Effect of Preirradiation of Transplantation Site on Growth Characteristics and Hypoxic Fractions in Human Colon Tumor Xenografts

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ABSTRACT

The volumetric growth curves and hypoxic fractions of seven different human colon tumor lines (clone A, clone D, WiDR, SW480, SW620, DLD-2, and HCT-8) xenografted into the flank regions of either unirradiated nude mice or mice that had received 17.5 Gy of 250-kVp X-rays 1 day prior to implantation were biomathematically analyzed using the Verhulstian equation. Significant variation was found among tumors with respect to both initial growth rates (r, days⁻¹) and theoretical final volumes (carrying capacities, X, mm³). In radiation-damaged normal tissue, tumors grew relatively well for about the first 2 wk postimplantation, attaining volumes of about 70 to 155 mm³. Then, tumor growth rates altered. This effect varied from relatively minor effects on growth rate (tumors of clones A and D) to inhibition of growth, with actual decreases in tumor volume (e.g., WiDr, SW480, SW620, HCT-8, and DLD-2). After this short-term transience in growth kinetics, neoplasms began to steadily regrow at about 3 wk postimplantation, albeit at a slower rate than that seen in controls. Tumor bed effect values were calculated using the ratio of times at which control tumors and tumors growing in the irradiation-injured tissue reached a volume of 75% of the X values derived from the respective control growth curves. Values for clone D, clone A, and WiDr, SW480, SW620, DLD-2, and HCT-8 tumors were, respectively, 1.89, 2.41, 3.48, 3.62, 2.82, 3.66, and 3.65, indicating that tumor bed effect responses varied by almost 100%, even for cancers of the same neoplastic class. Also, the hypoxic fractions of all tumors growing in radiation-damaged sites were increased as compared with levels in controls.

INTRODUCTION

Tumor cells transplanted into a normal tissue site damaged by ionizing radiation exhibit a decreased growth rate as compared with neoplasms growing in a pristine environment. This TBE is a classical phenomenon in radiation biology, first described by Frankl and Kimball in 1914 (1) and named in 1955 (2). Since 1955, there have been many publications describing the TBE, including time-dose response characteristics (3-5), implications for interpretation of tumor response to radiation or drugs (6-10), response to high linear energy transfer radiations (11), dependence on tumor histology (12, 13), influence on metastases (14), effects on intratumor hypoxic levels (15-17), extinction of clonal subpopulations in heterogeneous tumors (18-20), association with altered in situ cell kinetics (21), biomathematical modeling efforts (18), and descriptions of altered tumor vascular physiology resulting from the normal tissue damage (22-24). Based on these previous data, we investigated the TBE with regard to two specific objectives.

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2 To whom requests for reprints should be addressed, at Brown University School of Medicine, Radiation Research Laboratories, Box G, Room B-003, Providence, RI 02912.

The abbreviations used are: TBE, tumor bed effect; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor; TGF-α, -β, transforming growth factors α and β.

(a) To date, the magnitude of the TBE in various tumor systems has been empirically derived. Different indices of effect have been used, and in retrospect, some studies have been incomplete. Consequently, in our examination of the shapes of the growth curves of seven different xenografted human colon tumors transplanted into unirradiated and irradiated sites, we analyzed these volumetric data using the Verhulst equation. This equation is particularly amenable to consideration of neoplasms as quasi-ecological systems (25). Our intent was to develop a biomathematically appropriate way to analyze TBE data that would also potentially be a global method by which investigators could intercompare results.

(b) Several investigators have shown that the hypoxic fraction is elevated in neoplasms growing in irradiated sites (15-17). Because we have previously defined the levels of hypoxia in these xenografted human colon tumors in the unperturbed state (26), we measured hypoxic fractions in tumors after preirradiation of the normal tissue as another index of the effect of radiation injury.

