The Irradiation Effects on the Cytoskeletons of C3H/He Mouse Mammary Tumor Cells and Vascular Basement Membrane in Relation to Vascular Invasion: A Model of Intraoperative Radiotherapy

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KOBAYASHI, M. *The Irradiation Effects on the Cytoskeletons of C3H/He Mouse Mammary Tumor Cells and Vascular Basement Membrane in Relation to Vascular Invasion: A Model of Intraoperative Radiotherapy.* Tohoku J. exp. Med., 1988, **154** (1), 71-89 — We investigated the short-term effects of a single high-dose radiation upon transplanted MM46 tumor cells in mice by means of immunohistochemistry and electron microscopy. The irradiation induced: 1) giant cell formation from the 3rd day, 2) arrest of tumor cell mitosis in prophase and metaphase due to the disorganization of the mitotic spindles, 3) changes in immunoreactivity of laminin and cytoskeletons, and 4) multilayering of the vascular basal lamina and perivascular fibrosis. The above findings suggest a decrease in tumor cell compliance, growth and invasiveness and the potentiation of defensive host responses against vascular invasion after irradiation. The analysis of the temporal sequences of the events indicates that the time lapse between the optimal host response, tumor growth and invasion constitutes a critical period. ——— tumor; intraoperative radiotherapy; vascular invasion; cytoskeleton; laminin

Intraoperative radiotherapy (IORT) is designed to sterilize the cancer cells that evade a surgical resection by irradiation with a single high-dose of electron beams. The morphologic changes have been studied in various tissues after such

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irradiation (Sindelar et al. 1982a, b; Tepper et al. 1983; Kinsella et al. 1985). They were, however, investigated only by routine histological methods. In order to elucidate the mechanisms of the beneficial effects of IORT on tumor cell eradication, we investigated, by means of immunohistochemistry and electron microscopy, the effects of a single high-dose radiation applied to the transplanted MM46 tumor cells. Special attention was given to the alteration of cytoskeletons and vascular invasiveness.

MATERIALS AND METHODS

Forty-five to six week old female C3H/He NJcl-MTV + mice obtained from Clea Japan Inc. (Tokyo) were kept in cages and fed ad lib with standard laboratory diet and water. The animals were divided into 4 groups, each consisting of 10 mice (2 for control, 5 for 30 Gy irradiation, and 3 for 35 Gy irradiation) according to the experimental design (vide infra).

MM46 tumor cell suspensions (Irie 1971) (spontaneous mammary adenocarcinoma of C3H/He mice, kindly supplied by Dr. Y. Ogawa, Department of Radiology, Kochi Medical School) were maintained by serial transplantations in the peritoneal cavity of C3H/He mice. 5×10^6 viable tumor cells (checked by dye exclusion test and counted with a hemocytometer counter) were injected into the thigh of the left hind limb of the mice. In 7 to 8 days, the implants formed a solid tumor mass measuring up to 8 mm in diameter.

After 7 to 8 days of the implantation, the mice were fixed onto the apparatus designed by Dr. Ogawa for local irradiation of mice. The mice were locally irradiated with 30 and 35 Gy electron beams under general anesthesia by intraperitoneal injection of 0.65 mg sodium pentobarbital. A 6 MeV linear accelerator (NELAC-1006D, NEC, Tokyo) was used with a source-to-surface distance of 100 cm. The mice were sacrificed under ether anesthesia on the 1st, 3rd, 7th, and 14th day after irradiation. The tumor tissues were immediately excised, and divided into two pieces, one for light microscopy and immunohistochemistry and the other for electron microscopy. The specimen for light microscopy and immunohistochemistry was fixed in 15% phosphate-buffered formalin solution (pH 7.4) for 12–24 hr and embedded in paraffin. Three μm sections were cut and stained with hematoxylin-eosin (H-E) and by the peroxidase-antiperoxidase (PAP) method. Primary antibodies and their dilutions with their staining reactions are tabulated in Table 1. These antibodies required pretreatment of the tissue sections with trypsin. The PAP method employed in the present study was as follows:

TABLE 1. *Antibodies used for immunoperoxidase technique and staining reactions*

Antibodies	Dilutions	Trypsinization	Staining reactions	
			control	irradiation
Laminin*	1 : 200	20 min	+	+
Keratin†	1 : 400	10 min	+	+
Tubulin‡	1 : 188§	10 min	+ ~ +	+
Actin‡	1 : 150§	10 min	± ~ +	± ~ +

Staining reaction :

±, equivocal; +, weakly positive; +, moderately positive; ++, strongly positive.

