Regular paper

Metastasis inhibition after proton beam, β- and γ-irradiation of melanoma growing in the hamster eye*

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Standard ocular tumor treatment includes brachytherapy, as well as proton therapy, particularly for large melanoma tumors. However, the effects of different radiation types on the metastatic spread is not clear. We aimed at comparing ruthenium (^{106}Ru, emitting β electrons) and iodine (^{125}I, γ-radiation) brachytherapy and proton beam therapy of melanoma implanted into the hamster eye on development of spontaneous lung metastases. Tumors of Bomirski Hamster Melanoma (BHM) implanted into the anterior chamber of the hamster eye grew aggressively and completely filled the anterior chamber within 8–10 days. Metastases, mainly in the lung, were found in 100% of untreated animals 30 days after enucleation. Tumors were irradiated at a dose of 3–10 Gy with a ^{106}Ru plaque and at a dose of 6–14 Gy using a ^{125}I plaque. The protons were accelerated using the AIC-144 isochronous cyclotron operating at 60 MeV. BHM tumors located in the anterior chamber of the eye were irradiated with 10 Gy, for the depth of 3.88 mm. All radiation types caused inhibition of tumor growth by about 10 days. An increase in the number of metastases was observed for 3 Gy of β-irradiation, whereas at 10 Gy an inhibition of metastasis was found. γ-radiation reduced the metastatic mass at all applied doses, and proton beam therapy at 10 Gy also inhibited the metastastic spread. These results are discussed in the context of recent clinical and molecular data on radiation effects on metastasis.

INTRODUCTION

Particle radiotherapy, such as proton beam, yields promising clinical results worldwide, but the mechanisms of its action are still unclear. Ocular melanoma is one of the tumors treated by protons with excellent clinical results (Fuss et al., 2001; Höcht et al., 2004; Gragoudas, 2006;). The advantage of particle beams over photons is due to the physical characteristics and results in superior distribution of the radiation dose, which allows to spare normal tissue close to the target (Levin et al., 2005; Fokas et al., 2009). Proton beam therapy is also applied in other cancer treatments (Mizumoto et al., 2010; Mizumoto et al., 2011; Lorentini et al., 2012). However, the effects of particle beams on metastatic spread of cancer are not yet well understood. There are only a few publications on this subject. It was shown that particle beams might inhibit cellular metastatic potential (Takahashi et al., 2003). Proton ion irradiation was also demonstrated to decrease cell migration and invasion in a dose-dependent manner and strongly inhibit matrix metaloproteinase-2 activity in two preclinical models (Ogata et al., 2005). These results suggest that particle irradiation suppresses metastatic potential at a low dose, whereas another report shows that photon irradiation promotes cell migration and invasive capabilities at a low dose level.

Enhancement of migratory properties of different tumor cell lines were found after 1-3 Gy of gamma irradiation (Wild-bode et al., 2001; Goetze et al., 2010). Several factors associated with increased migratory properties have been found to be induced by gamma irradiation, such as HGF receptor/c-Met expression (Qian et al., 2003), integrin expression (Onoda et al., 1992; Goetze et al., 2010) or MMP-2 activity (Wild-bode et al., 2001, Park et al., 2006; Martinou et al., 2011). In particular there are suggestions that it is the interaction with tumor microenvironment that influences cell migration and invasiveness (Ohuchida 2004; Madani et al., 2008).

These findings taken together indicate that ion beam radiotherapy may be superior to conventional photon beam therapy in possible preventive effects on tumor cell metastasis (Girdhani et al., 2013). In our animal model we can observe both immediate effects, such as inhibition of the growth of the radiation treated tumor, and long- term effects of the therapy, such as reduction in the number of metastases (Urbanska et al., 2000; Matuszak et al., 2000).

The goal of this study was to compare the effects of β- and γ-radiation using brachytherapy and proton beam radiation of melanoma tumor growing in the eye on the development of metastases in the lung. In this study we show, for the first time, a substantial inhibition of lung metastasis after a single dose of 10 Gy (ie. 11.1 CGE Cobalt Grey Equivalent) of proton beam irradiation of the melanoma tumor growing in the hamster eye.

