Original Article

Metastases Development Following Local Tumour Treatment

(metastases / experimental tumour / tumour irradiation / tumour surgery / mice)

A. JURIN[1], T. JUKIĆ[2], S. IVANKOVIĆ[3], M. JURIN[3]

[1]County Hospital Čakovec, Čakovec, Croatia
[2]Faculty of Medicine Osijek, JJ Strossmayer University, Osijek, Croatia
[3]Department of Molecular Medicine, Ruđer Bošković Institute, Zagreb, Croatia

Abstract. Transplantable mouse methylcholanthrene-induced fibrosarcoma (CMC4 tumour growing in CBA/HZgr mice), characterized by lung metastases developing shortly after local tumour cell transplantation, was used as an experimental model to investigate the problem of tumour metastases after local tumour treatment. Surgery and/or irradiation were performed on locally growing tumour of particular size. Further, heavily irradiated, viable but not dividing tumour cells, imitating the situation in treated tumour-bearing organism, were injected intraperitoneally in a parallel group of treated tumour-bearing mice. The animals were killed 35 days after tumour transplantation and the number and volume of lung metastases were determined. Depending on the treatment performed, when the tumour mass was reduced or even eliminated, the number of lung metastases and their volume were significantly lower than in control mice, but the addition of tumour mass (injection of heavily irradiated tumour cells) resulted in a significant increase in lung metastases parameters, pointing to a possible role of the host's immune reaction against the tumour. Further, the release of a simple molecule, such as nitric oxide, from tumour mass seems to be detrimental for the survival of tumour cells and subsequently their metastases through the induction of angiogenesis and possible suppression of immune reaction. Thus, complex mechanisms could be involved when a locally growing tumour is exposed to a particular therapeutic approach.

Introduction

Numerous approaches have been attempted in controlling tumour growth. Some of them, such as surgery, irradiation, or chemotherapy are still main clinical approaches either alone or in particular combinations. However, the fate of distant metastases after the treatment of locally growing tumour, particularly by using surgical treatment and/or tumour irradiation, is still an open question (Baum and Badwe,1994; Demicheli, 2001; Demicheli et al., 2001; Fidler, 2002; Baum et al., 2005; Khokha and Voura, 2005). Actually, numerous clinical reports point to the problem that metastasis growth could be accelerated following local tumor treatment (Demicheli et al., 2001; Benouchan and Colombo, 2005; Khokha and Voura, 2005; Mocellin et al., 2006). It should be mentioned that the immune reactivity of a tumour-bearing organism is detrimental either for tumour growth or for host survival (Heppner and Miller, 1998; Bubenik and Šimova, 2005; Indrova et al., 2008).

This reactivity depends upon tumour mass and therapeutic approaches affecting local and/or disseminated tumour growth (Jurin and Suit, 1974; Heppner and Miller, 1998; Bubenik and Šimova, 2005; De Visser et al., 2006; Indrova et al., 2008). Further, it has been well documented that removal of the primary tumour reduces angiogenesis inhibitors, and a sharp spike in angiogenesis stimulators and growth factors is known to be involved in wound healing (Baum and Badwe, 1994; Baum et al., 2005). Thus, it is not surprising that tumour angiogenesis and its proliferation occur after (surgical) removal of locally growing tumour, and this act could be a trigger for "kick-starting" the growth of earlier established micro-metastases. Thus, the complex situation in a tumour-bearing organism could be further aggravated by anti-tumour therapy.

In this study, transplantable, methylcholanthrene-induced fibrosarcoma in mice, characterized by lung metastases developing shortly after local tumour cell transplantation, was investigated. The surgical approach and/or irradiation were performed on a locally growing tumour of a particular size. To increase tumour mass (viable, but not growing tumour), heavily irradiated tumour cells were injected intraperitoneally (ip) in a parallel

group of treated tumorous mice, which also seemed critical for the fate of metastases. The animals were killed 35 days after local tumour cell injection and the number and volume of metastatic lung nodules were determined.

Material and Methods

Animals

Male mice of the CBA/HZgr strain were used in the experiments. The animals were about three months old and weighed 20–23 g. They were provided standard diet (Mucedola, Settimo Milanese, Italy) and tap water *ad libitum*. The light regime was natural. Animals were treated according to the Animal Welfare Regulations.