*E-Y Laboratories, USA; †DAKO Corporation, USA; ‡BioGenex Laboratories, USA; §personal communication with BioGenex Laboratories.

Deparaffinize and rehydrate the sections. Inhibit endogenous peroxidase activity with fresh 0.75% H_2O_2 in methanol for 20 min. Treat the slides with 0.07% trypsin (1:250 Trypsin, DIFCO, Detroit, MI, USA) (0.07% trypsin, 0.1% CaCl_2 in distilled water, adjusted to pH of 7.8 with 0.1 N NaOH) at 37°C for 10 to 20 min. After washing in Tris buffer saline (TBS pH 7.6), incubate the sections with normal swine serum (DAKO, Santa Barbara, CA, USA) (1:20 in TBS) for 20 min. Incubate with primary antibody for 30 min, then rinse. Incubate with swine antirabbit IgG (DAKO) diluted to 1:30 in TBS for 30 min, then rinse. Incubate with peroxidase-rabbit anti-peroxidase (DAKO) diluted to 1:200 in TBS for 30 min, then rinse. Stain with fresh 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) solution (0.06% DAB, 0.03% H_2O_2 in TBS) for 5 to 7 min. Counterstain with Gill's hematoxylin for 10 sec. Rinse, tighten, dehydrate, clear and mount. All procedures were carried out at room temperature except the steps of trypsinization. In case where keratin absorption was required, the newly developed technique of absorption using the stratum corneum of the human skin was employed (Kobayashi 1987 submitted for publication). In order to test the specificity of the commercially available antisera, the test sera containing the primary, link, and tertiary antibody were substituted respectively with the TBS. The test serum containing the primary antibody was substituted with the non-immune serum. The positive, negative, substitution controls were stained in parallel with the test material.

The specimen for electron microscopy was fixed in 0.5% paraformaldehyde + 1.0% glutaraldehyde solution in 0.067 M cacodylate buffer (pH 7.4) for 2 hr at 4°C, then postosmicated in 1% osmium tetroxide buffered with 0.067 M cacodylate solution for 2 hr. The specimens were embedded in Epon. One μm thick sections were cut with an ultramicrotome (OMU2, Reichert, Vienna, Austria) and stained with polychrome epoxy tissue stain (Eastham and Essex 1969; Miller and Brown 1981) for screening. Thin sections were cut, stained with uranyl acetate and lead citrate and examined with an electron microscope (EMU 4A, RCA, Detroit, MI, USA).

Statistical analysis of the foci of vascular invasion in the two experimental groups was carried out with Mantel-Haenszel's chi-square test (Mantel 1963).

RESULTS

The tumor growth rate was essentially similar to that described by Ogawa et al. (1983) and Hasegawa (1985). At the time of sacrifice, the tumor implants showed no difference in size between the control and test animals. The irradiation group, however, had macroscopic foci of necrosis in the central portions of the tumor. Neither group had distant metastases at any time during the period of the experiment.

Histology

The foci of vascular invasion in the control and test groups are tabulated in Table 2 according to the postirradiation days and the number of the light microscopic slides examined. The blood vessels were difficult to identify in the routinely processed H-E sections. The sections stained for laminin and 1 μm thick sections stained with Paragon were most suitable to detect vascular invasion. The histologic differences between the two experimental groups are summarized in Table 3.

TABLE 2. *Foci of vascular invasion*

Day after irradiation	Control group	Irradiation group
1st day	3/28 (10.7%)	7/ 73 (9.6%)
3rd day	5/28 (17.9%)	10/ 73 (13.7%)
7th day	2/28 (7.1%)	10/ 73 (13.7%)
14th day	2/28 (7.1%)	9/108 (8.3%)

(Number of slides with foci of vascular invasion/number of slides examined).

