Retardation of Tumor Growth in Mice Caused by Radiation-induced Injury of Tumor Bed Stroma: Dependency on Tumor Type

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ABSTRACT

Dependency on tumor type of tumor growth retardation caused by the radiation-induced damage of tumor bed stroma, a phenomenon known as the tumor bed effect (TBE), was investigated using two mammary carcinomas designated MCA-4 and MCA-K and two fibrosarcomas designated FSA and NFSA, all syngeneic to C3Hf/Kam mice. Inoculations of tumor cells were given s.c. into the right hind thighs of mice either treated or not treated 1 day earlier with graded doses of γ-rays; tumor latency and growth rate were determined. Tumor latency was prolonged and tumor growth was retarded, but the magnitude of these two features of TBE greatly depended on radiation dose and tumor type. TBE began to appear at doses of 5-10 Gy and then sharply increased as the dose of radiation was increased up to between 20 and 30 Gy, at which point a plateau was achieved. TBE was also significant after 40 and 60 Gy total dose given in daily fractions of 2 Gy 5 times per week, a schedule commonly used in radiotherapy treatment of cancer patients. Carcinomas exhibited more pronounced TBE than fibrosarcomas, with NFSA showing only minimal TBE. Radiation-inactivated MCA-4 and FSA cells admixed with viable MCA-4 cells reduced tumor latency, but not the tumor growth delay, of resulting MCA-4 tumors in preirradiated legs. In contrast, admixture of irradiated NFSA and viable MCA-4 cells abolished growth delay but did not influence tumor latency of the TBE phenomenon. Thus the type of a tumor growing in the irradiated tissue is a very important factor that determines the expression of TBE.

INTRODUCTION

The growth and the therapeutic response of tumors are greatly influenced by normal tissue stroma that surrounds or infiltrates tumors. Small aggregates of tumor cells can be adequately nourished by oxygen and nutrients that diffuse from the microenvironment (1, 2), but a sustained tumor growth beyond that phase requires formation of tumor vasculature (3). Formation of tumor vasculature is accomplished by the stroma of the tumor bed under the influence of angiogenic factors released by tumor cells. However, the formation of blood vessels usually lags behind the proliferation rate of tumor cells, leading to inadequate blood supply to certain parts of tumors, which then become hypoxic and frequently undergo necrosis (1, 4). If, in the initial phase of tumor growth, blood vessels fail to develop, small tumor cell aggregates may persist in a dormant state for a long time (3). It is well established that the inadequacy of blood supply compromises drug delivery to tumors (5) and reduces sensitivity of tumors to ionizing radiation because it leads to the emergence of radioresistant hypoxic cells (4).

To study the role of tumor stroma in tumor growth and therapy, an approach can be taken that involves the assessment of growth parameters of tumor transplants in the s.c. tissue previously damaged by ionizing radiation. It has been found that tumor cells transplanted into preirradiated s.c. sites require more time to form palpable tumors than cells transplanted into nonirradiated tissues, and during further progression, tumors in the irradiated tissue exhibit a reduction in the growth rate (6-13). This phenomenon, termed TBE,4 is considered to be a result from the reduced ability of irradiated tissue to provide vascularization for tumors. The blood flow in such tumors is impaired, and it can be as low as 50% of that in tumors growing in the nonirradiated bed (10). It has been shown that the irradiated stroma can promote metastatic spread of solid tumors (14, 15), and that the radiation response of such tumors may be impaired (16, 17). We recently reported that an irradiated tumor bed makes a murine fibrosarcoma more responsive to local tumor irradiation or cyclophosphamide when the effect was measured by tumor growth delay, but less responsive to these agents when tumor curability was the end point of the response (17). Thus integrity of the stroma surrounding the tumor is an important factor that influences both the growth behavior and the response of tumors to treatment.

The experiments reported here were designed to determine whether the expression of radiation-induced tumor bed stroma injury is dependent on tumor type and whether it is expressed after doses of radiation commonly used in the clinic. Prior studies on TBE invariably used large single doses of irradiation, which are above doses used in clinical radiotherapy (6-13). In this study the experiments were performed with two fibrosarcomas and two mammary carcinomas in mice.

MATERIALS AND METHODS

Mice. Male inbred C3Hf/Kam mice bred and maintained in our own specific-pathogen-free mouse colony were used. They were 10–12 wk old at the beginning of the experiments and were housed 4 or 5 per cage.

