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Comparison of Metastatic Disease After Local Tumour Treatment with Radiotherapy or Surgery in Various Tumour Models*

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Abstract—*Spontaneous metastases in lymph nodes and/or the lung were obtained after tumour cell inoculation of four mouse tumours and one rat tumour into the foot-pads of syngeneic animals or their F_1 hybrids. Following local radiotherapy with doses of* 45–80 Gy, *significantly more mice died with metastases than following local amputation of the tumour-bearing foot when the* 2661 *carcinoma was involved. No significant difference was observed after these treatments for the other tumours. The enhancement of metastastic growth after local radiotherapy in the* 2661 *carcinoma seems not to be due to incomplete killing of tumour cells in the foot. The presence of irradiated normal structures and tumour tissue after radiotherapy promoted the outgrowth of* 2661 *carcinoma cells which were outside the radiation field at the time of treatment. Evidently, even under similar experimental conditions, radiotherapy may enhance the growth of metastases from some tumours and not from others.*

INTRODUCTION

THERE is controversy over the influence of the treatment of mammary carcinoma with radiotherapy on the expression of metastatic disease [1]. Preliminary findings suggested that a larger number of mice died with evidence of metastatic disease when the 2661 carcinoma transplanted in the foot pad was treated with radiotherapy than was found following surgery. This led us to study this phenomenon in more detail.

Increase in metastases has been shown in other experimental models after radiotherapy, but a reduction as well as no effect have also been described [2–11]. The validity of extrapolation of experimental data to the clinical situation is very limited due to the unavoidable wide difference in dose–time relationships dependent on the high tumour growth rate and early dissemination in most experimental systems. Nevertheless, it appeared useful to determine the mechanism(s) responsible for the detrimental effect of radiotherapy on the 2661 carcinoma.

Studies on this subject are presented in this report. In addition, the effects of radiotherapy and surgery were compared in other metastasizing tumours employing similar procedures to those used for the 2661 carcinoma.

MATERIALS AND METHODS

Tumours and their hosts

One rat and four mouse tumours were used in this study. The well-known Lewis lung carcinoma was transplanted in (C57BL/Rij × CBA/Rij) F_1 hybrid mice [12]. All other tumours tested originated spontaneously at Rijswijk. The mouse tumours were transplanted in their host of origin and the rat tumour in its F_1 hybrid. The 2661 carcinoma [13] and the 3641 mammary carcinoma were transplanted in CBA/Rij mice. The C22LR osteosarcoma [14] was transplanted in (C57BL/Rij × CBA/Rij) F_1 hybrid mice and the R1 rhabdomyosarcoma [15] in (Wag/Rij × Bn/Bi/Rij) F_1 hybrid rats. Tumour cell suspensions were prepared by the trypsinization method described by Reinhold [16]. With the exception of the Lewis lung carcinoma, these

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tumours had never given evidence of a metastasizing capacity when transplanted into the flank of the host. However, metastases developed if they were transplanted into the foot pad and removed at a suitable time afterwards, as has been previously observed for other tumours[13]. Animals were inoculated with $2–4 \times 10^5$ tumour cells in 0.01–0.02 ml Hanks' BSS supplemented with 5% calf serum. For the animals dying with evidence of metastatic disease, Table 1 shows the distribution of metastases in either lymph nodes, lungs or both organs, as found on gross examination at necropsy for each of the investigated tumours. It is evident that differences in localization of metastases occur for the various tumours. No local recurrences or macroscopic metastases were observed in other organs. The effect of treatment of the foot-pad tumours on the expression of metastatic disease is evaluated by consideration of the percentage of animals dying with metastases and the time of death. Animals which were alive at 120 days after treatment were assumed to be tumour-free, except for the 3641 mammary carcinoma, which was followed for 150 days.

Local treatment of foot-pad tumours

This was performed at a suitable size of the tumour. The mean tumour size in each experiment varied between 50 and 250 mm^3, except for the experiments with the Lewis lung carcinoma, for which the size varied between 100 and 500 mm^3. At this size the tumour was still located below the ankle joint, and surgical removal at the knee joint could be performed without local recurrences. A similar area, the tumour-bearing leg up to the knee joint, was treated with radiation. For both surgery and local radiotherapy the mice and rats were anaesthesized with Pentobarbital.