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Abbreviations: BHM, bomirski hamster melanoma; CGE, cobalt grey equivalent; HGF, hepatocyte growth factor; MMP2, matrix metalloproteinase 2; PMMA, poly(methyl methacrylate); SOBP, spread out bragg peak.

MATERIALS AND METHODS

Animals and tumors. The total of 60 female hamsters, weighting 80–120g, derived from the breeding facility at the University of Silesia, Katowice, Poland, were maintained on the standard laboratory diet with free access to drinking water. The study was performed in compliance with European policies and was approved by the Bioethics Committee for Animal Experiments (no 280/96, 384/99 and 35/2011 from April 20th 2011). A spontaneous skin melanoma, Bomirski Hamster Melanoma (Bomirski *et al.,* 1988), was used as a tumor model. Tumor implantation was performed as described earlier (Kukielczak *et al.,* 1999; Urbanska *et al.,* 2000; Romanowska-Dixon *et al.,* 2001; Romanowska-Dixon *et al.,* 2003). Briefly, tumor fragments were implanted microsurgically into the anterior chamber of the hamster eye. Six to nine days later tumors were visible, growing in the anterior chamber on the surface of the iris as well demarcated, dome-shaped single nodules. To non-invasively monitor the tumor growth in the hamster eye *in vivo* we estimated two dimensions of the tumor on the surface of the iris, applying a microscopic ruler. This approach assumes that assessment of only two dimensions in the plane of the iris reflects tumor growth adequately, as the growth in the depth dimension is limited by the anterior chamber volume. Two perpendicular diameters "a" and "b" of the tumor were measured each day and the mean tumor dimension was calculated ($\sqrt{a \times b}$) in mm. Eyeballs were enucleated when the anterior chamber was completely filled with the tumor. Thirty days after the enucleation animals were sacrificed, and internal organs were examined for metastases, clearly visible as black dots. Lungs were isolated and weighted.

Brachytherapy. Tumors were irradiated at a dose of 3, 6 and 10 Gy with a ^{106}Ru plaque (model Rue. EBB, 11.8 Mbq, BEBIG GmbH, Germany) at 0.017 Gy/min and with a ^{125}I plaque (BEBIG GmbH, Germany) at 0.012 Gy/min. All radiation exposures were preceded by injections of Vetbutal (36 mg/kg body weight). Doses were fractionated into 4 equal fractions administered every 24 hrs. The time of irradiation for a single fraction was from 62 to 294 min. ^{106}Ru emitted electrons have the energy of 3.54 MeV and ^{125}I is a source of γ-rays with the energy of 35.4 keV.

Proton beam. Protons for the irradiation experiments were accelerated using the AIC-144 isochronous cyclotron, operating at 60 MeV, which was designed and constructed at IFJ PAN (Kraków, Poland). The beam delivery system, the formation of a primary proton beam, was described by Bakewicz and Swakon (Bakewicz *et al.,* 2003; Swakon *et al.,* 2010).

The proton beam had to be specially formed for the irradiation purposes. The uniform lateral dose distribution was realized by a passive scattering with single tantalum foil (Swakon *et al.,* 2010, Michalec *et al.,* 2010). The Spread Out Bragg Peak (SOBP) was formed by a dedicated rotating modulator wheel, made of PMMA. The proton beam range was controlled by a PMMA range shifter or a set of PMMA plates with different thickness.

For the irradiation of hamster eyeballs the proton beam was configured to irradiate only the anterior chamber of the hamster eye. The proton beam has been prepared to penetrate the tissue to a depth of 3.88 mm (4.07 mm in water). The diameter of the final collimator was equal to 7 mm in order to irradiate only the eyeball area with a small margin. The transversal profiles were measured in air using a one dimensional scanner with diode and presented in the inset of Fig. 1C.

