Vascular Changes in Neuroblastoma of Mice following X-irradiation

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SUMMARY

Vascular changes in a transplantable neuroblastoma of A/J mice following X-irradiation were studied. X-irradiation with doses lower than 500 rads in a single exposure caused a slight increase in the functional intravascular volume, as measured with $^{51}$Cr-labeled red cells 1 day after the irradiation, and a gradual decrease thereafter. X-irradiation with 1000 and 2000 rads, in contrast, induced a decrease in the vascular volume at 1 day postirradiation and a further gradual decrease thereafter. The extravasation of plasma protein, as measured with $^{125}$I-labeled plasma protein, increased soon after irradiation with doses of 250 to 2000 rads, and then decreased. Histopathological studies of tumors irradiated with 2000 rads revealed a progressive crowding or condensation of the vascular bed as the tumor cell population became reduced or tumor lobules receded. Direct irradiation-induced damage to the vessels and the disorganization of the architecture of vascular networks following irradiation appear to cause vascular occlusion and interfere with the blood flow in the tumors.

INTRODUCTION

It is generally believed that the response of tumors to radiotherapy is closely related to the change in blood circulation during the course of treatment. An increase in vascular function and blood supply may enhance oxygenation of tumors and thus increase the radiosensitivity of surviving tumor cells to subsequent irradiation, whereas extensive radiation-induced vascular damage would result in poor oxygenation of the tumors, making the tumor cells radioresistant. In spite of this important fact, little is known about the vascular changes in and around the tumors. We have been studying irradiation-induced circulatory changes in the Walker carcinoma 256 of rats using a quantitative radioisotopic method (15–18). In the present study the effect of irradiation on the vasculature of the transplantable neuroblastoma of A/J mice was studied. Along with the quantitative measurement of the functional intravascular volume and the vascular permeability, histopathological changes in the vascular patterns were investigated.

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MATERIALS AND METHODS

Tumors. The tumor used in this study is a neuroblastoma of mice (Jackson Laboratory C 1300), which has been maintained in our laboratory by serial transplantations into A/J male mice. After the flank hair was clipped, a small fragment of the tumor (2 x 2 mm) was inoculated s.c. by a trocar needle into the flank of each animal. The tumors transplanted by this method became palpable within a few days and were 8 mm in diameter in 10 to 13 days. Two perpendicular diameters of each tumor were measured with a vernier caliper 2 to 3 times a week and the mean of the measurement was obtained.

Irradiation. When the tumors grew to about 8 mm in diameter, the mice were lightly anesthetized with sodium pentobarbital (0.05 mg/g) and the tumors were locally irradiated, with the rest of the body shielded with a lead sheet. The irradiation factors were 220 kV, 15 ma, added filtration of 0.25 mm Cu plus 1.02 mm Al, source surface distance of 27.5 cm, and a dose rate of 250 rads/min. The dose ranged from 250 to 2000 rads in a single exposure. The control tumors were treated similarly except for the irradiation.

Functional Vascularity. At various time intervals after the irradiation, the functional intravascular volume in the tumor was determined with $^{51}$Cr-labeled red blood cells, and the rate of extravasation of plasma protein was determined with $^{125}$I-labeled human serum albumin as follows. The heparinized mouse blood, drawn by cardiac puncture, was incubated with sodium chromate-5'Cr (200 $\mu$Ci/ml of blood) at 37° in a water bath for 30 to 40 min with gentle shaking. After the blood was washed with 0.9% NaCl solution 4 times to remove free $^{51}$Cr, $^{125}$I-labeled human serum albumin was added (20 $\mu$Ci/ml of blood) and the volume was adjusted with 0.9% NaCl solution to the original volume. About 0.05 ml of this mixture was injected in the tumor-bearing mice through a tail vein. Exactly 1 hr later, the skin over the tumors was cut open with scissors. The tumors were then excised and the nontumorous tissue was removed. Since the neuroblastoma is well capsulated, nontumorous tissue could be easily identified. No loss of blood from the tumors occurred during the operation. Blood from the same animals was withdrawn by cardiac puncture in heparinized syringes and 0.05 ml of whole blood was transferred into a test tube. The rest of the blood was centrifuged for 10 min at 1500 rpm and 0.05 ml of the plasma was obtained. The radioactivities of $^{51}$Cr and $^{125}$I in the tumor, blood, and plasma were counted with a well-type $\gamma$-scintillation counter with a 2-channel analyzer. The weight of the tumors was obtained after drying overnight at 110°.