TABLE 3. *Sequential histological changes of transplanted tumors in control and irradiation groups*

	1st day	3rd day	7th day	14th day
Tumor growth	Solid sheets invasive /Solid sheets invasive	Solid sheets invasive /Solid sheets loss invasive	Solid sheets invasive /Loss of cohesion	Solid sheets invasive /Loss of cohesion solid sheets*
Tumor cell	##/##	##/+	##/±	##/## /Regrowth
Giant tumor cell	-/-	-/+ /Some cells	± ~ +/## /Almost all cells	+ / ## /Some cells
Mitosis	##/## (Various phases)	##/+ (Prophase) (Metaphase)	##/+ (Prophase) (Metaphase)	##/## (Various phases)
Necrosis	±/±	± ~ +/+ ~ ##	+ / ##	+ ~ ##/##
Inflammatory cells	±/± ~ +	± ~ +/+ ~ ##	+ / ##	+ / ##
Fibrosis	-/-	-/-	-/+ ~ ##	- / ##

Control group/Irradiation group

Code: -, none; ±, equivocal; +, few or slight; ##, some or moderate; ###, many or marked.

*Regrowth area.

Control group

In each group, the skeletal muscle tissue was replaced with solid sheets of tumor cells without any histologic differentiation. Tumor cells had pleomorphic, hyperchromatic nuclei with single or multiple prominent nucleoli (Fig. 1a, 1c, 1e, 1g). The tumor had stellate areas of necrosis in the deeper portions. There were no cytoplasmic vacuoles or signs of degeneration except in the vicinity of necrosis. Many tumor cells were present in the different phases of mitosis. There were a few multinucleated cells on the 7th and 14th day. Inflammatory cell infiltrates increased in intensity in the peripheral portions on the 7th day.

Fig. 1. Light micrographs of transplanted tumor.

Left column, the control group; right column, the irradiation group. Row 1, 2, 3, and 4, the 1st, 3rd, 7th, and 14th day, respectively. Note giant tumor cells in d, f and h. F, fibrosis (H-E stain: a-f, $\times 300$; g and h, $\times 100$)

Irradiation group

The 1st postirradiation day. There was no morphologic change compared with the control group except for swelling of some, but not all, tumor cells (Fig. 1b).

The 3rd postirradiation day. Almost all tumor cells were swollen and degenerated. Some multinucleated giant cells appeared (Fig. 1d). There were broad, irregular areas of necrosis. There was an inflammatory infiltrate consisting mainly of lymphocytes and plasma cells. The inflammatory cells formed rosettes around some tumor cells.

The 7th postirradiation day. Some tumor cells had cytoplasmic vacuoles. Multinucleated giant cells were most numerous at this stage (Fig. 1f). Some giant tumor cells were in the prophase of mitosis and a few in the metaphase (Fig. 2a). The areas of necrosis became broader and more irregular. The inflammatory cell infiltrates extended from the periphery of the tumor to the deeper portions and were most extensive as reported (Imanaka et al. 1982).

The 14th postirradiation day. The periphery of the tumor showed many mitoses of various phases, including anaphase and telophase. Fibrosis became apparent in the areas of necrosis and inflammatory cell infiltration (Fig. 1h). The perivascular areas also showed mild fibrosis. Multinucleated giant cells became less numerous and were replaced by solid sheets of tumor cells. The tumor tissue showed evidence of regrowth (Fig. 2b).

Immunohistochemistry

Immunohistochemical stains are listed in Table 1. The irradiation affects on laminin, keratin, tubulin and actin are noted below :

Laminin

Control group. Tumor cells showed diffuse cytoplasmic stains (Fig. 2c). The control group demonstrated a continuous linear staining of the basement membrane (BM) of the blood vessels, skeletal muscles and Schwann cells. The blood vessels surrounded by tumor cells demonstrated less intense staining of the basement membrane than those abutting directly on the perivascular connective tissue. A few blood vessels were bordered by a thickened or multilayered BM (Fig. 2f). Some blood vessels were collapsed or compressed by the surrounding tumor cells. Some tumor cells showed foci of intravascular invasion (Table 3).