The proton beam monitoring system and the beam dosimetry. The set of ion chambers with dedicated Ergen electrometers was used for monitoring the proton beam parameters. The proton beam intensity has been measured by two PTW transmission ionization chambers type 7862. During irradiation the type 7862 ionization chambers carried out the function of a dose monitor. The time and spatial stability of the beam was controlled on-line by a six sector dedicated ion chamber, which consisted of four ion chambers in the form of quarters, a circle ion chamber and a ring one.

The PTW UNIDOS electrometer and the Markus chamber PTW type 23343 have been used to calibrate the dose. Dose measurements were performed in the middle of SOBP using the solid phantom (PMMA). Overall uncertainty of dosimetry was about 3%, the precision of dose delivery was better than 0.5%. The average dose rate of the proton beam during irradiation was between 0.07 and 0.08 Gy/s.

Hamster eye positioning and proton beam irradiation. Prior to the animal experiments, a phantom of the hamster head was used to develop the animal positioning system and hamster eye positioning. The analysis took into
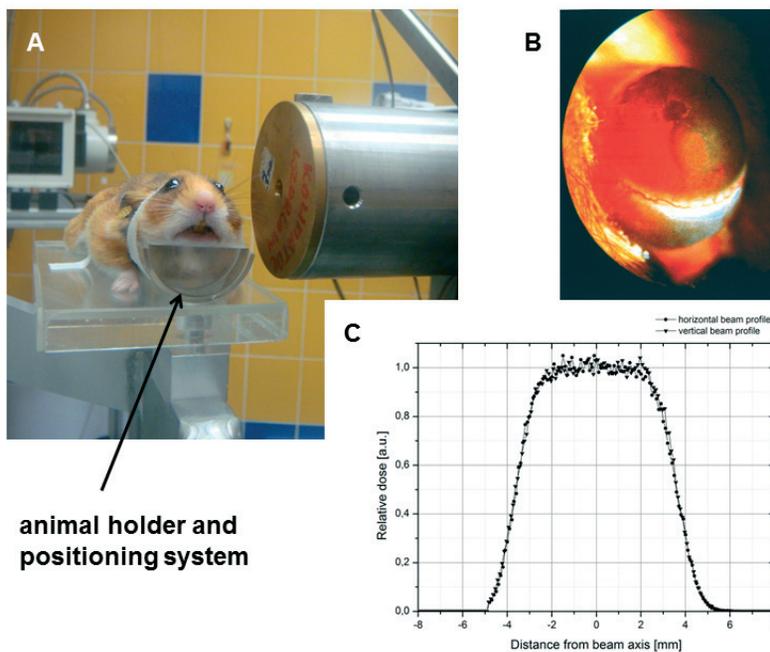

