2. Adhesion of endothelial cells to Matrigel® and gelatine 24 hours after the irradiation with X-rays; C – sham-irradiated (control) cells, 0.2 Gy – cells irradiated with 0.2 Gy, 1.0 Gy – cells irradiated with 1 Gy, 4.0 Gy – cells irradiated with 4.0 Gy. Bars represent means ± SD.

increase in adhesion of both cancer and normal cell lines to the ECM substrates following exposure to 1 to 10 Gy of X-rays; this effect was accompanied by the elevated expression of several adhesion molecules on the surface of the tested cells.

As indicated by the results shown in Fig. 3, irradiation with 0.2 Gy of X-rays led to inhibition of the migration of the isolated endothelial cells, the effect being detectable 24 hours after the exposure. In contrast, irradiation with 1 or 4 Gy stimulated the cells' capacity to migrate across the nick (Fig. 3). Similarly, Wild-Bode *et al.* [15] reported that exposure to 6 Gy of γ-rays promoted migration of the glioma cells. However, in view of the findings of Cordes *et al.* [3] that irradiation with such a dose of X-rays inhibits migration of the glioblastoma cells *in vitro*, this effect must await further clarification.

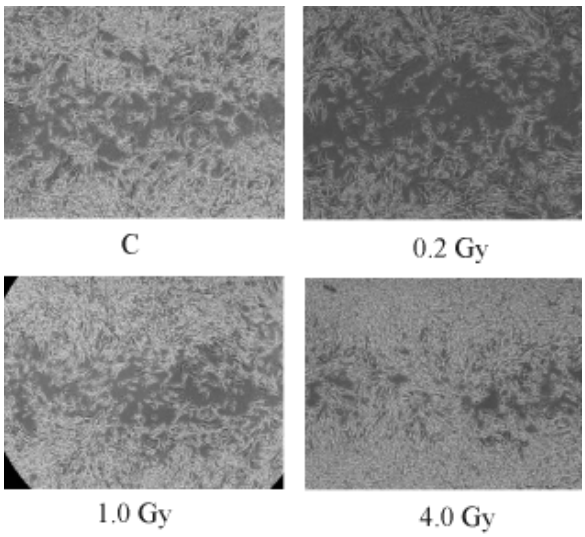

Fig. 3. Migration of endothelial cells across the artificial "wound" 24 hours after irradiation with X-rays; C – sham-irradiated (control) cells, 0.2 Gy – cells irradiated with 0.2 Gy, 1.0 Gy – cells irradiated with 1 Gy, 4.0 Gy – cells irradiated with 4.0 Gy.

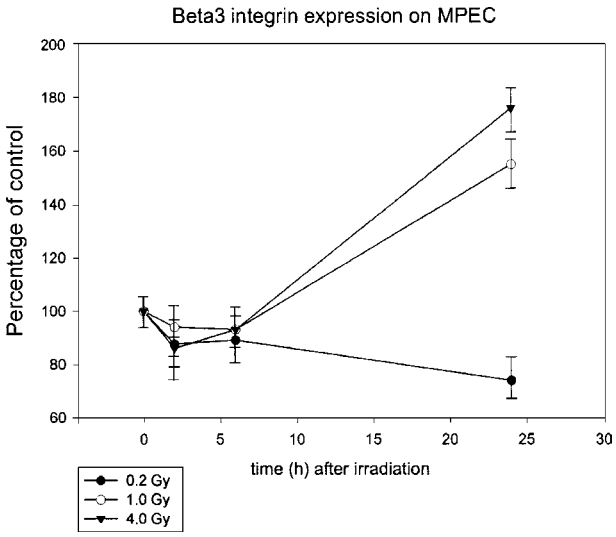

Fig. 4. Expression of the β_3 integrin subunit on the surface of endothelial cells irradiated with various doses of X-rays. Points represent means ± SD.

Finally, Fig. 4 shows that two hours after exposure of the isolated endothelial cells to 0.2 Gy of X-rays the surface expression of the β_3 integrin subunit was significantly suppressed and the effect was detectable throughout the first 24 hours post-exposure. In contrast, irradiation with 1 or 4 Gy led to a transient decrease, followed by a significant, dose-dependent stimulation of the expression of this integrin subunit. Similar elevation of the expression of the selected integrin receptors on cancer cells was reported by Cordes *et al.* [2]; however, no studies have been reported on the effects of doses lower than 1.0 Gy on the expression of integrin molecules on endothelial or other cell types.

In summary, the similar inhibition of proliferation at 48 and 120 hours after irradiation with both 0.2 Gy and 1 Gy and significant alterations of adhesion, migration and β_3 integrin subunit expression, i.e. inhibition after low and stimulation after higher doses, were detected. Based on the results obtained in this study, we have concluded that low compared to higher doses of X-rays differently affects selected pro-angiogenic parameters. In consequence, it could provide new insights into the mechanisms underlying the effects of the low-LET ionizing radiation on the events associated with neoangiogenesis in tumors. These effects may be possibly used in the low-level radiation therapy of cancers, such as proposed by Sakamoto [11].

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