Irradiation

This was carried out with a Philips–Müller X-ray generator (300 kVp, 10 mA, HVL 3 mm Cu). For local foot-pad irradiation, the dose rates were 3.5 Gy/min for the mouse and 2.6 Gy/min for the rat. During irradiation of the hind foot, the remainder of the body was protected by lead shields. The mean scattered radiation dose to the body of the animals was approximately 1% of the local dose for the mice [17] and less than 0.5% for the rats. The dose to the regional lymph nodes in the mice was approximately 3% of the local dose to the foot. Whole-body irradiation was accomplished with a [^{137}Cs] source at a dose rate of 1.15 Gy/min in unanaesthesized mice.

Cell survival assay

Tumour cell suspensions were prepared from a pool of five identically treated tumours which were excised directly after various doses of whole-body irradiation. The cell survival assay used is similar to the one described by Kallman *et al.*[18]. This technique involves titration of tumour cells by s.c. inoculation of graded tumour cell numbers subcutaneously at four sites in isogenic recipient mice; 250,000 heavily irradiated cells (100 Gy) were always added to each inoculum. The tumour incidence was scored for up to 120 days after tumour cell inoculation. Evaluation of cell survival was made on the basis of the number of cells needed to produce tumours in 50% of the inoculation sites in treated tumours vs control tumours.

Statistical evaluation

Comparison of the percentage of animals dying with metastases with their survival times among various groups was performed by the log-rank test[19], except for the results in Fig. 2. These results were compared with the chi-square test. The slope of the cell survival curve was calculated by least squares analysis fitting the experimental points. The results of the

Table 1. Distribution of metastases in animals autopsied after death from metastatic disease

Tumour	Percentage of animals with metastases in: Lymph nodes + lung	Lymph nodes only	Lung only	No. of animals autopsied
2661 Carcinoma	66	27	7	15
Lewis lung carcinoma	12		88	17
C22LR Osteosarcoma	43		57	30
3641 Mammary carcinoma	38	38	25	16
R1 Rhabdomyosarcoma	87	13		16

endpoint dilution studies were calculated by probit analysis.

RESULTS

Estimation of the curative radiation dose for the 2661 *carcinoma*

For comparison of the incidence of metastases after total eradication of the primary tumour by surgery or radiotherapy, it is necessary to know the radiation dose which will cure the local tumour. For that reason, a cell survival assay was performed after whole-body irradiation of tumour-bearing mice. To get an impression of the presence of hypoxic cells in the tumour, a similar assay was performed in mice which had been killed with nitrogen just before irradiation. The cell survival curves are presented in Fig. 1. The slopes of the two curves are similar, indicating that the survival of tumour cells at doses over 10 Gy is largely determined by the hypoxic subpopulation in the tumour. On the basis of these experimental data, and assuming that 10^9 clonogenic tumour cells are present per gram of tumour, it can be calculated that radiation doses of 60, 70 or 80 Gy can cure 99% of tumours with tumour masses of 0.3, 22 and 1675 g respectively. Extrapolation from results on cell survival to tumour cure data has been shown to be a valid method for a rat rhabdomyosarcoma [20].

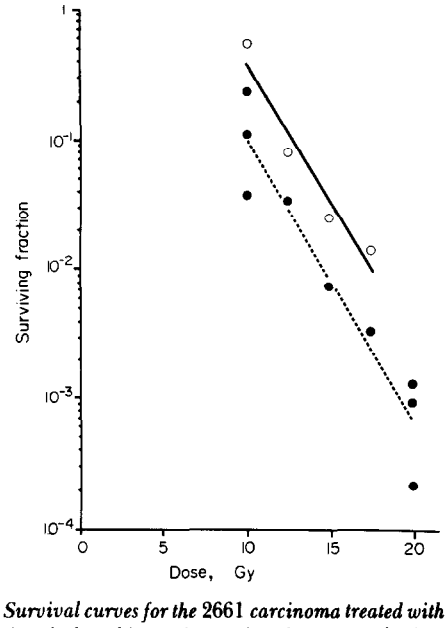

Fig. 1. Survival curves for the 2661 *carcinoma treated with X-rays in situ in air-breathing mice or in nitrogen-asphyxiated mice.* —○— *Cells exposed in dead mice* $D_0 = 2.00$ Gy; n = 50; -●- *cells exposed in living mice* $D_0 = 2.05$ Gy; n = 14.

