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Irradiation of C6 Glioma Cells with X Rays Increases the Expression of Angiogenic Genes and their Angiogenic Potential *in vitro* and *in vivo*

A. Parthymou[1], D. Kardamakis[2] and E. Papadimitrou [1]

[1]Lab of Molecular Pharmacology, Dept of Pharmacy, University of Patras, GR 26504, Greece, [2]Lab of Radiotherapy, Dept of Medicine, University of Patras, GR 26504, Greece

S u m m a r y. Malignant glioma is considered to be the most invasive and the most resistant to irradiation form of gliomas. In the present work, we showed that even though irradiation of C6 glioma cells decreased their cell numbers in vitro, it caused an increase in the secretion of MMP-2 and MMP-9 in the culture medium and induced the mRNA expression of MMP-2, MMP-9 and α_v integrin by C6 cells. Moreover, it enhanced their angiogenic activity in vivo, in the chicken embryo chorioallantoic membrane model of angiogenesis, and in vitro, in cultures of human umbilical vein endothelial cells.

INTRODUCTION

Rapid cell proliferation and high invasive capacity of glioma cells are crucial for tumour development and progression. Moreover, induction of angiogenesis is an essential prerequisite for tumour growth, as well as spread and precedes malignant tumour formation (1). Metalloproteinases 2 and 9 (MMP-2 and MMP-9 respectively) play a crucial role in cancer cell growth, migration and invasion and regulate tumour angiogenesis (2,3,4,5). Moreover, integrin $\alpha_v\beta_3$ is expressed during blood vessel formation, promotes endothelial cell survival (6) and through interaction with MMP-2 plays a key role in tumor angiogenesis (7).

We have previously shown that X rays enhance angiogenesis induced by C6 glioma cells inoculated onto the chicken embryo chorioallantoic membrane (CAM), immediately after irradiation of the CAM (8). In the present work, we report the effect of irradiation of C6 glioma cells on their proliferation, metalloproteinase secretion, expression of angiogenic genes and glioma cell-induced angiogenesis *in vivo* and *in vitro*.

METHODS

C6 rat glioma cells (ATCC) were grown routinely, as previously described (8). Human umbilical vein endothelial cells (HUVEC) were grown as previously described and used at passages 1-3 (9). C6 glioma cells were irradiated in air with graded single doses of X-rays generated at 6 MV (SL75 Philips linear accelerator) and at a dose rate of 4.5 Gy min^{-1}. Dosimetry measurement was performed with an ionization chamber. The effect of conditioned media of irradiated C6 cells on the proliferation of HUVEC was determined by direct cell counting or the MTT assay. Migration assays were performed in 24-well microchemotaxis chambers as previously described (9) The amounts and the activation of MMPs after irradiation of C6 cells was estimated by zymography. RT-PCR reactions were performed in total RNA using specific, already published rat primers. C6 glioma cells were transfected with the pEGFP-C1 construct (Clontech) using a solution of linear polyethylenimine polymers (EXGEN 500), according to the manufacturer's instructions. EGFP-transfected, non irradiated C6 glioma cells were inoculated onto CAMs, as previously described (8). 48 h later, CAMs were irradiated using a RT mobile contact therapy and a single X-ray dose of 10 Gy was used. 48 h after irradiation, CAMs were fixed, embedded in paraffin, cut, placed on positively charged glass slides and stained with standard haematoxylin-eosin staining. CAM sections were also examined using fluorescence

microscopy for the visualization of tumor cell growth. The significance of variability between the results from various groups and the corresponding controls was determined by unpaired t-test or ANOVA.

RESULTS

X-rays caused a dose dependent decrease in the number of C6 glioma cells 48 h after irradiation and this decrease was evident only at doses greater than 5 Gy. Interestingly, MMP-2 and MMP-9 expression and secretion, as well as the expression of the integrin α_v, were increased with elevated doses of X rays 48 h after irradiation and were mostly evident at the higher doses used. The increase was significant within the first 6 h after irradiation and further increased at 24 h. When C6 glioma cells were inoculated onto CAMs, they followed their distinctive pattern of growth (1,10) and finally caused the development of new small blood vessels in the area. When C6 glioma cells were irradiated with 10 Gy X rays and then inoculated onto CAMs, tumour growth and angiogenesis were significantly increased compared with CAMs where non-irradiated cells were inocculated. When C6 glioma cells were irradiated with 40 Gy X rays, there was an even greater enhancement of tumour induced vessel formation in the total area of the CAM, although tumour growth was insignificant. Similar results were obtained when C6 cells were irradiated 48 h after their inocculation onto non-irradiated CAMs. Conditioned medium of irradiated C6 cells caused a statistically significant increase in HUVEC proliferation and migration, in a manner dependent on the dose of X rays, which was reversed by o-phenanthroline.

CONCLUSIONS

Our results support the combined use of anti-angiogenic therapy with radiotherapy to improve the clinical outcome of malignant gliomas.

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