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Such treatment was found to be sufficient to remove all the moisture from tumors of the size used in this study. The intravascular volume and the extravasation of plasma protein were calculated using the following formula (17).

\[
\text{Vascular volume: ml of blood/g of tissue = } \frac{(^{51}\text{Cr activity/g of tissue})/(^{51}\text{Cr activity/ml of blood})}{1 - \text{hematocrit/100}}
\]

Extravasation rate: ml of extravasated plasma in 1 hr/g of tissue = Total plasma volume in g of tissue - intravascular plasma volume in g of tissue

where total plasma volume is \((^{125}\text{I activity/g of tissue})/(^{125}\text{I activity/ml of plasma})\) and intravascular plasma volume is vascular volume \(X (1 - \text{hematocrit/100})\).

**Histological Studies.** Eighteen tumors were examined histopathologically. Tumors of about 8 mm in diameter were irradiated with 2000 rads in a single exposure as described above. Some tumors were irradiated partially by covering a part of the tumor with a piece of lead sheet 3 mm thick. At 3 and 6 days after the irradiation the tumors were excised, fixed in 10% formalin, and embedded in paraffin in the usual manner of histological preparation. The tumor tissue sections were stained with hematoxylin-eosin and azocarmine for the evaluation of cellular changes and vascular stroma. For a detailed evaluation of vascular stroma a modified Bielschowsky’s silver stain was used. This stain demonstrated the blood vessels and connective tissues well.

**RESULTS**

The growth curves of control tumors and the tumors irradiated when they were 8 mm in diameter are shown in Chart 1. The rate of growth of control tumors was constant from about 6 to 20 mm in diameter. A significant decrease in tumor growth rate was observed thereafter. X-irradiation with doses of 250 rads and 500 rads resulted in only slight retardation of tumor growth. While the control tumors grew from 8 to 20 mm in 10 days, the tumors irradiated with 250 and 500 rads grew to 20 mm in 11 and 13 days, respectively. The irradiation with 1000 rads significantly inhibited the tumor growth; the time needed to grow from 8 to 20 mm was 20 days, doubling that for unirradiated control tumors. The tumors irradiated with 2000 rads continued to grow for 3 days after irradiation at a somewhat decreased rate, regressed temporarily, and started to regrow from 8 to 10 days after irradiation. They became 20 mm in diameter about 30 days after the irradiation.

The functional intravascular volume and extravasation of plasma protein of unirradiated control tumors are shown in Table 1. Since the vascularity of some tumors has been reported to decrease as the tumor grows (2, 4, 11, 17), the unirradiated control tumors were arbitrarily divided into 3 groups on the basis of size, and the vascular volume and extravasation of plasma protein among these groups was compared. No systematic difference in the vascular volume and extravasation of plasma protein was found in the 3 groups of tumors, indicating that the vasculature or vascular function does not seem to change significantly in this tumor at least within the range of 5 to 15 mm in diameter.

Thus, the average functional intravascular volume and the average rate of extravasation of plasma protein in all of the unirradiated tumors of 5 to 15-mm size were used as the control values. These averages were compared with those of the irradiated tumors the size of which stayed within the 5 to 15 mm range during the time intervals studied. The average functional intravascular volume and the extravasation of plasma protein of 70 control tumors was 0.2733 ± 0.0166 (S.E.) ml/g (dry) and 0.5391 ± 0.0203 (S.E.) ml/g (dry)/hr, respectively.