Irradiation group. Tumor cells stained in the same manner as the control group. Giant tumor cells revealed three staining patterns. Some giant cells showed a strong diffuse cytoplasmic stain. Other giant cells were strongly stained only in the ectoplasmic regions (Fig. 2d). Still other giant cells were negative for laminin. These giant tumor cells were distributed at random. The tumor cells became strongly positive on the 3rd day of irradiation and reached the maximum staining intensity on the 7th day. The thickening and/or multilayering of the

Fig. 2. Histology (row 1) and immunohistochemistry (row 2, 3, and 4) of transplanted tumor of the control and irradiation groups. a, a mitotic giant tumor cell (irradiation, the 7th day); b, an area of tumor regrowth (irradiation, the 14th day); c, laminin, control, the 7th day; d, laminin, irradiation, the 7th day; e, vascular invasion by tumor cells (laminin, irradiation, the 3rd day) with BM disruption (arrows); f, multilayered BM (arrows) (laminin, control, the 7th day); g, keratin, control, the 7th day; h, keratin, irradiation, the 7th day. (a, b and e, $\times 300$; c and d, $\times 500$; f and g, $\times 600$)

vascular BM became more prominent. The blood vessels resembled those of the control group on the first postirradiation day. Some tumor cells demonstrated intravascular invasion (Table 3).

The tumor cells of both control and irradiated animals demonstrated invasion of blood vessels and muscle cells. In this situation, the BM of both elements was abruptly disrupted and negative for laminin (Fig. 2e).

Fig. 3. Immunohistochemistry of transplanted tumor of the control and irradiation groups. a, tubulin, control, the 7th day; b, tubulin, irradiation, the 14th day. Note that giant tumor cells show peripheral cytoplasmic stain. c, tubulin, control, the 7th day. Note positive stain of mitotic spindles (arrow). d, tubulin, irradiation, the 14th day. Note globular stain of mitotic giant tumor cells (arrows). e, actin, control, the 7th day; f, actin, irradiation, the 7th day. (a, c and e, $\times 600$; b and f, $\times 400$; d, $\times 500$)

Keratin

Control group. Tumor cells showed diffuse cytoplasmic stains (Fig. 2g).

Irradiation groups. The three cytoplasmic staining patterns were distinguished in the giant tumor cells (Fig. 2h), i.e. 1) diffuse, 2) peripheral and 3) negative pattern.

Tubulin

Control group. Tumor cells were uniformly stained (Fig. 3a). The mitotic cells were strongly positive. The mitotic spindles were especially prominent in the anaphase (Fig. 3c). Tubulin was particularly well demonstrated on the 7th and 14th day.

Irradiation group. Tumor cells were positive for tubulin. Some giant tumor cells demonstrated a rim of positive staining in the ectoplasm after the 7th day (Fig. 3b). Globular or granular reaction products were also present in some tumor cells. The positive reaction was, in general, stronger in the test animals than in the control. The mitotic cells were strongly positive in the areas of regrowth on the 14th day. The mitotic giant cells were, however, weakly positive in contrast to the mitotic cells in the control (Fig. 3d).

Actin

Control group. Tumor cells were generally weakly positive (Fig. 3e). There was no difference in intensity of staining between the deeper portion of the tumor and the invasive areas. Tumor cells were only faintly positive on the first day in the control group and became moderately positive on the 7th day.

Irradiation group. The tumor cells showed a peripheral cytoplasmic staining pattern (Fig. 3f). Actin staining was slightly weaker in the test animals than in the control.

Electron microscopy

Tumor cells

Control group. The morphology of tumor cells among the controls was unaffected by the date of sacrifice. Tumor cells had irregularly outlined nuclei with abundant euchromatin and multiple prominent nucleoli. The subcellular organelles and cytoskeletons were, in general, sparse (Fig. 4a). A few thin and intermediate fibers could be identified in the cell periphery. Microtubules were inconspicuous in the tumor cells except in the mitotic cells. There were abundant diffusely distributed polysomes with a few swollen mitochondria, sparse rough endoplasmic reticulum, poorly developed Golgi complex and some lysosomes. Some tumor cells contained large membrane-bound electron-dense granules resembling secretory granules and/or lysosomes (Fig. 5d). A few tumor cells had

Fig. 4. Electron micrographs of the tumor cells. a, intracytoplasmic gland formation. The cytoskeletons are sparse. (control, the 7th day) Note the increased intermediate filaments in the ectoplasm of the intracytoplasmic gland. (inset, irradiation, the 7th day) b, Note the increased intermediate filaments and microtubules in the cell periphery. (irradiation, the 7th day) (a, $\times 26,500$; inset, $\times 8,500$; c, $\times 30,000$)