Tumors. Experiments were performed with two mammary carcinomas and two fibrosarcomas syngeneic to C3Hf/Kam mice; the spontaneous

4 The abbreviation used is: TBE, tumor bed effect.
mammary carcinomas MCA-4 and MCA-K; the spontaneous fibrosarcoma NFSA; and the 3-methylcholanthrene-induced fibrosarcoma FSA. Single-cell suspensions from fibrosarcomas were prepared by trypsin digestion of necrotic tumor tissue (18). Viability of the cells was more than 95% as assessed by trypan blue dye exclusion and phase-contrast microscopy. Cell suspensions of cells from carcinomas were prepared by a mechanical method (19). The viability of MCA-4 was approximately 30%. About 80% of those cells were single, and the remainder were in clumps of 2–5 cells. The suspensions of MCA-K cells were composed of single cells, approximately 60% of which were viable.

Assessment of the TBE. Tumor latency and the tumor doubling time were the end points of the TBE. To generate solid tumors, we gave mice s.c. injections of $1.5 \times 10^5$ FSA or NFSA tumor cells, $3 \times 10^5$ MCA-K cells, or $2–5 \times 10^5$ MCA-4 cells into the right thighs, which were either normal or preirradiated with $\gamma$-rays in graded doses, ranging from 5–60 Gy. In most experiments, single doses of $\gamma$-rays were given 1 day before tumor cell transplantation. In one experiment, legs were irradiated with fractionated doses of $\gamma$-rays, consisting of daily fractions of 2 Gy 5 days a week for 4 or 6 wk to a total dose of 40 and 60 Gy, respectively. In this situation the tumor cells were injected 1 day after the last radiation dose. The MCA-4 tumor, which exhibits strong TBE (9), was used in most experiments. Irradiation was delivered from a dual-source $^{137}$Cs unit at a dose rate of 8.85 Gy/min. During irradiation mice were not anesthetized but were immobilized in a jig. The thighs were irradiated in a circular irradiation field 3 cm in diameter. The possibility that the irradiation affected the total body was minimal, since only 1% of the delivered dose was received at a distance of 1 cm from the radiation field.

Mice were inspected for tumor appearance and growth 3 times weekly or, if needed, more frequently. The time needed for tumors to enlare to 5 mm in diameter was arbitrarily used as the time of tumor appearance (latency period). Tumor growth was determined by measuring three mutually orthogonal tumor diameters with a vernier caliper. The number of days tumors needed to grow from 6–12 mm in diameter was used as the tumor diameter doubling time.

Admixture of Heavily Irradiated and Viable Tumor Cells. In experiments in which we tested the effect of heavily irradiated tumor cells on TBE, $2 \times 10^5$ MCA-4 or $2 \times 10^5$ FSA tumor cells irradiated with 100 Gy $\gamma$-rays were mixed with $2 \times 10^5$ viable MCA-4 cells prior to injection of the cell mixture into legs of mice. In a separate experiment, $5 \times 10^6$ NFSA cells irradiated with 100 Gy were mixed with $5 \times 10^5$ MCA-4 viable cells and injected s.c. Thus the ratio of heavily irradiated to viable tumor cells was 10:1. Irradiation of the cells was performed with a dual-source $^{137}$Cs unit within 30 min prior to injection of the mixture into mice.

RESULTS

Dependency of TBE on Radiation Dose. This experiment was performed with MCA-4. The right hind thighs of mice were exposed to graded single doses of radiation ranging from 5–30 Gy and 1 day later given s.c. inoculations of $4 \times 10^5$ tumor cells. Controls were nonirradiated mice that received the same number of tumor cells. Fig. 1 shows the dependency of both tumor latency and growth rate on the radiation dose. Tumor latency was not affected by doses of 10 Gy and lower. A significant TBE was evident after 15 Gy and increased steeply with a further increase in radiation dose up to about 30 Gy. In comparison with tumor latency, the growth rate of already established tumors was affected by smaller radiation doses. As little as 5 Gy slowed the growth of tumors. The effect increased with an increase in radiation dose approaching a plateau at approximately 15 Gy.