Comparison of the effects of local surgery or radiotherapy on survival of mice

Mice bearing a foot tumour of the 2661 carcinoma were either amputated or treated locally with various doses of X-rays. The number of mice dying with metastases is presented in Fig. 2. In all irradiated groups, a significantly higher percentage of mice died with metastases than in the amputated group. No indication was found for a more pronounced negative effect in either the lower or the higher dose ranges.

Figure 3 shows the time of death in the group treated with either surgery or 80 Gy of X-rays. It is evident that not only the number of mice which died with metastases is influenced, but also the mean time of death is significantly earlier in the irradiated group than in the amputated group.

Tumour growth in pre-irradiated legs

To study whether tumour growth was altered in an irradiated leg, normal viable 2661 carcinoma cells were inoculated into the foot-pad of control mice or mice in which this leg was pre-irradiated. The results were evaluated for growth of the tumour in the inoculated foot or as metastases. The results in Table 2 show that foot tumours were observed after inoculation of 625 cells and higher numbers in control mice. With these cell doses, almost no tumours were detected macroscopically in mice which had been pre-irradiated with 70 Gy either one or seven days before inoculation of tumour cells. Only one tumour developed in the group irradiated one day before inoculation with 125 tumour cells. Although almost no local tumours developed in the feet of irradiated mice, a large percentage of these mice died with metastases. The TD_{50} for metastases is even significantly lower if the foot is irradiated one day before tumour cell inoculation.

Inoculation of viable cells of the Lewis lung carcinoma into control mice and mice in which the inoculated leg was irradiated with 70 Gy one day before tumour inoculation resulted in tumour growth in both control and irradiated legs. The TD_{50} for control and irradiated mice was 3138 and 8265 respectively, which were not significantly different. No mice died with metastases in the absence of a tumour in the foot-pad.

Radiotherapy followed by surgery in the 2661 *carcinoma*

To investigate whether the increase in metastases after radiotherapy also occurs if the

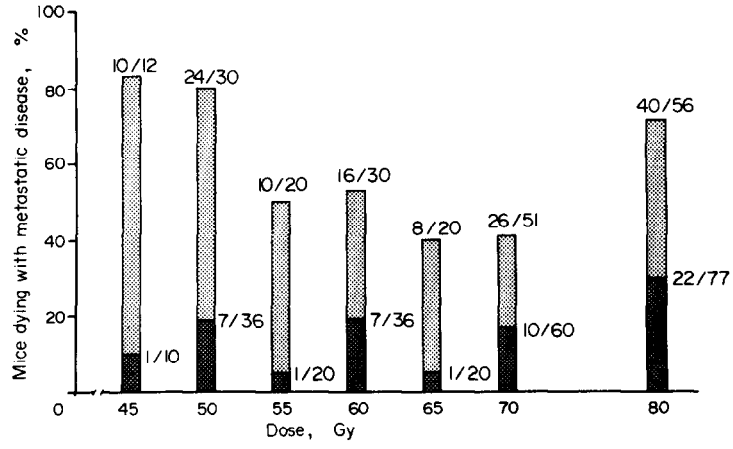

Fig. 2. Number of mice dying with metastases from the 2661 carcinoma. Local therapy was given with various doses of X-rays or amputation at 7–10 days after tumour cell inoculation. Each set of blocks represents the results of surgery (dark blocks) performed in experiments carried out simultaneously with treatment with a certain dose of radiation (light blocks). A significantly larger number of mice died with metastases after all doses of radiation than was found following amputation.

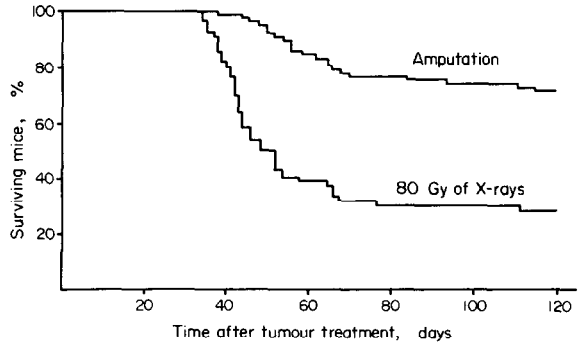

Fig. 3. The survival curves of mice treated by amputation or with 80 Gy of X-rays to a 2661 carcinoma transplanted into the foot-pad. All mice died with metastases without macroscopic evidence of local regrowth in the foot pad.

irradiated leg is removed shortly after irradiation, tumour-bearing mice received local radiotherapy on the tumour-bearing leg followed one hour later by amputation of the irradiated leg. The results, as presented in Table 3, group 1C, show that amputation abrogates the enhancement of metastatic growth after local radiotherapy. The combination of the two treatments is even better than amputation alone, although the difference is not significant.