Tumour

The tumour was a fibrosarcoma (CMC-4) induced in a young CBA/HZgr female mouse by an injection of methylcholanthrene (in olive oil) into the flank subcutaneous tissue. Fragments from this original tumour had been implanted in syngeneic recipients, first-generation transplant tissue was obtained and stored in liquid nitrogen. By injecting these fragments into recipient mice, second-generation transplant tissue was obtained. Thus, first- to fourth-generation transplant tissue was obtained and kept in liquid nitrogen. For this experiment, fourth--generation transplant tissue fragments were implanted into recipient-source mice. Freshly excised tumours from these source mice comprised fifth-generation transplant tissue, and were used in the study.

Tumour cell suspension

Large intact pieces of the tumour, removed from the source mice, were minced and the mash was poured into a beaker containing cold Hank's solution and mixed with a 10 cm³ syringe. The beaker was placed on ice to allow large particles to settle. The supernatant was removed 5 min later using a syringe with 22-gauge needle and passed through Swinney filter (Millipore, Billerica, MA) into test tubes. The specimen was spun down at 150 g for 10 min, the supernatant was discarded and the pellet resuspended. All visible clumps were removed using a Pasteur pipette so that cells were dispersed as a single-cell suspension. The viability was tested by Trypan blue exclusion: > 90 % of tumour cells were scored as viable.

Tumour cell inoculation

CBA/HZgr mice were subcutaneously injected into the right thigh with 5×10^5 CMC-4 fibrosarcoma cells in 100 mm³ RPMI using a tuberculin syringe and a 25-gauge needle. Ten days later, when the tumours were 8 mm in diameter, particular tumour treatments were performed, i.e. irradiation or/and surgical removal.

The treatment of the mice with growing tumour

Tumour growth dynamics was followed by measuring three orthogonal tumour diameters (a,b,c) with a caliper (Lange Skin fold Caliper, Cambridge Scientific Industry, Cambridge, MD) starting on day 10 after inoculation, and subsequently on every 2nd or 3rd day until day 35. Tumour volume was calculated by using the formula a×b×c×0.526. The relative tumour volume (RTV) was calculated based on the results of the measurements. The formula for calculating RTV was as follows: RTV = V_t/V_0. V_0 represents the tumour volume when the particular tumour treatment was applied to the mice. V_t represents the tumour volume each time a measurement was taken.

Tumours were irradiated with 10, 30, and 60 Gy, respectively, when they had reached about 8 mm in diameter. Mouse right thigh (with growing tumour) was irradiated by using linear accelerator Varian Clinac 1800 (OSI, Lakewood, NJ) with a 20 MeV electron beam, dose rate 2 Gy/min at room temperature. The other leg was protected with lead blocks. Homogeneous tumour irradiation was obtained by using 1 cm bolus. Surgical removal of the growing tumour was performed on anaesthetized (chlorethyl 25 mg/kg) mice. Shaved skin was opened with scissors, tumour totally removed and skin closed with sutures. After local tumour treatment the mice of particular groups were ip injected with four doses of 2×10^7 heavily irradiated (100 Gy) tumour cells on days 16, 20, 24, and 28 after tumour transplantation.

All the mice were killed on 35th day after tumour transplantation. The lungs were removed, washed in water, dissected in lobes and placed in Bouin's fixative (70% saturated picric acid, 24% formaldehyde and 6% glacial acetic acid). The number and the size of tumour nodules on lung surfaces were determined under the optical system (magnification 10×) with a scale in the ocular.

Statistical analysis

The differences between effects of particular treatments were statistically evaluated by an unpaired Student's *t*-test. This test was performed using a standard statistical package, STATISTICA for Windows.

Results

CBA/HZgr mice were injected with CMC-4 fibrosarcoma cells into the right thigh.

It should be noted that locally growing fibrosarcoma, used in these experiments, metastasized in the lungs rather early. Ten mice in a separate group (not presented in this study) had been killed 10 days after local tumour transplantation (the growing tumour was about 8 mm in diameter); tumour nodules were visible (by naked eye) in three of them and, under the microscope, in the lungs of all other animals. When the tumour had reached 8 mm in diameter, local irradiation (10, 30, or 60 Gy, respectively) or surgical removal of the tumour was performed.