TBE with "Clinical" Radiation Doses. Earlier studies on TBE commonly used single doses of radiation, which are much larger than the radiation doses used per fraction in the clinical treatment of malignant tumors (6–13). Consequently it appeared important to establish whether fractionated irradiation delivered in a clinical fractionation schedule produces TBE. Total doses of 60 Gy and 40 Gy were delivered to the right thighs of mice in daily fractions of 2 Gy (5 fractions/wk). An additional group of mice was treated with a single dose of 16.7 Gy, which is estimated to be equivalent to 60 Gy in 27 fractions on the basis of an $\alpha/\beta$ model for late responding tissues, with an assumed $\alpha/\beta$ value of 3.7 (20). However, in the present study, 60 Gy were delivered in 30 fractions. One day after the last dose of radiation, mice were given injections of $3 \times 10^5$ MCA-4 cells, and tumor growth was followed. Table 1 shows tumor latency and tumor doubling time. All three radiation treatment schedules produced significant TBE. While tumor latency was affected most by a single dose, a similar increase in tumor doubling time was produced by the single dose and by the 60-Gy fractionated treatment. Therefore, a fraction-
TUMOR TYPE AND TUMOR BED EFFECT

The right hind thighs of mice were exposed to single 20-, 40-, or 60-Gy doses of \( \gamma \)-rays and 1 day later given s.c. inoculations of \( 3 \times 10^6 \) MCA-4 or MCA-K cells, or \( 1.5 \times 10^6 \) FSA or NFSA cells. The results, presented in Fig. 2 and Table 2, show that the extent of TBE greatly depended on tumor type. Tumor appearance of carcinomas was prolonged more than that of fibrosarcomas, but the degree of the radiation effect on the two carcinomas was similar within the range of doses used here. Interestingly a very small TBE was observed for NFSA. The tumor diameter doubling time was increased for MCA-4, MCA-K, and FSA by a factor of approximately 2, and as with the latency period, the extent of TBE did not vary greatly within the range of radiation doses used. Table 2 shows the tumor diameter doubling times for the 20-Gy point. NFSA tumor exhibited no change in the doubling time within the tumor size range used here, if it grew in the preirradiated tissue. However, the NFSA tumor does exhibit some degree of TBE, but only when it reaches a size larger than 12–13 mm in diameter (17).

**Influence of Heavily Irradiated Tumor Cells on TBE.** The growth of recurrences following tumor irradiation is slower than the growth of nonirradiated tumors, which is also largely attributed to TBE (11, 13, 21, 22). Since tumor cells that survive irradiation are in contact with radiation-killed cells, it was of interest to investigate whether the radiation-inactivated tumor cells influence the extent of TBE. Two separate experiments were performed using the MCA-4 tumor. In one experiment, \( 2 \times 10^6 \) MCA-4 viable tumor cells were mixed with \( 2 \times 10^6 \) heavily irradiated tumor cells from the same tumor or from the FSA tumor (which exhibited a smaller TBE by the latency end point; see Fig. 2) and injected s.c. into the right hind thighs of nonirradiated mice or mice irradiated with 30 Gy 1 day earlier. Results presented in Table 3 show that irradiated MCA-4 cells did not affect tumor appearance in normal mice and that irradiated FSA tumor cells prolonged it only slightly. However, both types of irradiated cells greatly reduced tumor latency in preirradiated tissue, with FSA cells being more effective. In contrast to the effect on tumor appearance, heavily irradiated cells from neither MCA-4 nor FSA tumors influenced tumor diameter doubling time of MCA-4 tumors. Since the NFSA tumor shows only minimal TBE, we tested the ability of cells from this tumor to influence the growth of MCA-4 in preirradiated tissue. Viable MCA-4 cells (\( 5 \times 10^6 \) /mouse) admixed with \( 5 \times 10^6 \) irradiated NFSA cells were injected s.c. into the right thighs irradiated with 15 Gy 1 day earlier. Table 3, Experiment 2, shows that heavily irradiated cells only slightly reduced tumor latency in preirradiated tissue, but it totally abolished any radiation-induced increase in tumor diameter doubling time. Also irradiated NFSA cells in normal mice somewhat enhanced the growth rate of MCA-4 tumors. Thus both tumor latency and diameter doubling time features of TBE

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**Table 2**

Diameter doubling time of tumors growing in preirradiated tissue: dependency on tumor type