Figure 1. Hamster eye irradiation with proton beam.
(**A**) Hamster in the animal positioning system, (**B**) hamster eye with the tumor, magnification 10x and (**C**) proton beam dose depth distribution and lateral profiles of the beam used for hamster eye irradiation.
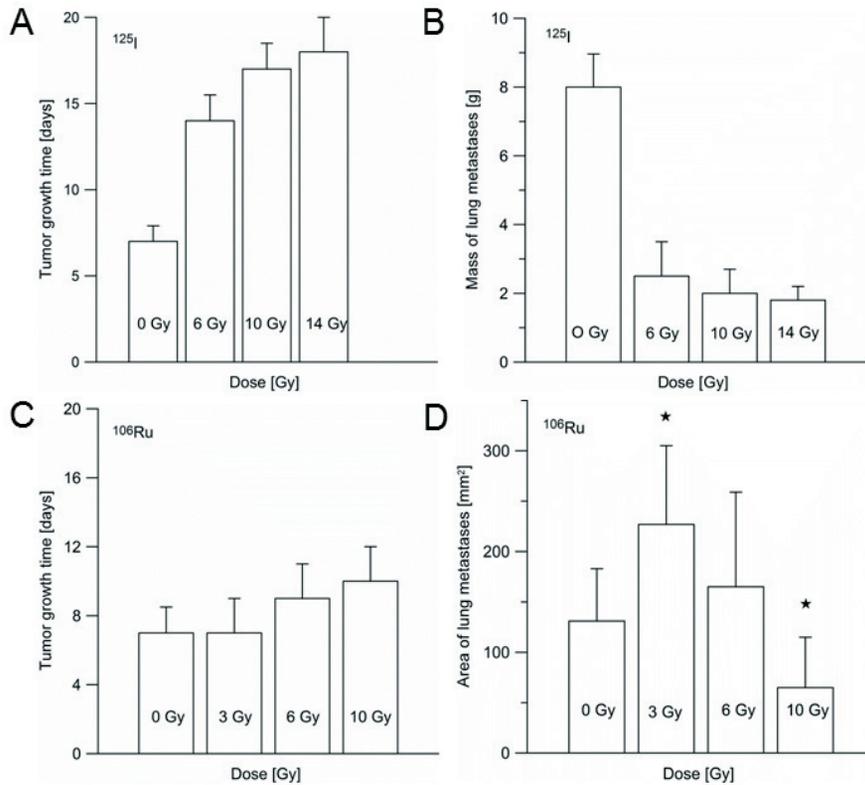
Figure 2. The effects of γ- and β-irradiation of the BHM melanoma tumor growing in the hamster eye.
(**A**) Growth inhibition of primary tumor implanted in the eye by ^{125}I irradiation. (**B**) Inhibition of lung metastasis after a fractionated dose of 6-14 Gy of ^{125}I irradiation of BHM melanoma tumors growing in the hamster eye. (**C**) ^{106}Ru slightly affected tumor growth in the eye. (**D**) The effect of fractionated doses of ^{106}Ru irradiation on lung metastasis. The experimental group consisted of 5–8 of animals. Asterisks indicate statistical significance of $p < 0.05$ in comparison with control.
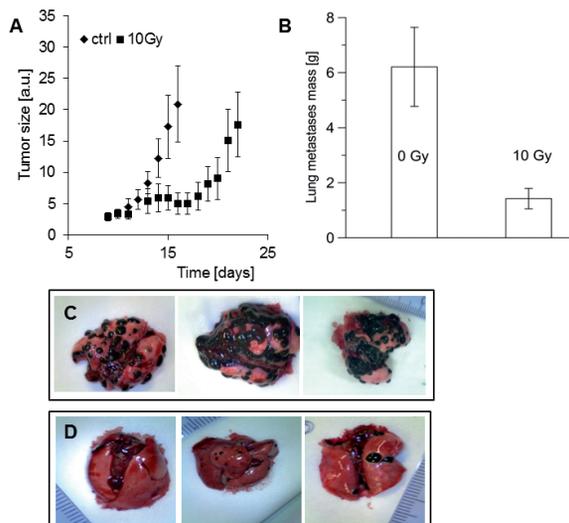

Figure 3. (A) Inhibition of BHM melanoma tumor growing in the hamster eye, irradiated with a proton beam at a single dose of 10 Gy (n = 7, black square), in comparison with untreated control (n = 6, black diamond). (B) The mass of metastases to the lung decreased 4.35 times after proton beam irradiation (10 Gy) of BHM melanoma tumor growing in the hamster eye (p = 0.0052). Average mass with SEM is shown. The number of control animals was 6, and the number of irradiated animals was 7. Representative isolated lungs with metastases from untreated (C) and irradiated (D) animals.

account the protruding shape of the hamster eye and the size of the anterior chamber. The animal positioning system consisted of an animal holder, attached to the frame of the 3D positioning system, movable at 0.01 mm steps. A laser positioning subsystem and entrance field simulation light have been used for an accurate hamster eye positioning in the proton beam.

A single dose of 10 Gy (11 CGE) has been delivered within a maximum irradiation time of 150 s. Animals were anesthetized for about 30 min, for both the positioning and irradiation, using 100 mg/kg ketamine (Bioketan, Vetoquinol, Biowet, Poland) and 10 mg/kg xylazine (Sedazin, Biowet, Poland).

Statistical analysis. The significance of the difference between the experimental groups was determined using Student t-test, with $p < 0.05$.