MATERIALS AND METHODS

Tumor Lines. The human colon tumor lines used were WiDr, SW480, SW620, clone A, clone D, DLD-2, and HCT-8. Clone A, clone D, and DLD-2 lines were originally isolated and subcloned at the Roger Williams Cancer Center, Providence, RI, and have been in our laboratory for many years. For these experiments cultures were generated from stock frozen in liquid nitrogen. The WiDr, SW480, SW620, and HCT-8 lines were obtained from the American Type Culture Collection, Rockville, MD, as frozen stock. Cell lines were maintained as exponentially growing stock cultures in RPMI-1640 medium containing 10% fetal bovine serum, 1% anti-pleuropneumonia-like organisms agent, 1% sodium bicarbonate, and 0.04% Gentamicin (all reagents from the Grand Island Biological Co., Grand Island, NY; GIBCO).

Generation of Solid Tumors and Measurement of Volumetric Growth Curves. To produce tumors, exponentially growing cells were trypsinized (0.03% trypsin-EDTA, 5 min, 37°C; GIBCO) from 175-cm² plastic flasks (B-D Labware, Trenton, NJ), centrifuged (7 min, 1000 rpm), and resuspended as single cells in Hanks' balanced salt solution (GIBCO) (5 x 10⁷ cells/ml). Then, 0.2 ml of suspension were injected into the right flank regions of male nude mice (nu/nu genotype) (26). Young adult male mice (nu/nu genotype) were purchased from the Charles River Breeding Laboratories, North Wilmington, MA, and were kept in isolation for 1 wk before any treatments were undertaken. Mice were ear tagged for identification and were housed 5 per large cage (with dust covers) in a dedicated animal room in the Animal Care Facilities of the Biomedical Center of Brown University within a laminar flow animal hood (Thorin Industries, King of Prussia, PA). Food and water were available ad libitum.

Tumor Bed Effect Protocol. For TBE experiments, implantation sites received 17.5 Gy of 250-kVp X-rays as previously described (19) 1 day prior to tumor cell injection. Tumor volumes in control and TBE groups were calculated using the formula for a prolate ellipsoid (26). All measurements were made by a single individual. There were typically 10 to 12 mice per group.

Biomathematical Analysis. We used the Verhulst equation to analyze...
the growth curves, in which the volume at any time \( t \) is given by

\[
V(t) = \frac{V_0}{K - e^{-r(t-t_0)}}
\]

where \( V_0 \) is the theoretical tumor volume at time zero, \( r \) is the Malthusian growth rate (days\(^{-1}\)), and \( K \) is the environmentally determined asymptotic final volume or carrying capacity (mm\(^3\)) (25, 27). \( V_0 \) was arbitrarily set to 100 mm\(^3\) to represent the cell volume injected at time zero. We have previously described the use of this equation for tumor growth analysis (18, 27).

Hypoxic Fraction Bioassay Using the Paired Survival Curve Technique. For determination of hypoxic fractions, the parallel line excision bioassay technique was used, as we have previously described (26). Complete survival curves were generated, and the hypoxic fractions were determined from the relative survival of cells irradiated with high doses in either air-breathing or nitrogen gas-phytiated (10 min) tumor-bearing mice. Typically, there were 8 to 10 individual tumors assayed per dose point per condition (i.e., oxic or hypoxic) for each tumor line.

RESULTS

Volumetric Growth. In Fig. 1, we show volumetric growth curves for the clone A, clone D, WiDR, SW480, SW620, HCT-8, and DLD-2 xenografted tumors after a dose of 17.5 Gy of 250-kVp X-rays to the normal tissue 1 day prior to tumor cell implantation (A to G, respectively). The curve shapes, while similar overall, varied with respect to the individual tumor type. The initial maximum TBE volumes attained were about 100 mm\(^3\) (tumor diameter, 5 to 6 mm) and were reached at roughly the same time (about 17 to 18 days postimplantation) for tumor lines in which the maximum was clearly defined (e.g., lines WiDR, SW480, SW620, and HCT-8) and DLD-2 (Fig. 1). There is some variability in these initial maximum volumes, as volumes ranged from about 150 mm\(^3\) (clone A, clone D, WiDR) to about 75 mm\(^3\) (SW620, HCT-8). After these initial maximum volumes were reached, an inflection of varying extent and duration in growth rate occurred, which lasted to about Day 21 to 22 (Fig. 1). Clone A and clone D tumors did not show regression per se at the 17.5-Gy dose level, in contrast to the SW480, SW620, and HCT-8 tumors which exhibited marked negative growth rates. After this 3- to 4-day period, tumors began to regrow, albeit at rates significantly different from controls of the same volume.