The growing tumour was irradiated with 10 Gy in one of the experimental groups 24 h prior to the surgical approach. Additionally, separate groups of the above-treated mice were injected ip with heavily irradiated (100

Table 1. The treatment of CBA/HZgr mice following transplantation of viable CMC-4 fibrosarcoma cells into the right thigh

Group	The treatment on a particular day						
	1	10	16	20	24	28	35
1	tumour transplanted						killed
2	tumour transplanted	irradiation 10 Gy					killed
3	tumour transplanted	irradiation 30 Gy					killed
4	tumour transplanted	irradiation 60 Gy					killed
5	tumour transplanted	surgical removal					killed
6	tumour transplanted	irradiation 10 Gy + surgical removal					killed
7	tumour transplanted		100 Gy irradiated cells ip				killed
8	tumour transplanted	irradiation 10 Gy	100 Gy irradiated cells ip				killed
9	tumour transplanted	irradiation 30 Gy	100 Gy irradiated cells ip				killed
10	tumour transplanted	surgical removal	100 Gy irradiated cells ip				killed
11	tumour transplanted	irradiation 10 Gy + surgical removal	100 Gy irradiated cells ip				killed
12			100 Gy irradiated cells ip				killed

Gy) tumour cells. Local tumour growth was observed until 35th day after tumour transplantation into the thigh, when the mice were killed and the number as well as the volume of lung metastases were determined. All experimental groups are presented in Table 1.

Local tumour irradiation

As presented in Fig. 1, tumour regression followed local irradiation regardless of the dose applied. However, tumour re-growth occurred after about 10 days following 10 or 30 Gy dose, but the irradiated tumour volume was significantly ($P < 0.01$) smaller than in controls until the end of the experiment. At that time there was no any visible tumour if the 60 Gy dose had been applied. In a separate group of five mice with transplanted tumour, irradiated locally with 60 Gy, there was no local tumour re-growth even after 150 days (not shown here).

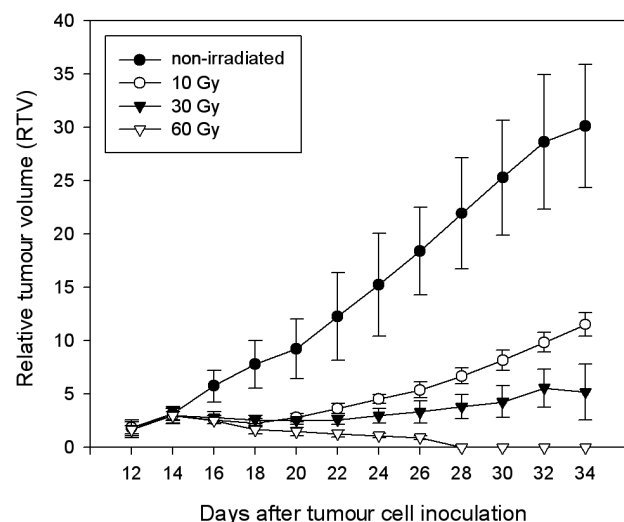All the mice were killed 35 days after tumour transplantation and the number as well as the volume of lung metastases was determined and results presented in Table 2. Tumour metastases were noticed in all control mice and in those locally irradiated with 10 Gy (Groups 1 and 2). The nodule incidence and total volume were lower in local tumour-irradiated mice than in the control group but the differences were not significant. Further, when the local tumour was irradiated with 30 Gy, the number of lung nodules was significantly lower ($P < 0.01$) as well as the volume ($P < 0.05$) than in controls (Groups 1 and 3) and 1/10 mice was even without visible lung metastases. Following 60 Gy local irradiation only 2/10 animals had lung metastases and the nodule number and volume were significantly lower ($P < 0.01$) than in the controls (Groups 4 and 1).

Surgical removal of growing tumour

There was no tumour re-growth after the surgery until the end of the observation period.

As presented in Table 2, only 2/10 mice were without tumour nodules in the lungs. The number of nodules was significantly lower ($P < 0.01$) than in controls (Groups 1 and 5) but significantly higher ($P < 0.05$) than in 60 Gy tumour-irradiated group (Groups 5 and 4). Further, metastasis volume was significantly ($P < 0.05$) lower than in the control group but significantly ($P < 0.05$) higher than in the 60 Gy locally irradiated group.