Fig. 5. Electron micrographs of vascular endothelial cells. a and b, endothelial cell ballooning (control, the 14th day); c, endothelial cell reaction (irradiation, the 7th day); d, widening of endothelial cell gap (control, the 3rd day); e, critical period after irradiation. Note that the endothelial cell is destroyed but the basal lamina is intact. (irradiation, the 14th day) (a, $\times 8,100$; b, $\times 9,000$; c, $\times 5,400$; d, $\times 13,500$; e, $\times 16,200$)

intracytoplasmic cysts equipped with a few short microvilli (Fig. 4a). There were a few aggregates of tumor cells forming a poorly defined lumen with microvilli. Desmosomes were rare. The basal lamina is present around the tumor cells in several areas.

Irradiation group. Tumor cells had bizarre nuclei with much more euchromatin and larger multiple nucleoli than in the controls. Many tumor cells demonstrated severe degeneration. Mitochondria showed stage IV degeneration (Trump et al. 1971). The cytoplasm of many cells showed ballooning. Giant tumor cells had multinucleated and/or multilobed nuclei with abundant euchromatin and multiple macronucleoli. Many tumor cells and some giant tumor cells were in mitosis. Some giant tumor cells demonstrated an increase in intermediate filaments and microtubules in the cell periphery (Fig. 4b) and intracytoplasmic gland formation with increased intermediate filaments in the ectoplasm (Fig. 4a, inset). The changes were essentially identical in the intravascular as well as extravascular tumor cells (Fig. 6f).

Electron microscopy also revealed significant changes in the capillary endothelial cells and the vascular basal lamina.

Capillary endothelial cells

The capillary endothelial cells were enlarged or protruded into the lumen with prominent nuclei in the irradiation group (Fig. 5c). Some showed mitoses on the 7th day. Some demonstrated intracellular edema and disruption of the plasma membrane. These cells showed a patchy distribution irrespective of the location in the tumor. The cytoplasmic ballooning was more frequent in the control animals than in the test animals (Fig. 5a, 5b). Cytoplasmic condensation, i.e., cellular dehydration, was also observed in both groups but was more prominent in the irradiation group. The intercellular gaps were widened between the endothelial cells with cytoplasmic condensation (Fig. 5d). Some blood vessels were lined by juxtaposed degenerating and regenerating endothelial cells.

Vascular basal lamina

The vascular basal lamina showed various changes in both control and test animals. Some blood capillaries had indistinct or disrupted basal lamina while others demonstrated multiple layers (Fig. 6a). The overlying endothelial cells were markedly swollen and ballooned. Some areas showed subendothelial edema (Fig. 6b) with widening of the gap between the endothelial cells and the basal lamina. These changes were much more extensive in the irradiation group.

Fibrosis occurred around some blood vessels especially in the irradiation group (Fig. 6e). The fibrotic areas showed accumulation of collagen fibers with about 64 nm cross bandings and most conspicuous at the places where the vascular basal lamina had disappeared.

Tumor cells invaded the subendothelial space in both control and test

Fig. 6. Electron micrographs of the blood vessels. a, the multilayered basal lamina (irradiation, the 7th day); b, the subendothelial edema (irradiation, the 7th day); c and d, tumor cell invasion into the subendothelial space. Note the disruption of the basal lamina and the cytoplasmic projection of the tumor cell. (c, control, the 14th day; d, irradiation, the 7th day); e, perivascular fibrosis (irradiation, the 7th day); f, intravascular tumor cell (irradiation, the 3rd day). (a, $\times 16,200$; b, $\times 13,500$; c, $\times 10,800$; d, $\times 11,700$; e, $\times 4,000$; f, $\times 2,700$)

animals. In this situation, the basal lamina was disrupted in contact with the invading tumor cells. These tumor cells demonstrated cytoplasmic projections into the subendothelial areas (Fig. 6c, 6d). Some blood capillaries and venules were filled with noncoherent tumor cells. These tumor cells showed cytoplasmic degeneration in the irradiation group (Fig. 6f). Although the endothelium had desquamated in some blood vessels, the basal lamina was preserved (Fig. 5e).

DISCUSSION

Liotta (1986) elaborated a three-step hypothesis in respect to the early phase of tumor invasion: 1) tumor cell attachment via specific surface receptors with the extracellular matrix, 2) digestion of the matrix with hydrolytic enzymes, 3) active tumor cell locomotion mediated by cytoskeletons. In this hypothesis, laminin and cytoskeletons play an important role in tumor invasion.