Verhulstian Growth Parameters. We list the Verhulstian tumor growth parameters for the control and TBE conditions in

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Irradiation (Gy)</th>
<th>Verhulstian growth parameter</th>
<th>Hypoxia (%)</th>
<th>TBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone A</td>
<td>0</td>
<td>0.212</td>
<td>6606</td>
<td>3.0 (1.8-5.0)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.112</td>
<td>5615</td>
<td>18.8 (10.8-32.8)</td>
<td>2.41</td>
</tr>
<tr>
<td>Clone D</td>
<td>0</td>
<td>0.209</td>
<td>7528</td>
<td>0.12 (0.02-0.72)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.149</td>
<td>6775</td>
<td>3.5 (2.2-5.8)</td>
<td>1.89</td>
</tr>
<tr>
<td>WiDR</td>
<td>0</td>
<td>0.098</td>
<td>6366</td>
<td>14.0 (8.3-23.7)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.045</td>
<td>4000</td>
<td>47.7 (29.5-77.2)</td>
<td>3.48</td>
</tr>
<tr>
<td>SW480</td>
<td>0</td>
<td>0.044</td>
<td>1413</td>
<td>15.3 (8.3-28.8)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.025</td>
<td>NE</td>
<td>32.7 (19.5-54.8)</td>
<td>3.62</td>
</tr>
<tr>
<td>SW620</td>
<td>0</td>
<td>0.084</td>
<td>2828</td>
<td>13.9 (5.3-36.4)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.062</td>
<td>1555</td>
<td>55.5 (32.2-95.6)</td>
<td>2.80</td>
</tr>
<tr>
<td>DLD-2</td>
<td>0</td>
<td>0.091</td>
<td>7010</td>
<td>44.0 (38.0-50.0)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.036</td>
<td>NE</td>
<td>72.1 (52.0-106.0)</td>
<td>3.66</td>
</tr>
<tr>
<td>HCT-8</td>
<td>0</td>
<td>0.054</td>
<td>2170</td>
<td>82.1 (46.8-144.2)</td>
</tr>
<tr>
<td>17.5</td>
<td>0.035</td>
<td>NE</td>
<td>88.5 (48.6-161.0)</td>
<td>3.65</td>
</tr>
</tbody>
</table>

SEs on the \( r \) and \( K \) values were typically about 4.8 and 6.5%, respectively.

Hypoxic percentages for all unperturbed tumors except for DLD-2 have been previously reported (26).

Numbers in parentheses, range.

TBE values have been calculated in an isoeffect manner by using the ratio of times needed in each tumor system to reach volumes of 0.075 x \( K_{\text{mm}} \) (mm\(^3\)).

NE, not estimable.

Table 1. Both the \( r \) and the \( K \) parameters were decreased in the TBE. In some instances (SW480, DLD-2, HCT-8), \( K \) values were not estimable from the TBE growth curves.

Tumor Cell Yields. The average cell yields (cells/mg x 10\(^4\), mean ± SEM) for the clone A, clone D, SW480, SW620, WiDR, DLD-2, and HCT-8 control tumors after enzymatic disaggregation were, respectively, 1.74 ± 0.10, 2.20 ± 0.13, 3.01 ± 0.32, 3.44 ± 0.67, 1.65 ± 0.38, 2.89 ± 0.30, and 4.09 ± 0.51. There were, therefore, differences in cell yield among the various neoplasms. Cell yields for the various tumors in the
TBE situation were not significantly different from their respective control values.