<table>
<thead>
<tr>
<th>Tumors tested</th>
<th>Nonirradiated tumor bed</th>
<th>Irradiated (20 Gy) tumor bed</th>
<th>( P ) (Mann-Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA-4</td>
<td>7.4 ± 0.5(^a)</td>
<td>15.3 ± 2.0</td>
<td>0.001</td>
</tr>
<tr>
<td>MCA-K</td>
<td>9.4 ± 0.7</td>
<td>16.6 ± 0.5</td>
<td>0.001</td>
</tr>
<tr>
<td>FSA</td>
<td>8.3 ± 0.3</td>
<td>19.6 ± 0.5</td>
<td>0.001</td>
</tr>
<tr>
<td>NFSA</td>
<td>7.5 ± 0.3</td>
<td>7.4 ± 0.3</td>
<td>NS(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SE.

\(^b\) NS, not significant.

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**Table 3**

Effect of irradiated tumor cells on TBE

<table>
<thead>
<tr>
<th>HITC source</th>
<th>Latency Diameter doubling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>No HITC</td>
<td>Nonirradiated</td>
</tr>
<tr>
<td>MCA-4</td>
<td>23.0 ± 0.9(^d)</td>
</tr>
<tr>
<td>FSA</td>
<td>22.2 ± 0.6</td>
</tr>
<tr>
<td>FS2</td>
<td>26.4 ± 0.9(^d)</td>
</tr>
<tr>
<td>NFSA</td>
<td>13.5 ± 1.0</td>
</tr>
</tbody>
</table>

\(^d\) Mean ± SE.

\(^a\) HITC, heavily irradiated tumor cells.

\(^b\) The right hind thighs were irradiated with 30 Gy (Experiment 1) or 15 Gy (Experiment 2) single dose of \( \gamma \)-rays 1 day before the thighs were given s.c. injections of \( 2 \times 10^6 \) viable MCA-4 cells, alone or mixed immediately prior to injection with \( 2 \times 10^6 \) MCA-4, or FSA cells irradiated with 100 Gy (Experiment 1) or s.c. injections of \( 5 \times 10^6 \) viable MCA-4 cells, alone or mixed with \( 5 \times 10^6 \) irradiated NFSA cells. Tumor latency is expressed as the time in days from tumor cell injection to enlargement of tumors to 5 mm in diameter. Groups consisted of 7–8 mice each.

\(^P < 0.05\) (Mann-Whitney U test) compared to the corresponding "No HITC" group.

\(^P < 0.001\) (Mann-Whitney U test) compared to the corresponding "No HITC" group.
can be influenced by the presence of heavily irradiated cells, but which one will be affected depends on the type of irradiated cell.

DISCUSSION

Since the initial observation by Frankl and Kimball (6) of reduced growth of tumors in irradiated tissue, a number of articles have considered various features of, including possible mechanisms for, TBE (7–13). It is generally considered that the major mechanism for TBE is the reduced ability of irradiated stroma of the tumor bed to vascularize the tumor (7, 10). An example of evidence in support of this mechanism comes from Van den Brenk et al. (23), who found that formation of capillary sprouts in the tumor bed in response to tumor cell implants was greatly inhibited by prior irradiation of the tumor bed. Inhibition of tumor vascularization by irradiation can be so severe as to reduce tumor blood flow to as low as 50% of that of tumors growing in nonirradiated tissue (10). It is generally considered that TBE is the expression of the late-responding tissues, which basically are tissues that proliferate slowly, and includes the endothelium of blood vessels. However, a recent analysis of the TBE phenomenon using the quadratic model showed that the α/β ratio is 6.2, the value which is on the border of α/β values for acutely responding tissues (24). This implies that TBE may not entirely be the expression of late-responding tissues. As will be discussed further in the text, the interaction between tumor cells and the irradiated stroma is an important component in the development of TBE, suggesting that the TBE phenomenon is more complex in its pathogenesis than previously considered.