Local tumour irradiation prior to surgery

The local tumour had been irradiated with 10 Gy and surgical removal was performed the following day. There was no local re-growth until the end of the observation period. As presented in Table 2 (Group 6), all the mice developed lung metastases. The number of lung nodules and their volume were significantly ($P < 0.01$) higher than in the group without previous local irradiation, i.e. surgery only (Groups 6 and 5). There was no significant difference in comparison to either control mice or those locally irradiated with 10 Gy.

Fig. 1. The growth of CMC-4 fibrosarcoma transplanted in CBA/HZgr mice following local tumour irradiation.

Table 2. The incidence of lung metastases (nodule number and volume) in CBA/HZgr mice with the growing tumour locally exposed to irradiation and/or surgery when the tumour had reached 8 mm in diameter. The mice were killed 35 days following injection of CMC-4 fibrosarcoma cells into the right thigh

Group	Mice with metastases Total number of mice	Number of lung metastases			Metastases volume (mm^3)
		Incidence	Mean	Median	Mean ± S.E.
1	10/10	4×5, 3×6, 7, 8, 9	6.3	6.0	14.05 ± 3.53
2	10/10	2×2, 2×3, 4×4, 6, 11	4.3	4.0	8.78 ± 3.28
3	9/10	0, 1, 2×3, 3×2, 5×4	2.7	3.0	3.99 ± 0.99
4	2/10	8×0, 1, 3	0.4	0.0	0.16 ± 0.14
5	8/10	2×0, 2, 3×3, 4×4	2.7	3.0	5.85 ± 1.78
6	10/10	4, 3×5, 6, 7, 8, 9, 10, 11	7.0	6.5	11.18 ± 3.25
7	10/10	4, 2×5, 2×6, 7, 2×8, 2×10	6.9	6.5	15.02 ± 3.92
8	10/10	4×2, 2×3, 3×5, 8	3.7	3.0	16.49 ± 4.51
9	10/10	4×3, 2×4, 4×9	5.6	4.0	7.83 ± 3.32
10	10/10	2×5, 3×6, 4×8, 10	7.0	7.0	16.55 ± 4.82
11	10/10	2, 2×3, 4, 2×5, 7, 2×8, 9	5.4	5.0	25.78 ± 5.60
12	0/10	0	0.0	0.0	0 ± 0

Addition of heavily irradiated tumour cells ip

Four doses (2×10^7 cells each) of heavily irradiated (100 Gy) tumour cells were injected ip (this represented tumour mass in the organism out of therapeutic approaches) in separate groups of mice treated as above. There was no influence on the growth of untreated tumour (Fig. 2), but the re-growth of irradiated tumours (10 and 30 Gy) was significantly ($P < 0.05$) stimulated if additional irradiated tumour cells were injected (Figs 3 and 4). It should be noted that normal mice injected ip with these tumour cells did not develop any tumour – local or metastases (Group 12, Table 2). Further, the number of tumour lung nodules and their volume was the same in the control group and in ip-injected mice if local tumour was not treated (Groups 1 and 7, Table 2).

However, local tumour treatment influenced the incidence and the volume of tumour lung metastases in the mice additionally injected with heavily irradiated tumour cells. As presented in Table 2, additional tumour ip injection did not influence the number of metastases when the tumour was irradiated with 10 Gy (Groups 2 and 8) but the volume was significantly ($P < 0.05$) higher in the mice from group 8. Further, when local irradiation with 30 Gy or surgical eradication were performed, the number and volume of the metastases significantly ($P < 0.05$) increased in the mice injected additionally with tumour cells (Groups 3 and 5 versus 9 and 10, Table 2). Finally, when combined local tumour treatment (irradiation with 10 Gy followed by surgery) was performed, the addition of tumour cells ip significantly ($P <$
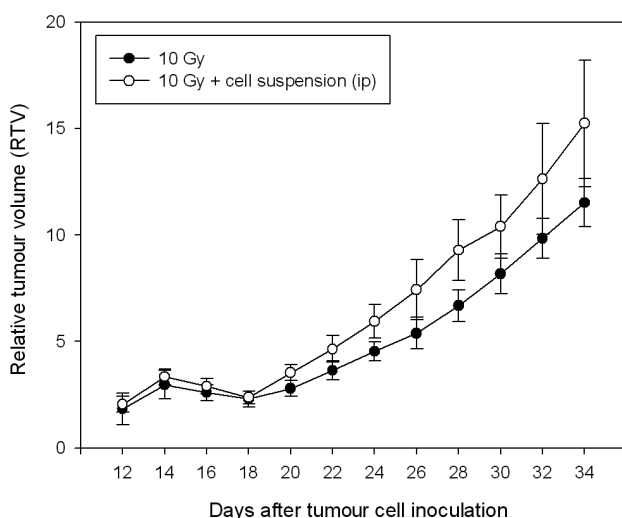