**TBE Values.** We used an arbitrary value of $0.075 \times K_{\text{on}}$ (mm$^3$; Table 1) to derive TBE values, which were defined as the ratio of times needed to reach that volume in control and TBE growth situations. The TBE defined in this manner is therefore an isoeffect comparison. The choice of this value was a compromise for two reasons. (a) While we wanted to compare tumors at as large a volume as possible in the regrowth period, the regrowth of SW480 and HCT-8 tumors was very slow, which severely constrained our choice of a maximum value suitable for comparison of the entire set of volumetric responses. (b) The comparison volume could not be too small, or problems would arise because of the early volume transient seen in the TBE situation (Fig. 1). TBE values for clone D, clone A, WiDR, SW480, SW620, DLD-2, and HCT-8 tumors were, respectively, 1.89, 2.41, 3.48, 3.62, 2.82, 3.66, and 3.65, indicating that there was almost a 100% variation in TBE expression, even within the same class of neoplasm.

**Hypoxic Fractions.** The hypoxic fractions for the control and TBE situations are listed in Table 1, and in Fig. 2, we have plotted the hypoxic fractions determined in unperturbed tumors and in tumors growing in the preirradiated sites against each other. The percentage of increase in hypoxic fractions in the TBE situation was most marked in the tumors that had the lower hypoxic fractions in the unperturbed state (e.g., clone A, clone D). We also present in Fig. 3a, a significant inverse correlation between the hypoxic percentages in both unperturbed and perturbed (TBE) tumors with their respective growth rates as represented by the $r$ parameter (Table 1), given by (log) hypoxic percentage $= -1.857 - 2.757 \log r$ for the controls only ($n = 7$, correlation coefficient $= -0.773, t = 2.72, P < 0.05$), and (log) hypoxic percentage $= -1.107 - 2.048 \log r$ when both controls and TBE values are considered ($n = 14$, correlation coefficient $= -0.760, t = 3.87, P < 0.01$) (28). While a tendency toward a similar inverse correlation between the percentage of hypoxia and the $K$ values (Table 1) is also present (Fig. 3b), this relationship is not statistically significant ($n = 11$, correlation coefficient $= -0.524, t = 1.84, 0.05 < P < 0.10$) (28).

**DISCUSSION**

The shapes of tumor growth curves (Fig. 1) were biomathematically analyzed to derive TBE values. Operationally, it was necessary to use the carrying capacities derived from control growth curves to select levels for comparison, because $K$ values for some neoplasms growing in irradiated tissue were difficult or impossible to estimate. The biomathematical approach generates reasonable results. For example, Verhulstian-derived TBE values for the xenografted colon tumors may be compared with values obtained using the approach of Milas (29), in which TBE values were described on the basis of a doubling in tumor diameter (from 6 to 12 mm). For tumors in which sufficient TBE volume data are available (clone A, clone D, and WiDR) (Fig. 1), Verhulstian TBE values are 2.4, 1.9, and 3.5, while TBEs derived from tumor diameter-doubling considerations are 2.7, 2.2, and 4.3. Verhulstian TBEs are very similar to the volumetric TBE values and exhibit the same rank ordering.

The data also show that large differences exist in the expression of the TBE among xenografted human colon tumors (i.e., 1.9 to 3.6) at an X-ray dose of 17.5 Gy. Similar variability in the magnitude of the TBE has also been documented in other tumor systems. For example, at a dose of 10 Gy, Begg and Terry (12) noted that the TBE was 1.6 in the rodent SA F round cell sarcoma and 2.2 in the poorly differentiated CA NT adenocarcinoma. At a dose level of 20 Gy, Milas (29) found that TBE values ranged from 1.0 to 2.4 in 5 rodent sarcomas and from 1.3 to 2.1 in 5 rodent carcinomas.

