Residual Late Radiation Damage in Mouse Stromal Tissue Assessed by the Tumor Bed Effect

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Tumor bed effect/Stromal tissue/X-irradiation/Late radiation damage.

Irradiation of murine subcutaneous stroma before implantation of tumor cells leads to retarded tumor growth. This effect is called Tumor Bed Effect (TBE) and can be used to assess the sensitivity of stromal tissue to radiation. We tested the ability of stromal tissue to recover from X-ray-induced damage as a function of the time interval between X-irradiation and implantation of tumor cells over a period of 195 days. We also assessed the effects of a second test treatment of X-irradiation before implantation to assess residual damage by the first radiation treatment. The tumor bed effect in C57Bl10×DBA2 mice observed after X-ray treatment and implantation of M8013 cells (from a transplantable mouse mammary carcinoma) declines with the time that elapses between X-rays and implantation. Implantation of tumor cells 195 days after initial irradiation of 10 or 20 Gy resulted in a considerably smaller TBE. The half-time of the decay is estimated as about 50 days. The extent of the recovery was then tested in two-fraction experiments, with radiation fractions separated by intervals of 30 or 180 days. In the experiment with re-irradiation at an interval of 30 days after the first radiation dose of 20 Gy hardly any recovery was observed, whereas at an interval of 180 days a considerable recovery was observed. We presume that the recovery in TBE that was observed a long time after the irradiation results from a proliferative stimulus to endothelial cells which takes place during the post-irradiation period. The proliferative response leads to cell death of the X-ray damaged endothelial cells and thereafter these are replaced by healthy cells.

INTRODUCTION

When cells from transplantable tumors are inoculated into pre-irradiated tissues, tumors appear later and grow at a lower rate. Many studies have shown a reduced growth rate of the tumors.1–10 This growth rate reduction is called the “Tumor Bed Effect” (TBE). The TBE has been shown to be dependent on the radiation dose2–4,7,11 and very persistent; Hewitt and Blake4 observed a retarded growth of implanted tumor tissue 450 days after irradiation of the tumor bed. In most experimental studies on TBE, subcutaneous tissue has been chosen as the site for implantation of tumor cells. The subcutaneous stroma of the implantation site on the thigh of mice consists of a loose type of connective tissue and is provided with a vascular network. To enable the tumor to grow, implanted tumors have to initiate the formation of new capillaries originating from host tissue. Irradiation inhibits the outgrowth of capillaries which support the tumor transplant. This leads to impairment of blood supply and thus to a lack of nutrients and oxygen.12,13 The decreased growth rate of tumors implanted in previously irradiated beds is mainly the result of retarded blood vessel growth.3 TBE is not only observed after implantation of tumor cells in previously irradiated normal tissue, but also after irradiation of solid tumors themselves. For example, the retarded re-growth rate in mouse tumors after a high radiation dose relative to control tumors, in the results reported by Sugie et al.14 and by Amano et al.,15 is a result from the TBE. It is caused by retarded re-growth of vasculature, and not from radiation effects on tumor cells.

Evaluation of the growth rate of untreated tumors implanted into irradiated beds can provide information about the sensitivity and recovery of stromal vasculature with time. According to the report by Terry et al.,10 the tumor bed undergoes recovery after irradiation similar to other late responding normal tissues. These authors presume that a
major route of recovery is by repopulation, starting as early as 1 day after the first irradiation. They observed that with increasing fraction intervals of 6 and 12 weeks (in two fraction experiments) a further dose sparing effect occurred. From their experiments, there was no evidence that recovery was completed by 12 weeks after irradiation. As these results conflicted with old data of Hewitt and Blake, we investigated residual radiation damage of the stroma using the tumor bed effect assay with intervals up to 195 days after irradiation. The extent of “memory” of a first X-ray treatment was investigated in two fraction experiments. For this purpose the tumor bed was irradiated with 10 or 20 Gy of X-rays. Thirty or 180 days after the initial X-ray treatment the bed was re-irradiated. To test the extent of TBE, tumor cells were inoculated 15 days after the second X-ray treatment.