Experiments to investigate the mechanism(s) responsible for enhancement of metastatic growth after radiotherapy in the 2661 carcinoma

The only visible difference between radiotherapy followed by amputation of the irradiated leg and radiotherapy alone is that, in the latter case, the mice remain alive with an irradiated tumour-bearing leg. This might be of prime importance for the outgrowth of tumour cells outside the field of treatment. For

Table 2. Growth of 2661 carcinoma cells in control and pre-irradiated legs and development of metastases

Number of inoculated cells	Control		70 Gy on day −7		70 Gy on day −1	
	Foot tumour	Metastases	Foot tumour	Metastases	Foot tumour	Metastases
1	0/5	0/5	0/5	0/5	0/5	1/5
5	0/3	0/3	0/5	0/5	0/5	0/5
25	0/5	0/5	0/4	0/4	0/5	3/5
125	0/5	0/5	0/5	1/5	1/5	3/5
625	2/5	2/5	0/4	3/4	0/5	4/5
3125	5/5	5/5	0/4	3/4	0/4	3/4
15625	4/5	3/3	0/5	5/5	0/5	5/5
TD_{50}*	1100	666	> 15625	471	> 15625	54
95% limits	413–2926	574–772		179–1238		7–396

*Number of tumour cells required to obtain tumour growth in 50% of the inoculated mice.

that reason, the question was raised of whether the increased incidence of metastases could be related to (a) the high local radiation dose, which results in very pronounced normal tissue damage; (b) the presence of irradiated tumour tissue.

To investigate the first possibility, the local radiotherapy was applied not to the tumour-bearing leg but to the other hind leg, while the tumour-bearing leg was removed by amputation. The data shown in Table 3 indicate that irradiation of the other leg (group 2C) increased the number of mice dying with metastases, although not significantly. To study the second possibility, the tumour-bearing legs of mice were amputated and an irradiated tumour was subsequently implanted within the amputation wound. The implanted tumours were flank tumours of the same stage of development as the foot-pad tumours which were removed from donor mice and irradiated *in vitro* with 100 Gy of X-rays. As shown in Table 3, the presence of irradiated tumour tissue within the amputation wound (group 3C) did not change the number of mice dying with metastases. A significant increase in that number is observed when both situations mentioned above concurred [an irradiated tumour-free hind leg and an irradiated tumour implanted within the amputation wound (group 3D)]. However, the increase is not as great as that observed after local irradiation of the tumour-bearing leg (group 3B).

During irradiation of the tumour-bearing leg, shielded parts of the body receive some scattered radiation. Since no negative effect of radiotherapy was observed if it was followed by surgery, it was originally concluded that the scattered radiation was not responsible for any enhancement of metastatic growth after radiotherapy. Since it now appears that a combination of factors may account for the negative effect of local radiotherapy, the combination of scattered radiation and the presence of irradiated tumour tissue might also be responsible for the increase in metastases formation. To study this possibility, mice were subjected to 0.8 Gy of whole-body irradiation with gamma rays (the approximate dose of scattered radiation), while the tumour-bearing leg was removed by amputation. In one group, an irradiated tumour was subsequently transplanted within the amputation wound. The results in Table 4 show that these treatments did not influence the number of mice dying with metastases.

Comparison of surgery and radiotherapy in other tumours

The comparison of surgery and radiotherapy was also made for a number of other metastasizing tumours. The results are presented in Table 5. No significant differences in the percentage of animals dying with evidence of metastatic disease or in the time of death was observed after treatment of the foot tumours by amputation or by irradiation. No local recurrences were observed after either treatment modality.