Besides changes in volumetric tumor growth, levels of hypoxia are increased in the TBE situation (Fig. 2). Our results are consistent with other published information. Milas et al. (16) have reported that hypoxic fractions in the SA-NH rodent sarcoma increased from 3% to 12% after a preirradiation dose of 30 Gy of X-rays, while Penhaligon et al. (17) found an increase from 2.5% to 4.6% in the RIF-1 sarcoma after a dose of 15 Gy. Apparently then, a generic consequence of radiation damage to normal tissue is the production of increased hypoxic fractions in tumors. These increases in the percentage of hypoxia were most marked in the neoplasms that exhibited low
hypoxic fractions in the unperturbed state. Also, there appears to be a significant inverse correlation of tumor growth rate as represented by the $r$ parameter (Table 1; Fig. 3A) and intratumor hypoxia. This relationship predicts that hypoxic percentages are actually less in faster growing tumors than in slower growing neoplasms. In this regard, a similar tendency has been reported by Moulder and Rockwell (30) and Rockwell and Moulder (31), who summarized hypoxia levels in rodent and xenografted human tumors. However, these tendencies were not statistically significant, likely because hypoxic fractions were correlated against tumor volume doubling times, a less robust parameter of tumor growth rate than the Verhulstian $r$ parameter. This inference, i.e., that hypoxic fractions are larger in slower growing neoplasms is, however, in accordance with results from growth factors experiments on tumor-bearing mice in which such alterations in tumor growth rates and hypoxic percentages have been noted (see below).

It is important to discuss possible mechanisms by which such differential TBE and hypoxic fraction results might arise. A fundamental study of TBE and hypoxic fraction physiology has been performed by Okunieff et al. (24), using the mouse FSA-II tumor system. After 16 Gy, about a 25% decrease in the number of blood vessels per tumor was seen, particularly due to selective loss of microvessels less than 40 μm in diameter. Okunieff et al. noted that differences in blood flow between control and TBE tumors were small "until tumors grew larger than 100 mm" in which case blood flow was reduced by about 70%. Similarly, reductions in blood flow of about 50% were found in rat mammary cancers by Jirtle et al. (22) after preirradiation of the normal tissue bed with about 14.4 Gy. Therefore, endothelial cell killing with resultant interference with tumor blood flow and covariates of blood flow, such as tumor growth rate and steady-state hypoxic fractions, must be a central underlyjing feature of the TBE. This, however, does not explain differences in TBE among neoplasms, particularly in situations where equivalent radiation doses to the normal tissue have been given and where, presumably, equivalent endothelial cell killing has been produced.

We hypothesize that such differences in volumetric growth, TBE expression, and hypoxic fractions among the xenografted human colon tumors (Figs. 1 to 4) reflect the complex autocrine/paracrine/endocrine relationships between tumor cells and host stroma (32). There is evidence to support this hypothesis.

(a) Administration of the angiogenic growth factor EGF to mice bearing xenografted human A431 tumors markedly increases tumor growth (33-35) and concomitantly decreases hypoxic fractions (36). Conversely, sialoadenectomy, which removes the major source of EGF in the mouse, produces decreased tumor growth rates and increased tumor hypoxic fractions. Additionally, Gross et al. (37) have shown that daily injections of FGF-2 will also increase the growth rates of xenografted DLD-2 tumors, a result similar to that produced by EGF. Whether changes in hypoxic fractions in DLD-2 tumors would occur after FGF-2 administration has not been investigated. Summers et al. (38) noted that ovariectomy of female C3H mice bearing syngeneic mammary tumors would increase the TBE (28.5 Gy) by a factor of about 1.8 as compared with intact mice, suggesting that endocrine status may affect the TBE. Also, Penhaligon et al. (39) have shown that angiogenic stimulation of a TBE site prior to transplantation of the Lewis lung carcinoma decreased the extent of the subsequent TBE.

(b) Solesvik et al. (40) have shown that the microvascular patterns of human melanomas xenografted into nude mice exhibit individual characteristics. Because the vascular system originates from the host, such individuality in microvascular response must be determined by some aspect of the implanted tumor cells. Hypoxic fractions have also been shown to vary significantly in these melanomas (40).