MATERIALS AND METHODS

Mice and tumors

Male DBA2xC57BLJ0 mice were obtained from Harlan-CPB, Zeist, The Netherlands. The animals were, when the experiments started, 10–12 weeks old.

The M8013X tumor cell line is derived from a mammary adenocarcinoma in an estrogen stimulated male C57BL mouse at the “Netherlands Cancer Institute” in 1950. This tumor is only slightly antigenic and is poorly differentiated with cells of cuboid shape, sometimes arranged in cords. The macroscopic doubling time ranges from 1.6 to 1.8 days and the tumor can give rise to metastases. To obtain a tumor cell suspension, an aseptically excised tumor of approximately 2 g was minced with scissors in about 35 ml Eagle’s minimum essential medium. The tumor debris was separated and the resulting supernatant cell suspension was used. The cells were counted in a hemocytometer using dye exclusion as a test for viability and treated further as described previously. To obtain tumors, 10^5 viable M8013X cells in a volume of 25 μl were inoculated subcutaneously into the thigh of the right hind leg of each mouse. At this inoculation site unrestricted tumor growth to volumes up to 1 cm^3 was observed. The implantation of tumor cells, to assess TBE, was always done at least 15 days after irradiation. In this way contribution of fast phases of repair of radiation damage was excluded.

Assay

The response of the subcutaneous stromal tissue of the right hind leg was assessed by differences in growth time of M8013X tumors implanted into the centre of the previously treated field. The size of the tumors was measured 3 to 5 times a week using calipers to determine three perpendicular diameters (d1, d2, d3). The volume (V) was calculated using the formula V = (π/6) × d1 × d2 × d3. Before each measurement, the animals were briefly anesthetized with ether. Mean tumor volumes (± s.e.m.) were plotted as a function of the time after implantation. The time taken to reach a volume of 1.0 cm^3 was determined for each tumor. The time interval for tumors growing in untreated beds to the endpoint size of 1.0 cm^3 was then subtracted from the equivalent times for tumors in pre-treated beds in order to calculate the retardation of the tumor growth. This is the Tumor Bed Effect (TBE) and TBE has units of days.

Irradiation

The right hind legs of the mice were irradiated with an X-ray generator (Siemens Stabilipan), operated at 250 kV and 14 mA, yielding a dose rate, after filtration of the beam by 0.5 mm Cu, of 1.89 Gy/min at the position of the right hind leg. The mice were individually placed in perspex boxes as described by Wondergem et al. Mice were anesthetized 15 min before treatment with pentobarbitone sodium, (Nembutal ®), 50 mg/kg i.p.).

In ‘two fraction’ experiments, the first fraction was given when the animals were 10–12 weeks old, which corresponds to the zero time point in the ‘one fraction’ experiments (cf. Fig. 1). The second fraction of X-rays was given after 30 days or after 180 days. The longer intervals used excluded any contribution from early acute phases of repair. Implantation of tumor cells was done 15 days after the second fraction of irradiation.

RESULTS

The results plotted in Fig. 1 show that the TBE gradually declines as a function of the time after a single dose of X-radiation at the tumor bed when implantation of tumor cells was delayed to test this effect. From these results it can be
deduced that this decline takes place with a half time of about 50 days. At an interval of 195 days between irradiation and implantation of tumor cells, the extent of remaining TBE after a dose of 10 or 20 Gy was about 30 per cent of the level at an interval of 14 days. A dose of 20 Gy in young animals yields a TBE of approximately 17 days. As we showed previously that the TBE does not increase further at doses larger than 20 Gy,\textsuperscript{11} we did not extend the doses to above 20 Gy. Although experiments in age-matched control animals show that the maximum extent of TBE is somewhat smaller in older animals (see Fig. 3), the decline in TBE during the 195-day interval between X-rays and implantation is an indication of a considerable recovery of the tissue between day 14 and 195.