The postirradiation change in the functional intravascular volume is shown in Chart 2. On the 1st day after 250 rads, the vascular volume appeared to increase slightly. No noticeable change was observed 1 day after 500 rads. On the 3rd day after these doses, the intravascular volume was slightly smaller than that of the control tumors; and on the 6th day, the decrease was more pronounced although not statistically significant (t test). The study was not carried out beyond 6 days in animals with 250 and 500 rads because the tumors irradiated with these doses grew continuously and became

### Table 1

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>No. of tumors</th>
<th>Vascular volume [ml/g (dry)]</th>
<th>Extravasation of plasma protein [ml/hr/g (dry)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01–0.05 g&lt;sup&gt;a&lt;/sup&gt; (5.3–9.2 mm)</td>
<td>30</td>
<td>0.2753 ± 0.0310&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5187 ± 0.0345</td>
</tr>
<tr>
<td>0.05–0.10 g (9.2–11.4 mm)</td>
<td>20</td>
<td>0.2819 ± 0.0287</td>
<td>0.5706 ± 0.0300</td>
</tr>
<tr>
<td>0.10–0.23 g (11.4–15.0 mm)</td>
<td>20</td>
<td>0.2618 ± 0.0194</td>
<td>0.5381 ± 0.0387</td>
</tr>
<tr>
<td>0.01–0.23 g&lt;sup&gt;c&lt;/sup&gt; (5.3–15.0 mm)</td>
<td>70</td>
<td>0.2733 ± 0.0166</td>
<td>0.5391 ± 0.0203</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dry weight.

<sup>b</sup> Mean ± S.E.

<sup>c</sup> Pulled data of all the tumors used.
rather necrotic and hemorrhagic. When the dose was increased to 1000 and 2000 rads, the vascular volume decreased promptly. The vascular volume 1 day after irradiation with these doses was smaller than that of the control tumors, although the decrease by 1000 rads was not statistically significant. It continued to decrease and by the 12th day the vascular volume of these tumors was about one-third of that of the unirradiated control tumors. The extravasation of plasma protein in the irradiated tumors gradually declined thereafter and was about one-half of the control value by the 12th day after 1000 and 2000 rads.

**DISCUSSION**

Contrary to the reports of others (2, 4, 11), as well as our own observation in Walker tumors (16, 17), the functional vascularity in the neuroblastoma of mice is rather constant in tumors of sizes from 6 to 15 mm in diameter as shown in Table 1. The neuroblastoma is a nodular tumor surrounded by a network of peripheral vasculature with uniformly distributed small blood vessels. Unlike the Walker tumor, in which massive necrosis tends to develop in the central part of the tumor, necrosis in the neuroblastoma occurs randomly in the tumor. Furthermore, necrosis is relatively moderate until the tumor grows to about 15 mm in diameter. The moderate necrosis and lack of obstruction of circulation may account for the rather constant functional vascularity in the nonirradiated control tumors.
The blood vessels of the neuroblastomas of mice appear to be rather susceptible to irradiation, as indicated by the prompt changes in their functions. The cause of the initial increase in the intravascular volume 24 hr after 250 rads (Chart 2) is not clear, although a similar increase in other tissues has been attributed to vasodilation (8, 13). The decreased functional intravascular volume at 24 hr after 1000 and 2000 rads is similar to our previous observation with the Walker tumor of rats (15, 16, 18, 24). In the Walker tumor, however, the decrease in the intravascular volume after 1000 and 2000 rads was actually biphasic; the intravascular volume decreased immediately after irradiation, increased slightly thereafter, and then decreased again at 24 hr (24). Whether such biphasic changes occur in the neuroblastomas of mice was not investigated in the present study. The decrease in the vascular volume from 3 days after 250 and 500 rads and from 1 day after 1000 and 2000 rads appears to be due to permanent vascular damage rather than to a temporary vasoconstriction, since the decrease persists.