Fig. 3. The growth of CMC-4 fibrosarcoma transplanted in CBA/HZgr mice following tumour irradiation with 10 Gy without any additional treatment, or in tumour-irradiated group injected ip with 100 Gy irradiated tumour cells on days 16, 20, 24 and 28 after local tumour transplantation
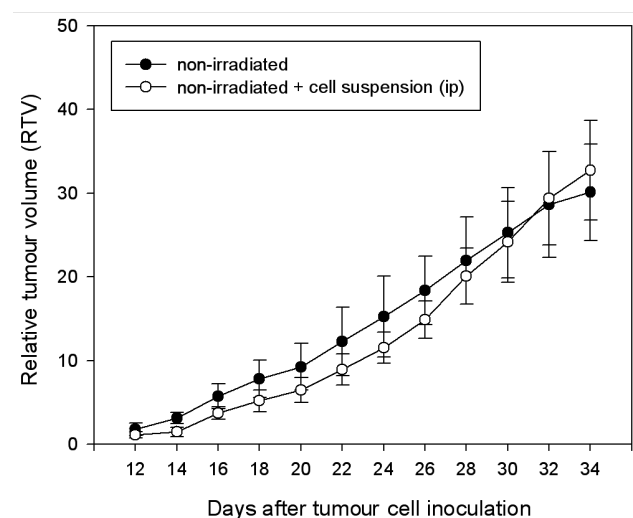

Fig. 2. The growth of CMC-4 fibrosarcoma transplanted in CBA/HZgr mice without any additional treatment, or in the group injected ip with 100 Gy irradiated tumour cells on days 16, 20, 24 and 28 after local tumour transplantation
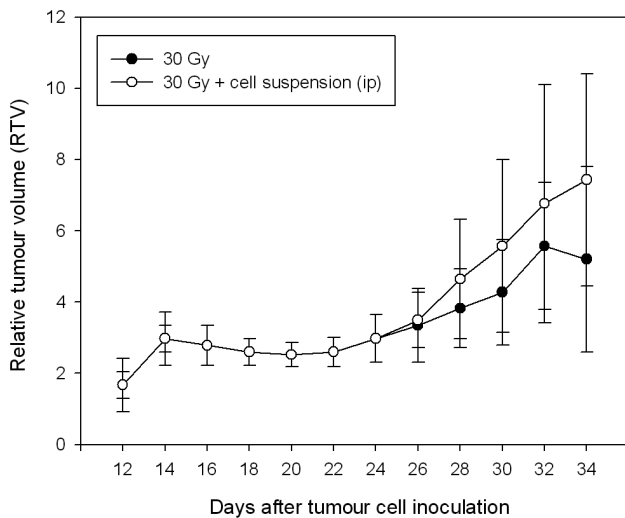
Fig. 4. The growth of CMC-4 fibrosarcoma transplanted in CBA/HZgr mice following tumour irradiation with 30 Gy without any additional treatment, or in tumour-irradiated group injected ip with 100 Gy irradiated tumour cells on days 16, 20, 24 and 28 after local tumour transplantation

0.01) increased the metastasis volume but not the number of lung nodules (Groups 6 and 11, Table 2).