The results shown in Fig. 1 suggest that at a longer time interval after irradiation the re-irradiation tolerance of the tissue responsible for the TBE would increase. This was tested using two-fraction experiments, with a first fraction of 20 Gy X-rays. Fractions of X-rays were separated by an interval of either 30 days or 180 days. The results of these experiments are presented in Figs. 2 and 3. The TBE observed after two fractions with an interval of 30 days shows its maximum value of approximately 17 days; while a second fraction of 5 Gy leads to a TBE of 14 days, which is not significantly different from the maximum level of 17 days. This indicates a considerable "memory" of damage on the stromal tissue caused by the first fraction. The experiments with an interval of 180 days show a TBE of approximately 8.5 days after a second dose of 5 Gy and this is well below the maximum extent of TBE in these experiments of 15 days. With a longer interval, there is apparently less memory caused by the first treatment. This agrees with the observed decline in TBE with time between irradiation and implantation observed in Fig. 1. The results in Figs. 2 and 3 (0 Gy group) also show dose dependent increase of TBE in age-matched control animals. Namely, in older animals, the extent of the TBE is slightly less than in younger animals, and the growth rates of control tumors were a little faster than those in younger animals: 1.8 vs 2.1 days doubling time (Fig. 4). The growth rate of tumors in an irradiated bed of older animals did not differ significantly from young animals: 3.1 vs 3.2 days doubling time.

![Fig. 2. Tumor bed effect (TBE) in mice that were irradiated 30 days after an initial 20 Gy radiation treatment (▲), as well as in age matched mice without prior treatment (■). The interval between the last X-ray irradiation and implantation of tumor cells is 15 days. Treatment groups consisted of 10–18 mice.](image1)

![Fig. 3. Tumor bed effect (TBE) in mice that were irradiated 180 days after an initial 20 Gy radiation treatment (▲), as well as in age matched mice without prior treatment (■). The interval between the last X-ray irradiation and implantation of tumor cells is 15 days. Treatment groups consisted of 10–18 mice.](image2)

![Fig. 4. Growth curves of M8013 tumors in control mice of two different age groups as used in the experiment described in fig. 1 (10–12 weeks + 45 days: ○ and 10–12 weeks + 195 days: ▦). Corresponding growth curves of tumors in irradiated mice (20 Gy, when the mice were 10–12 weeks old) are also shown: implantation of tumor cells 45 days after irradiation (▲), or 195 days after irradiation (▲). Error bars indicate ± s.e.m. Treatment groups consisted of 55 (○), 45 (●), 18 (△) and 25 (▲) mice.](image3)
DISCUSSION

Our results show that the X-ray-induced TBE is strongly dependent on the time interval between irradiation and implantation of tumor cells up to 195 days. Namely, the TBE at an interval of 195 days declined to about 30% of the value observed at an interval of 14 days. A half-time for the decline in TBE was estimated as about 50 days. Although a considerable recovery of the irradiated tissue responsible for the tumor bed took place after treatment, the TBE had not completely disappeared even after 195 days. Two-fraction experiments confirmed these findings. The experiment where the “memory” of the first 20 Gy fraction was tested using a second radiation fraction separated by 30 days showed a large “memory”. With a longer interval of 180 days (Fig. 3), however, we observed considerably less memory. This is in agreement with the results in Fig. 1.

In an earlier study, we showed that when endothelial cells were forced to proliferate by another treatment (hyperthermia) X-ray damage came to expression earlier and therefore damage to endothelial cells did not contribute to TBE when stromal tissue was stimulated later by implantation of tumor cells.11,20) The results of the two-fraction experiments in the present study show that the TBE is very persistent. This indicates that endothelial cells normally proliferate only slowly during the period of up to 180 days. We presume that the recovery that still takes place during the 180 day-interval after 20 Gy results from a proliferative stimulus to endothelial cells which takes place during the post-irradiation period. The proliferative response leads to cell death of the X-ray damaged cells. These dead cells are then replaced by either proliferation of neighboring survived endothelial cells or by cells from the circulation, which compensates for the cell loss that proceeds with time after irradiation.11,20)

The age effect in control animals (0 Gy group in Fig. 3) showing a lower TBE after a dose of 20 Gy suggests some recovery of the tumor bed with age, which is difficult to explain. It is speculated that recruitment of endothelial cells from outside the irradiated volume (via the circulation) may play a role to replace X-ray damaged cells, when these cells are stimulated by implantation of tumor cells. This recruitment, during the microscopic growth phase of tumors, may be for some unknown reason then more efficient in older animals.