Our histological study indicated that the vascular stroma became progressively more abundant and crowded in areas where the original lobular pattern was obscured (Figs. 1 and 2), despite the decreased functional vascular volume. Although the volume of individual tumor cells increased remarkably, the tumor lobules became progressively smaller in many areas and contained much fewer cells per lobule. The change in the tumor size is not commensurate with the remarkable cellular enlargement in these irradiated tumors, probably because of the decreased number of cells per lobule. This indicated a progressive loss of the tumor cells. The shrinkage of the lobules in the irradiated tumors is in good agreement with the report of Tannock and Howes (21), who observed a decrease in tumor cord size in an irradiated spontaneous C3H mouse mammary adenocarcinoma. Should the vascular stroma persist despite the cellular loss resulting in a shrinkage or disappearance of the tumor lobules, one would naturally expect a marked crowding or condensation of the vascular bed to occur. The degree of the cell loss and the shrinkage or disappearance of the tumor lobules varied considerably from area to area of the tumor as well as from tumor to tumor; the density of the vascular beds varied accordingly. In some tumors or some areas of the tumor, the density of the vascular bed was not increased if not reduced, but the vascular stroma surrounding the tumor lobules was markedly thickened. This suggests that the thickened vascular stroma may represent an aggregation or condensation of the vascular stroma of several of the original tumor lobules lost after irradiation. Unfortunately, the silver staining applied in this study does not adequately differentiate vascular from connective tissue. Thus, it could be argued that the thickened stroma observed with this method resulted not from a condensation of preexisting vessels, but from a proliferation of connective tissue. It appears rather unlikely, however, that proliferation of connective tissue was rapid enough to demonstrate the crowded and thickened vascular stroma to such an extent as early as 3 days after irradiation (Fig. 1D). We therefore conclude that the shrinkage of lobules, as a consequence of the disappearance of killed tumor cells, results in crowding and thickening of the stroma at least in early stages of vascular change. Other students (1, 12) have reported that the shrinkage of irradiated tumors was accompanied by an increased vascularity due to the loss of parenchyma cells and suggested that the blood supply to the tumor might be increased in such tumors via the condensed vascularity. Although our histological data suggest a postirradiation condensation of vascular stroma, the quantitative measurement of blood volume clearly indicates that the functional intravascular volume of shrinking tumors after irradiation is less than that of the unirradiated control tumors. A reasonable explanation for the increased blood supply in the studies of other workers (1, 12) lies in the possibility that the loss of killed parenchyma cells may be much greater than the decrease in functional vascularity, resulting in a net increase in the blood supply: tumor ratio.

The progressive decrease in the functional intravascular volume despite the marked condensation in the vasculatures could be attributed to a number of reasons. It might be that the extensive disorganization of the vasculature as a result of the lobular shrinkage causes vascular occlusion and disrupts blood flow resulting in the decreased functional intravascular volume. This assumption does not, however, rule out the possibility that the decreased vascular volume in the irradiated tumors also results from direct irradiation damage to the endothelium or to walls of the blood vessels. A decreased functional blood volume could be observed as early as 1 day after irradiation with 1000 to 2000 rads when the histological change in the vasculature was not yet evident. It could, therefore, be concluded that the 2 mechanisms, direct damage and disruption of vascular networks, must work hand in hand in the irradiated tumors to cause the decreased intravascular volume. In support of our contention that direct damage occurs to the endothelium is the work of Steamer and Christian (19) and Stearner and Sanderson (20). These workers reported that irradiation of chick embryos and 3-week-old chickens with 1100 to 1200 rads caused vascular occlusion as early as 3 hr after the irradiation and attributed this to extensive endothelial damage, lysis, necrotic disruption of endothelial barriers, and endothelial thrombosis. The capillaries in tumors and chick embryos or young chickens may have similar features; they are formed hastily and grow rapidly. The disruption of original vascular stroma and a decrease in vascular function in irradiated tumors also have been reported by others (3, 5). McAlister and Margulis (9) and Rubin and Casarett (12) have observed microangiographic vascular damage in tumors following irradiation with doses of 680 to 3000 R in a single exposure. Using a transparent chamber, Merwin et al. (10) also noticed that irradiation of a transplantable mouse mammary tumor causes a narrowing and fragmentation in the tumor vessels soon after irradiation with 2000 to 5000 R. They attributed such changes to the regression of tumors.