DISCUSSION

This study clearly demonstrates that a much larger number of mice died with metastatic disease when the 2661 carcinoma in the feet of mice was locally treated with radiotherapy than with surgery. However, a similar comparison of radiotherapy and surgery in four other tumour models tested did not reveal a difference in the expression of metastatic disease for either treatment modality. Since it seemed of importance to determine the mechanism(s) responsible for the enhancement of metastatic growth after radiotherapy in the 2661 carcinoma, the involvement of various factors are successively discussed.

The most obvious mechanism to explain the enhancement of metastatic growth after radiotherapy of the 2661 carcinoma would be incomplete eradication of tumour cells in the primary tumour, which might result in migration of tumour cells out of the tumour. No direct evidence was obtained to show that the enhancement of metastatic growth was due to incomplete killing of tumour cells in the foot tumour. This was the result of the apparent inability of viable 2661 carcinoma cells to grow in an irradiated leg (Table 2). However, if the radiosensitivity of the 2661 carcinoma is compared with three other tumours tested, it is evident that the 2661 carcinoma is the most sensitive. Although its survival curve has the highest extrapolation number, it exhibits the lowest D_0 (Table 6). Calculation of the surviving fraction of tumour cells after a dose of 70 Gy of X-rays by the use of these values results in lower cell survival for the 2661 carcinoma by a factor of 10^3–10^4 than for the other two mouse tumours. The fact that enhancement of metastatic growth after radiotherapy is not observed with these two less radiosensitive mouse tumours gives indirect evidence for the conclusion that it seems very unlikely that the enhancement of

Table 3. Percentage of mice dying with metastases of the 2661 carcinoma after various treatments

Series	Group	Treatment of tumour-bearing leg	Treatment of tumour-free leg	Incidence*	Percentage of deaths	Difference with amputated group
1	1A	Amputation		39/85; 54(15–111)	46	
	1B	70 Gy X-rays		61/82; 42(23–102)	74	$P < 0.001$
	1C	70 Gy X-rays + amputation		28/90; 51(16–108)	31	n.s.
2	2A	Amputation	— —	27/79; 68(23–111)	34	
	2B	70 Gy X-rays	— —	62/77; 51(20–102)	81	$P < 0.001$
	2C	Amputation	70 Gy X-rays	39/86; 47(20–115)	45	n.s.
3	3A	Amputation	— —	23/79; 69(23–111)	29	
	3B	70 Gy X-rays	— —	59/74; 47(26–102)	80	$P < 0.001$
	3C	Amputation + irradiated tumour	— —	23/72; 61(36–120)	32	n.s.
	3D	Amputation + irradiated tumour	70 Gy X-rays	57/114; 47(17–114)	50	$P < 0.01$

*Mice dying with metastases/total number of mice observed; median survival time in days after treatment (range).

Table 4. Percentage of mice dying with metastases of the 2661 carcinoma after various treatments

Treatment of tumour-bearing leg	Total body gamma irradiation	Incidence*	Percentage of deaths	Difference with amputated group
Amputation	——	18/53; 69(23–111)	34	
Amputation	0.8 Gy	18/54; 62(39–117)	33	n.s.
Amputation + irradiated tumour	0.8 Gy	20/58; 57(30–116)	34	n.s.

*Mice dying with metastases/total number of mice observed; median survival time in days after treatment (range).

Table 5. Fraction of mice or rats dying with metastases after local treatment of tumours transplanted into the foot-pad*

	Rat	Mouse		
Treatment of tumour-bearing leg	R1 Rhabdomyosarcoma	Lewis lung carcinoma	C22LR Osteosarcoma	3641 Mammary carcinoma
Amputation	11/31; 89(21–105)	17/40; 46(24–73)	22/39; 54(20–111)	10/20; 101(15–125)
70 Gy X-rays		22/40; 39(27–87)	20/40; 56(35–94)	9/20; 97(7–132)
90 Gy X-rays	13/35; 75(50–113)			
Difference with amputated group	n.s.	n.s.	n.s.	n.s.

*Animals dying with metastases/total number of animals observed; median survival time in days after treatment (range).

Table 6. The radiosensitivity of various tumours

Tumour	D_0 in Gy (hypoxic)	*n*	Reference No.
2661 Carcinoma	2.0	50	Fig.1
Lewis lung carcinoma	3.1	8	[21]
C22LR Osteosarcoma	2.6	15	[14]
R_1 Rhabdomyosarcoma	3.8	10	[22]

metastatic growth after radiotherapy for the most sensitive 2661 carcinoma is due to incomplete killing of tumour cells in the foot tumour when doses of 70 Gy or higher are used. So, migration of surviving tumour cells from the primary tumour can be excluded as an explanatory mechanism.