Our data show that the development and magnitude of TBE depend on radiation dose. Both tumor latency and growth rate were dependent on radiation dose delivered to the tumor bed. TBE on MCA-4 began to appear at doses of approximately 10 Gy, showed a steep increase as the dose of irradiation was increased, and then plateaued at approximately 20–30 Gy. It was further observed that the MCA-4 growth rate was affected by lower doses of irradiation than was its appearance (see Fig. 1). This could be explained on the basis that larger tumors have a greater need for oxygen and nutrients than small cell aggregates. Therefore, the reduction in the supply of oxygen and nutrients caused by the deficiency in the development of blood vessels in irradiated tissue will be manifested sooner in larger tumors. Dependency of TBE on radiation dose has been investigated by a number of investigators and was recently reviewed by Begg and Denekamp (25). Most studies used radiation doses in the range of 5–60 Gy. Using reduction in the growth rate as the end point of TBE, the analysis showed that, in general, TBE was dose dependent, reaching a plateau for most tumors at doses between 20 and 30 Gy (25). In situations where it was possible to analyze the data, some tumors showed a threshold dose of 5–10 Gy. Thus the dose-response parameters observed for the MCA-4 tumor are within the range of doses producing TBE in other tumor systems.

An important finding here was that the development of TBE was greatly dependent on tumor type, with carcinomas exhibiting greater TBE than fibrosarcomas. NFSA did not show TBE at any radiation doses used here. Thus, regardless of the extent of tissue stroma damage, which in fact inhibits stroma’s ability to supply tumors with a vascular network, certain tumors can circumvent the restrictive effect of TBE on tumor growth. The reasons for the variation among tumors in TBE expression are not known, but they are likely related to angiogenic abilities of tumor cells. From the time when Folkman et al. (26) demonstrated that tumor cells secrete soluble factors that stimulate angiogenesis, many tumors have been shown to possess significant angiogenic properties (reviewed in Refs. 3, 27, and 28).

Since irradiated tumor cells do not lose the ability to induce angiogenesis (23), it was possible to investigate the role of tumor cells in the expression of TBE in the present study. Our data showed that heavily irradiated tumor cells can counteract the effect of the damaged stroma on both the latency and growth rate of tumors, but which of these two features of TBE was affected depended on the source of tumor cells. MCA-4 and FSA cells reduced the latency period but not the growth rate of the MCA-4 tumor. FSA cells were more potent in this respect. This effect of irradiated tumor cells can be attributed to the Revesz phenomenon (29, 30), which represents stimulation of tumor cell proliferation in the presence of heavily irradiated tumor cells. On the other hand, admixing irradiated NFSA cells with MCA-4 cells did not affect the latency of the MCA-4 tumor in the irradiated bed, but completely reversed the inhibitory effect of irradiated tissue on tumor growth rate. The NFSA tumor contains a large number of macrophages (approximately 75%) which secrete factors that stimulate tumor cell proliferation. Since macrophages are known to be good stimulators of angiogenesis (31), it is quite possible that the large number of macrophages that accumulate at the site of injection of NFSA cells may overcome the inhibitory effect of irradiation on blood vessel formation. In addition to macrophages, it is possible that other systemic circulating cells can contribute. NFSA causes a significant granulocytosis (32), and since granulocytes are a good source of angiogenic factors (33), it can also be assumed that granulocytes are a cell type that contributed to the abolition of TBE in the present study. However, it is puzzling why the latency period was not affected if angiogenesis was induced, unless one assumes that the initial cell inoculum containing heavily irradiated NFSA cells "repaired" the irradiated tissue in such a way that this tissue is now able to respond to demands for vascularization only when a stronger angiogenic stimulus is present. The stronger stimulus is likely to occur when tumor cell transplants achieve a certain tumor size adequate to release sufficient amounts of angiogenic factors. That this course of events might happen in this experimental setting is suggested by our recent observation (34) that the growth rate of already established tumors in the preirradiated bed was less inhibited as the number of tumor cells in the initial tumor inoculum was higher. In this particular case, irradiation of the tumor bed was performed 200 days before tumor cell inoculation. Therefore, the extent of TBE is dependent not only on the degree of local tissue damage, but also on the type of tumor cells present in the irradiated tissue. As discussed, some tumors may engage systemic factors in order to reduce or abolish TBE. Current studies are in progress to investigate the nature of the systemic influence on TBE expression.

As indicated above, the investigations on TBE have invariably been performed using large single doses of ionizing radiation,
much higher than those used in the clinic. Since our present data show that irradiation delivered in doses and fractionation schedules commonly used in the clinic can also induce a significant TBE, one can assume that TBE is a likely participant in the final outcome of tumor radiotherapy in clinical settings. As shown by our earlier study with FSA, TBE might negatively influence tumor radiocurability (17). If this observation with FSA is applicable to other tumors, protection against stromal damage during radiotherapy needs to be considered; that protection should be therapeutically beneficial.

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