It is generally known that irradiation increases vascular permeability in tissues (7, 13, 14). The transitory increase in the extravasation of newly injected 125I-labeled plasma protein soon after irradiation in the neuroblastoma may reasonably be attributed also to an increased vascular permeability. The increase of vascular permeability may result from direct damage to the capillary wall, an increase in hydrostatic pressure, an altered autonomic neural activity...
from the development of vasoactive compounds, or an alteration of the colloid osmotic pressure. It is, however, conceivable that other factors, such as an increased tissue space for the plasma protein as a result of loss of parenchymal cells and/or a decrease in the return of plasma protein to blood circulation via lymphatics, could also cause the increase of extravasation of the plasma protein. The subsequent progressive decline with time in the extravasation of plasma protein parallels the decrease in the functional vascular volume indicating the decrease in the extravasation results from a reduced blood volume as previously discussed.

The presence in tumors of hypoxic cells due to an inadequate blood circulation is regarded as one of the limiting factors of radiotherapy. It has been reported (6, 22, 23) that some of the hypoxic cell population undergoes reoxygenation following irradiation and becomes more susceptible to subsequent irradiation. The mechanism of reoxygenation is still obscure although it has been speculated that an increased vascular function and decreased oxygen consumption due to the death of oxygenated cells might increase oxygen supply to the hypoxic area and reoxygenate the hypoxic cells. Our observation of decreased intravascular volume in the irradiated tumors may appear to be contradictory to this hypothesis. However, the intravascular volume that we measured in the present study is the volume of blood per unit weight of tumor tissue. The tumor weight includes the living as well as killed tumor cells. The number of living cells in the tumors a few days after irradiation with 1000 to 2000 rads is most probably less than 1% of the original cell population. Since the decrease in the vascular volume per unit weight of tumor tissue is not equally as dramatic as that of the number of living cells, it could be concluded that the blood volume per living cell in the irradiated tumors may actually be greater than that in unirradiated controls. We have, however, no experimental evidence for reoxygenation in this tumor.

The interrelationships between the vascular changes, the concentration of living cells, oxygen consumption, and reoxygenation of hypoxic cells in irradiated tumors are under investigation in our laboratory.

REFERENCES

Fig. 1. Transplantable neuroblastomas of mice. A and B, nonirradiated control tumor showing regular lobulation by a network of delicate vascular stroma. C, D and E, tumor 3 days after 2000 rads, exhibiting cellular enlargement and pleomorphism (C), obscuration of lobular pattern and crowding of delicate vascular stroma (D), and preservation of lobular pattern by delicate vascular stroma (E). A and C, azocarmine stain; B, D, and E, Bielschowsky silver stain, modified. X 150.
Fig. 2. Transplantable neuroblastomas of mice, 6 days after 2000 rads. A, cellular gigantism and frequent karyorrhexis. B, obscuration of lobular pattern, crowding, and marked thickening of vascular stroma. C, preservation of lobular pattern but striking thickening of vascular stroma. D, unirradiated part (upper) consisting of small uniform cells similar to control tumor; part affected by irradiation (lower) with cellular enlargement and pleomorphism. E, lobular pattern and a delicate network of vascular stroma in unirradiated part (upper) and shrinkage of lobules and crowding of vascular stroma in irradiated part (lower). A and D, azocarmine stain; B, C, and E, Bielschowsky silver stain, modified. X 150.
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