This conclusion implies that the enhancement of metastatic growth after radiotherapy must be due to factors which promote the outgrowth of tumour cells already outside the radiation field at the time of irradiation. Promotion of metastatic growth has been described for an immunogenic tumour when rats were immunosuppressed by whole-body irradiation before or after tumour excision [23]. In our study, the mere fact that the mice received a low dose of scattered radiation in shielded parts of the body [17] is not responsible for promotion of metastatic growth, since no increase in the incidence of metastases was observed when the tumour was removed after irradiation (Table 3, group 1C). Furthermore, irradiation with 2 or 5 Gy whole-body irradiation 1 hr after amputation of the foot tumour did not increase the number of mice dying with metastases (19/30 died after amputation and 19/33 died after amputation followed by whole body irradiation; unpublished observations).

Another factor which has been shown to promote the growth of disseminated tumour cells is the presence of heavily irradiated tumour cells [5]. No effect of the presence of an irradiated tumour mass was observed in our

study (Table 3, group 3C). However, a significant increase in the expression of metastatic disease is observed in the simultaneous presence of an implanted tumour mass and an irradiated leg (Table 3, group 3D). Although the enhancement of metastatic growth in this (simulated) situation is not as great as after local radiotherapy in the tumour-bearing leg, these results indicate that apparently detrimental factors can promote the outgrowth of already disseminated tumour cells. However, the extent of the contribution of each of the factors can not be assessed.

Although the experimental set-up was as similar as possible for the other tumours studied, no greater increase in the incidence of metastatic disease after radiotherapy than after surgery was observed for these tumours. Apparently, the differences in results are due to some unknown property of the 2661 carcinoma which is not possessed by the other tumours tested. Immunogenicity seems not to be involved, since the nonimmunogenic osteosarcoma[24] responds quite similarly to the immunogenic Lewis lung carcinoma[25], but differs from the nonimmunogenic 2661 carcinoma[13]. Furthermore, no correlation with the susceptibility to the Révész effect[26] was noted, since with the addition of heavily irradiated cells to each inoculum a decrease in TD_{50} by a factor of 60 or more was found for the osteosarcoma[14], whereas this factor was 2 to 3 for the 2661 carcinoma (unpublished results). Differences in metastatic pathways might be reflected by the different localization of metastases for the various tumours (Table 1). However, if both lymph node and lung metastases develop, it is impossible to determine whether lung metastases result directly from haematogenous dissemination or indirectly from lymph node metastases. Nevertheless, the fact that, for the 2661 carcinoma, lung metastases only rarely develop without lymph node metastases might be considered as an indication that the lymphatic pathway is predominant for this tumour. This is also in accord with results described by Van de Velde *et al.*[13]. On the basis of the localization of metastases in the other mouse tumours, it may be deduced that the lymphatic pathway is of less predominance, although no further evidence is available to support this conclusion. From these data we tentatively postulate that the metastases-enhancing effect of radiotherapy in the 2661 carcinoma is due to a combination of detrimental factors, i.e., scattered radiation to the critical lymph node and the presence of irradiated normal structures and tumour tissue in the lymph node drainage region. These factors promote the outgrowth of tumour cells residing in that lymph node at the time of treatment. Rappaport and Brown[8] noted an increase in lymph node metastases when KHT tumour cells were inoculated into locally pre-irradiated feet. These findings were interpreted as being due to an increase in the dissemination rate of tumour cells into the lymphatics and a decrease in the latent period between tumour inoculation and the initiation of metastasis[27]. However, it is very difficult to make a distinction between the mentioned mechanisms and a decrease in the number of cells required to form lymph node metastases (which was found for the 2661 carcinoma). For that reason, a similar mechanism might be involved in our study and that of Rappaport and Brown. Although more experimental studies report metastases enhancement after radiotherapy, no effect or a reduction in metastases are also reported. These differences in results seem not to be due to differences in experimental procedures or endpoints since in our study, in which similar experimental conditions were established, differences in response were also found among a panel of tested tumours.

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