RESULTS AND DISCUSSION

Growth inhibition of the primary tumor

^{125}I irradiation led to inhibition of the tumor growth in the eye from 7 days in untreated animals to 14–17 days in animals irradiated with fractionated doses between 6–14 Gy (Fig. 2A). ^{106}Ru brachytherapy did not affect the primary tumor growth (data not shown). After several days, some regrowth of the primary tumor was observed. The inhibition after γ-radiation was similar to that achieved after 10 Gy of proton beam irradiation (Fig. 3B). It has to be pointed out that different radiation dose rates have been used: 0.017 Gy/min for ^{106}Ru, 0.012 Gy/min for ^{125}I and 4.2 Gy/min for proton beam irradiation. Therefore a direct comparison of the effects is not possible.

Enhancement of metastasis by very low doses of beta-radiation

Treatment with γ-irradiation strongly inhibited metastatic spread, as seen in Fig. 2B, however, it seems that the lowest dose of 3 Gy of β-radiation caused an increase in lung metastasis. The largest applied dose of 10 Gy of β-radiation led to inhibition of metastasis (Fig. 2C).

Our previous studies have shown that BHM melanoma growing in the hamster eye is heavily vascularized, and that this vascular network is highly pathological, with densely distributed, tortuous, and irregular vessels, with many sprouts and embolisms (Romanowska-Dixon *et al.*, 2001). One may speculate

that irradiation of such tissue will lead to destruction of endothelial cells, and subsequently to leakage of vasculature, thus promoting metastatic spread. One of the mechanisms involved in the endothelial cell death after radiation is activation of the ceramide-sphingomyelin pathway, although usually induced at the level of 10 Gy dose (Kolesnick & Fuks, 2003; Fuks & Kolesnick, 2005).

The clinical reports justifying the use of short-range electron emitting brachytherapy did not demonstrate any enhancement of metastasis (Lommatzsch et al., 2000). There was a suggestion, however, to treat patients with possibly low dose-rate plaques (Kleineidam & Guthoff 1994). The randomized trial of 125I brachytherapy did not find any differences in the metastasis rates (COMS 2006).

Inhibition of metastasis by proton beam irradiation of the primary tumor

A single dose of 10 Gy of proton beam irradiation delayed the growth of the BHM melanoma in the hamster eye by 10 days (Fig. 3B). Albeit the inhibition of the primary tumor growth was moderate, proton therapy strikingly reduced the mass of the metastases in the lungs in comparison with untreated animals. On average, 10 Gy of proton irradiation diminished the mass of metastases 4.35 times, even though there was a significant spread between individual animals (Fig. 3A, C, D). These results are in agreement with data for osteosarcoma published by Ogata et al. (2005). Proton ion irradiation decreased cell migration and invasion in a dose-dependent manner and strongly inhibited matrix metaloproteinase-2 activity in highly aggressive HT1080 human *fibrosarcoma* cells *in vitro* and significantly decreased the number of pulmonary metastases in mouse *osteosarcoma in vivo* (Ogata et al., 2005). Similarly, it was shown that in *in vitro* models cell properties such as adhesion, migration, invasion, and the expression level or activity of molecules related to metastasis such as $\alpha V\beta 3$, $\beta 1$ integrin, and MMP-2 were decreased after small doses of proton beam treatment (Takahashi et al., 2003).

Suppression of the growth of spontaneous metastases observed after proton beam therapy in hamsters may stem from numerous mechanisms, such as inhibition of cell motility, adhesion, and invasion, or perturbation of cell death pathways. To fully address these issues, we will continue to study these mechanisms on the cellular, molecular and submolecular level.

CONCLUSIONS

1. Small, sublethal doses of β-irradiation might increase the metastatic spread.

2. Proton beam irradiation at 10 Gy inhibited the growth of melanoma tumor in the hamster eye and diminished number of metastases in the lung more than four times.

3. The already established infrastructure (modernized AIC-144 cyclotron, the beam delivery system, the therapy room) as well as the equipment for the beam control and monitoring and the human eye positioning systems, prepared for treatment of human patients, were adapted and developed for irradiation of a very small, 4–6 mm in diameter, hamster eye.

4. The mechanisms of tumor growth inhibition after proton beam irradiation need further explanation.

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