(c) Responses of solid tumors in vivo to irradiation in situ can be used to gain insight into the TBE. In vivo, concomitant tumor cell killing as well as normal tissue injury occurs, leading to regression of tumor volume soon after irradiation. A TBE response may often be identified by a later transient volumetric inflection. Thomlinson and Craddock (41) described this inflection as a "second wave of regrowth delay," which should occur at a time when the volume of the regrowing tumor exceeds the capacity of the existing vasculature to support further growth. In the Thomlinson and Craddock work (41), rat RIB5 tumors were irradiated (20 Gy) at volumes of about 1150 mm$^3$. A plateau was seen at a volume of about 3625 mm$^3$, indicating an additional increase in tumor radius (shell thickness) of about 3 mm. In a similar study, Wurschmidt et al. (23) irradiated rat RIH rhabdomyosarcoma tumors (15 Gy) at volumes of about 250 mm$^3$ and observed a plateau at a volume of about 650 mm$^3$, indicating an additional increase in tumor radius of 1.5 mm. In the xenograft TBE work presented herein, maximal tumor volumes occurred at about 100 mm$^3$ (Fig. 1), a tumor radius of about 2.9 mm. Lastly, a similar critical tumor radius (about 3 mm) at which a decreased tumor growth rate occurred may be calculated from the TBE work of Penhaligon et al. (39).

Therefore, data from both the preirradiation (TBE) and the in vivo irradiation models indicate that solid tumors use the preexisting (radiation damaged) vasculature to increase in radius (shell thickness) by about 1.5 to 3 mm before neovascularization is absolutely required. Such a neovascularization process requires the involvement of an angiogenic stimulus, but it does not require that the potency of this stimulus be equivalent in different types of tumors. In this regard, production of angiogenically active substances (e.g., TGF-α, TGF-β, FGF-2) in the unperturbed state in vivo or release of such substances via tumor cell lysis because of a hostile microenvironment or radiation injury could directly affect either host or tumor cells and/or be sequestered by the local extracellular matrix, to be available at later times for neovascularization (42, 43). Growth factors such as FGF-2 have been shown to stimulate endothelial cell proliferation and migration in vitro (42, 43), and there is evidence documenting that the differential production of growth factors by different tumors within a given histological class should be considered a general phenomenon (e.g., Ref. 44). To recapitulate, transplantation of tumor cells that differentially produce angiogenic growth factors could cause proliferation and migration of endothelial cells to occur at different rates, resulting in differential TBE expression. Tumors with high levels of angiogenic ability would be predicted to exhibit rapid neovascularization with minimal growth inhibition and a low TBE (e.g., clone A, clone D). Conversely, tumors that have low angiogenic capabilities should show a marked inflection in their respective growth curves and a high TBE (e.g., SW480, SW620, HCT-8), which could be attributed to a slower neovascularization process.

(d) There is an additional mode of action of growth factors that directly supports the concept that such substances are involved in TBE expression, which arises from the work of Haimovitz-Friedman et al. (45) and Kwok and Sutherland (46,
ASSOCIATION OF THE TBE WITH INCREASED TUMOR HYPoxic FRACTIONS

These investigators have noted that, in vitro, the angiogenic polypeptides FGF-2 and EGF can, respectively, either protect or sensitize mammalian cells to ionizing radiation damage. In this regard, we have shown that TGF-α, which binds to the same receptor as EGF, will similarly produce radiosensitization of human colon tumor cells in vitro. Therefore, if neoplasms vary in the production of substances such as FGF-2 or EGF/TGF-α, with resultant quantitative differences in the in vivo survival of host and/or tumor cells upon exposure to ionizing radiation, this might explain at least part of the lack of uniformity in estimation of postirradiation repair and recovery parameters for the TBE by various investigators (3–5, 38).

In summary, evaluation of the "TBE" involves consideration of not only the implantation site and its local characteristics but also the characteristics of the individual tumor, with possible superimposition of host influences. In this regard, the TBE represents an excellent model to study such "contextual interactions" (48).

REFERENCES

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