Discussion

As presented above, the number and the volume of spontaneous tumour metastases in the lungs of tumour-bearing mice depended on the treatment of locally growing tumour. Keeping in mind that the animals at the time of local tumour treatment already developed metastases, it is of particular interest that local irradiation with 60 Gy, besides complete elimination of local tumour, significantly reduced lung metastases. Further, the mice sacrificed 150 days after this local tumour treatment were without local tumour and, which is particularly important, without its metastases. However, surgical elimination of locally growing tumour was not so effective as local tumour irradiation in reducing lung metastases. An increase in tumour mass, by injecting irradiated tumour cells ip, could be detrimental to the host in controlling lung metastases. We propose that the immune reactivity of the host against the tumour and the dynamics of vascular network development in growing tumour tissue could provide a valuable possible explanation.

Actually, despite improvements in diagnosis, therapeutic techniques, patient care, and adjuvant therapies, most deaths from cancer are due to metastases that are resistant to conventional therapies (Fidler, 1990). Several reasons account for the failure to treat metastases. Tumours are biologically heterogeneous with subpopulations of cells with different invasive, metastatic and angiogenic properties (Davies et al., 2002). Further, the process of metastasis affects a small subpopulation of cells pre-existing in original, locally growing, tumour (Fidler 2002). Finally, the outcome of metastasis de-

pends on mutual interactions of metastatic cells with host's homeostatic mechanisms (Rofstad et al., 2006). Angiogenesis in metastases could be a perfect example. The onset of angiogenesis involves a change in the local equilibrium between pro-angiogenic and anti-angiogenic molecules (Fidler, 1994). During the interaction of metastatic cells with host tissues, signals from autocrine, paracrine, or endocrine pathways influence tumour cell proliferation with the growth dependent on the net balance of positive and negative signals (Stiles et al., 1979). Further, autocrine or paracrine growth factors controlling organ repair and regeneration may also affect the proliferation of tumour cells. Accordingly, human renal carcinoma cells established as micrometastases in the lungs of nude mice underwent a significant growth acceleration subsequent to unilateral nephrectomy but not partial hepatectomy; human colon cancer cells implanted subcutaneously demonstrated accelerated growth in partially hepatectomized but not in unilaterally nephrectomized mice (Gutman et al., 1995). These results indicate that metastatic cells can respond to physiological signals produced when homeostasis is disturbed and that tumour cells either originating from or having an affinity for growth in a particular organ can also respond to these physiological signals (Fidler, 2002).

We presented in this study that the number of lung metastases and their volume were significantly lower than in control mice when tumour mass was reduced or even eliminated by local irradiation, pointing to a possible role of host's immune reaction against the tumour as one of possible explanations. The presence of tumour mass is detrimental for immune reaction against the tumour (Jurin and Suit, 1974; Heppner and Miller, 1998; De Visser et al., 2006). The increase in tumour mass by injecting heavily irradiated tumour cells ip (viable but not dividing tumour cells) in locally treated tumour-bearing mice resulted in a significant increase in lung metastasis parameters. It is generally accepted that tumour cells might disseminate from a locally growing tumour, but the establishment of metastases by tumour cells appears to be a very inefficient process, i.e. the growth of metastases is well controlled (Good et al., 1990; Holmgren et al., 1995; Heppner and Miller, 1998; Orr et al., 2000; Egeblad and Werb, 2002; Khokha and Voura, 2005). Tumour cells from the primary tumour are seeded in particular organs and a good balance between growth and apoptosis is present in these dormant micrometastases (Folkman, 1995; Baum et al., 2005).

The regulation of vascular network development seems to be crucial in metastasis growth. This is one of possible reasons which should be studied. A number of factors are known to inhibit angiogenesis and others that stimulate it. Inhibiting factors dominate locally to maintain a dormant state which can no longer be maintained when stimulating factors are increased or inhibiting factors are reduced. To conclude, depending on the treatment performed, when tumour mass was reduced or even eliminated, the number of lung metastases and their volume were significantly lower than in control

mice, but the addition of tumour mass (viable but not growing tumour) resulted in a significant increase in lung metastases parameters, pointing to a possible role of the host's immune reaction against the tumour. Further, the release of a simple molecule, such as nitric oxide, from tumour mass seems to be detrimental for the survival of tumour cells and subsequently their metastases through the induction of angiogenesis and possible suppression of immune reaction (Aleksandrova et al., 2001; Wai et al., 2006). Thus, complex mechanisms could be involved when a locally growing tumour is exposed to a particular therapeutical approach (Mikyškova et al., 2004; Bubenik and Šimova, 2005).

Acknowledgement

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