The laminin stain revealed the blood vessels, which were inconspicuous in the H-E sections. Thus, it made possible the investigation of vascular invasion by tumor cells. The foci of vascular invasion varied from area to area in the test animals and control and showed no statistical difference in number between the two groups ($p > 0.05$). Therefore, some tumor cells must have already invaded blood vessels before irradiation. The fact that intravascular tumor cells demonstrated the same morphological and immunocytochemical changes in both groups indicates that irradiation also affected both intra- and extravascular tumor cells simultaneously and to the same extent. Our experimental design did not allow us to estimate the exact time of vascular invasion by tumor cells or to explore the possibility of preventing vascular invasion by preceding local irradiation.

Tumor cells of the control group showed positive cytoplasmic stain for laminin. Albrechtsen et al. (1981) and Nielsen et al. (1983) demonstrated the positive cytoplasmic stain for laminin in human breast cancer cells, and stated that it indicated the synthesis of laminin took place in breast carcinoma cells. Foidart et al. (1980) observed a similar phenomenon in murine breast adenocarcinoma and gave the same interpretation as the above investigators. The tumor basal lamina, however, is rare in our material. In our control group tumor cells can produce laminin but may not be able to form the basal lamina because of lack in cell differentiation. Laminin probably acts as a growth stimulating factor of tumor cells and promotes their invasion and metastasis by the adhesion to type IV collagen or the secretion of type IV collagenase (Kleinman et al. 1985). In our irradiation group the changes in the laminin stain of giant tumor cells can be attributed to one of several causes. Irradiation might have caused cessation of laminin production or disturbance of the intracellular transport of laminin. In the former case, the peripheral positive stain for laminin may be explained on the basis of binding of the preformed laminin by laminin receptor on the cell surface. In the latter case, the laminin produced by the tumor cells accumulates in the cell periphery in combination with disturbed excretion. Focal cytoplasmic degrada-

tion products (Hruban et al. 1963) or the focal edema of the tumor cells may have physically pushed intracellular laminin to the cell periphery. The electron microscopic findings are compatible with each of these interpretations. The lack of production, redistribution or disturbed excretion of laminin in tumor cells may have changed the biological activities of laminin resulting in a decrease in tumor cell growth and invasion as postulated by Kleinman et al. (1985). Our study also demonstrated in both control and test groups that the BM was disrupted and negative for laminin at the site of vascular invasion by the tumor cells and that the vascular BM stained only faintly with laminin in the deeper portions of the tumor. These findings suggest the possibility that the tumor cells incited a mechanical damage of the BM or that undamaged invading tumor cells enhanced the production of the degrading enzymes (e.g., type IV collagenase) as Liotta et al. (1977) demonstrated. Thinning or disappearance of the BM around malignant cells was observed by Albrechtsen et al. (1981), Barsky et al. (1983) Papotti et al. (1983), Haglund et al. (1984) and Hashimoto et al. (1984). They also suggested the possibility of the proteolytic degradation of the BM by the tumor cells. Our observation resembles the mesenchymolysis of Takizawa (1966) and supports the observation of Araki (1979).

The diffuse cytoplasmic stain of the tumor cells for actin, keratin and tubulin changed to the peripheral pattern after irradiation. This alteration of the staining pattern may have resulted from the displacement of the cytoskeletons induced by irradiation. These morphologic changes most likely affect the functional properties of the cells such as cell compliance, motility and mitosis, etc.

The decrease in tumor cell compliance probably follows the redistribution of Keratin intermediate fiber (KIF) and tubulin since KIF maintains cell structures by forming a spherical network around the chromosomes and mitotic spindles in the metaphase of mitosis and by keeping the subcellular organelles coherent in the network (Lazarides 1980; Kitajima et al. 1985) and microtubules polymerized from tubulin (Fawcett 1981) are also related to the maintenance of cell structures (Tilney 1971).