The re-irradiation tolerance observed in two-fraction experiments in rapidly proliferating tissues is generally rather high. For example, Wondergem and Haveman21) studied the effects of two fractions of X-rays separated by an interval of 90 days on the skin reaction of the mouse foot. They observed an impaired tolerance in the acute skin reaction of only 25 per cent even after a high initial dose of 30 Gy.

By contrast, in a normally slow or hardly proliferating tissue such as spinal cord, slow recovery of irradiation damage is observed. Studies of Hornsey et al.22) and of Knowles23) on lumbar spinal cord showed a complete recovery from radiation damage (i.e., full gain of the tolerance) at an interval of 100 days and of 1 year, between the first radiation treatment and re-irradiation, respectively. According to Van der Kogel,24) no recovery, other than repair of sublethal damage, is observed in two-fraction experiments with the cerebral spinal cord at intervals up to 56 days. A significant additional recovery was observed at an interval of 112 days for the “early delayed” white matter necrosis. Recovery from the “late delayed” vascular damage started 16–20 weeks after the initial treatment and was completed in about 210 days.20) The author concluded that for the two distinct types of damage in the rat cervical spinal cord, recovery by repopulation was dependent on the type of target cell involved.

The reports by Stewart et al.25,26) on mouse kidney showed that in this tissue no additional recovery takes place in a 6-month interval. Even when a rather low first X-ray dose was given, no recovery other than repair of sublethal damage was observed in the two-fraction experiments. The authors concluded that stimulated proliferation in kidney by the first fraction is not sufficient to match the loss of functional cells or that proliferation does not lead to the required functional integrity in the kidney.

In two-fraction experiments on the mouse lung,27) it was demonstrated that 60 per cent of the effect of a first X-ray dose was repaired in one day presumably as a result of sublethal damage repair. With a 5-week interval, the residual damage had decreased to 25 per cent. The additional repair in lung is attributed to “slow repair” without a role for cell proliferation.27)

We conclude that substantial recovery in subcutaneous stromal tissue after a high first dose (20 Gy) is possible, provided that the interval between the two treatments is sufficiently long. Our results confirm those of Hewitt and Blake41) in that TBE is persistent; namely, they observed a high TBE after 20 Gy at a long interval of 450 days between radiation and implantation of test tumor cells, whereas no TBE was observed after a 350-day interval. The apparent inconsistency might be due to a very limited number of animals in the treatment groups (5–8), which may have precluded a statistically reliable assessment. It was noted earlier that the cells that are mainly responsible for the TBE are vascular endothelial cells.12,13) The slow turnover of these cells may explain the persistence of TBE over a rather long period. In this regard, Hobson and Denekamp28) estimated the potential doubling time of vascular endothelial cells in non-stimulated mouse skin, using continuous labeling with tritiated thymidine. However, because of very low labeling indices, calculated potential doubling times were not very precise and the estimated potential doubling times ranged from 93 to 2300 days. In the irradiated tumor bed, the endothelial cells are stimulated. Experiments by Wondergem et al.,11) applying hyperthermia after X-rays, show a complete recovery of the
TBE as a result of the heat treatment. They showed that hyperthermia led to a strongly enhanced proliferative activity of endothelial cells in the subcutaneous tissue.\(^{20,29}\) Also based on these results it is supposed that the recovery of TBE long after irradiation is the result of proliferative activity of endothelial cells. X-ray-induced damage comes to expression by the proliferation of the irradiated cells, even if the proliferation takes place long time after the initial radiation treatment. The lethally damaged cells have to be replaced, either by proliferation of survived endothelial cells or by circulating cells from outside the irradiated region. Recent results with (cuff-induced) mechanical damage to mouse vasculature clearly show a role of bone marrow-derived progenitor cells in the repair process after this type of damage.\(^{30}\) It is very likely that these progenitor cells are involved in the recovery of TBE after radiation-induced damage to vasculature, while further studies are required to address the issue.

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