The decrease in invasiveness and the disturbance of cell motility and mitosis may follow the redistribution of actin and tubulin, the former being important in cell motility (Hayashi et al. 1975) and cytoplasmic division (Mabuchi 1985) and the latter being polymerized to microtubules (Fawcett 1981) which form the cell processes and mitotic spindles (Tilney 1971). The disturbance of mitosis can also explain the formation of multinucleated giant tumor cells. Microtubules also serve as a vehicle of transport or secretion (Lacy 1977). Cell products, including laminin, type IV collagenase, etc. are transported by microtubules. The disturbance of these functions may also result in the decrease in invasiveness.

Sato et al. (1983) demonstrated that irradiation disturbed the normal rearrangement of centrosomes that form at the onset of mitosis. This results in the formation of irregular multicentric mitotic spindles. The globular or granular

stain of tubulin in some tumor giant cells after irradiation suggests malformation or derangement of microtubules. Our study revealed that mitotic tumor giant cells on the 7th day of irradiation were in the prophase or metaphase and not in the anaphase or telophase. The stain for tubulin was only weakly positive in these cells in contrast to the mitotic cells of the control animals. This finding may be interpreted as radiation-induced malformation of mitotic spindles or dysfunction of mitotic spindles in chromosomal movement.

In these manners, intraoperative single high-dose irradiation is effective in reducing local invasiveness and growth. The irradiation effects reach the peak on approximately the 7th postirradiation day.

It is of interest to note in our study that there was an apparent discrepancy in amount of KIF in the tumor cells as detected by immunohistochemistry and electron microscopy. This may be explained on the basis of the fact that some KIFs are in the form of discrete tonofilaments that cannot be readily resolved by electron microscopy (Saba et al. 1983).

Fajardo and Berthrong (1978) reviewed the general morphology of the irradiation effects. In general, our study reproduced his findings in blood vessels. We observed more frequent endothelial cell ballooning adjacent to invading tumor cells in control animals. The endothelial cell ballooning is, however, more intense in the irradiation group than in the control group. This may be explained on the basis of the endothelial cell reaction to the invading tumor cells rather than to the irradiation. The irradiation may have intensified the endothelial cell ballooning. The regeneration or recovery of the damaged endothelial cells explains the reduplication of the vascular basal lamina (McKinney and Panner 1972; Vracko and Benditt 1972; Vracko 1974). The defense against vascular invasion is generally ascribed to the BM. The multilayering of the vascular basal lamina must fortify the line of defense assisted by the irradiation-induced perivascular fibrosis. We stated in the previous discussion that the vascular BM stained only faintly with laminin in the deeper portions of the tumor and suggested the existence of the degrading enzymes. We did not observe the extensive disappearance of basal lamina in our electron microscopic study in contrast to the immunohistochemistry. The basal lamina of the blood vessel with perivascular fibrosis is, however, sparse or masked by fibrosis. This might have caused the decrease in immunoreactivity of laminin. On the other hand, the specimens for electron microscopy were taken from the rather superficial area of the tumor. The discrepancy of these findings between electron microscopy and immunohistochemistry might follow this sampling variation.

Liotta and Rao (1986) raised the possibility of the defective nature of newly formed tumor vessels resulting in easy intravasation by tumor cells. We did not make a quantitative assessment of the vascularity of tumor angiogenesis (Folkman 1971). Our findings, however, suggest the possible existence of a critical period after irradiation in which the selective destruction of the endothelial cells takes

place, leaving the basal lamina intact. This is not followed by prompt regeneration of the endothelial cells. During this period, the tumor cells easily penetrate the blood vessels. The extravascular and intravascular tumor cells were equally affected by irradiation. The degenerated intravascular tumor cells may not anchor the endothelial cells at the metastatic sites (Liotta and Rao 1986). Our experiments were not carried on long enough to determine the fate of intravascular tumor cells in the irradiated animals and controls. However, the formation of a multilayered basal lamina after irradiation would appear to be protective against further invasion. Our study indicates that the biologic competition between the tumor cell invasion and the host defense mechanisms is especially important during this critical period. Our experimental study which was designed to simulate the surgical cases of human cancers with the IORT was, however, complicated by the confounding factors. Future studies should be directed to the investigation of these confounding effects: the isolation of the irradiation effects on the tumor cells and the host, respectively. This should include the implantation of the previously irradiated tumor cells in the nonirradiated areas, the implantation of the nonirradiated tumor cells in the previously irradiated areas, the intravenous injections of the irradiated and nonirradiated tumor cells and the study of the metastatic sites, etc. and the long term effects of IORT.

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