The Proliferation of Capillary Endothelial Cells

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SUMMARY

Recognition of capillary endothelial cells was facilitated by a special staining procedure developed previously. Thymidine-labeling indices for endothelial cells were estimated in a number of unstimulated mouse tissues and were found to be in the range 0 to 2.4%. There was no increase in mean labeling index up to 3 weeks after 2000 rads or up to 2 weeks after 4000 rads irradiation of muscle, skin, or bone. After fracture of the femur, the labeling index of capillary endothelial cells in regenerating callus increased to about 10% at 3 to 5 days after fracture and decreased to zero by Day 16. The duration of DNA synthesis in the fractured femur was estimated in a double-labeling experiment to be 7 ± 2 hr. The results of a repeated labeling experiment suggested a turnover time of 80 ± 25 hr on the 6th day after fracture, with a wide distribution of intermitotic times about the mean value. At 3 days after injury, when the labeling index is higher, the corresponding estimate of turnover time is about 50 hr: this value is close to a previous estimate of turnover time for capillary endothelial cells of a mammary tumor growing in the same strain of mouse.

INTRODUCTION

The proliferation of capillary endothelial cells plays a central role in such diverse processes as wound healing, organ transplantation, tumor growth, and the response of tissue to radiotherapy. Indeed, the rate of extension of the capillary bed may be the limiting factor for each of these processes, inasmuch as the survival of any tissue is critically dependent on a supply of nutrients through the vascular system. However, despite the extensive studies of cell proliferation in many types of normal and malignant tissue, there have been few systematic investigations of the normal rate of proliferation of capillary endothelial cells or of their proliferative response to stimuli. In this study, we have used a special staining technique (29, 31) to obtain maximum contrast between capillaries and other tissue elements, and we have investigated the proliferation of endothelial cells in mice by the techniques of thymidine autoradiography. Data are presented for endothelial cells in a variety of normal tissues, in tissues damaged by irradiation, and in the callus formed after fracture of the femur.

PROLIFERATION OF ENDOTHELIAL CELLS IN UNSTIMULATED TISSUE

Materials and Methods. Specific-pathogen-free C3H/He mice were used in this and all other experiments. Mice of both sexes were used; they were 8 to 12 weeks old and weighed 25 to 30 g. The thymidine-labeling index was estimated for capillary endothelial cells in a number of normal tissues. Five mice were each given i.p. injections of 50 μCi of TdR\(^{-3}H\)\(^4\) and were killed 1 hr later. The following tissues were excised and placed in neutral formol-0.9% NaCl solution: liver, kidney, stomach, small intestine (jejunum), skin, muscle (both cut from the right leg), and right femur. Bones were decalcified, and 4-μ paraffin sections were cut on precleaned glass slides. Sections were dewaxed, stained overnight in Luxol fast blue solution, and then stained with the periodic acid-Schiff reaction (31). Autoradiographs were prepared by dipping in Ilford K5 emulsion, and exposure times of about 1 month were used. Slides were developed in Kodak D 19 developer and fixed in Kodak fixer. After being washed for 1 hr, the sections were stained with Harris’ hematoxylin; they were left unmounted because we have sometimes observed grain fading in mounted sections.

Endothelial cells usually were heavily labeled (> 20 grains/cell), and background was low (< 2 grains/cell). Endothelial cells were recognized and included in cell counts if they were within the walls of blood vessels which were only 1 cell thick (i.e., endothelium of arterioles and venules was excluded) and if the nuclei were long, thin, and completely surrounded by red-stained periodic acid-Schiff-positive cytoplasm. The presence of blue-stained erythrocytes within the lumen assisted with recognition of capillaries; however, we included empty capillaries in the counts if we were certain of their identity. Color photomicrographs of sections stained by the above method have been published elsewhere (29).

Results. Estimates of labeling index for each tissue are presented in Table 1; the low values imply a slow rate of proliferation. Endothelial mitoses were rarely recognized in the sections.

In many murine tissues, the duration of the S phase (T\(_S\)) has a relatively constant value in the range 7 to 9 hr (5, 24). If

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\(^{4}\)The abbreviation used is: TdR\(^{-3}H\), tritiated thymidine.

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Tissues were anesthetized with sodium pentobarbitol, and radiation was administered with a cesium irradiator, with the use of 2 opposed sources at a dose rate of 1050 rads/min. Thymidine-labeling indices for capillary endothelial cells in the skin, muscle, and femur were evaluated from groups of 3 or 4 mice that were killed at intervals up to 3 weeks after irradiation.

The techniques of histology and autoradiography were as above. In this and subsequent experiments, slides were randomized to prevent subjective bias when scoring autoradiographs.

Results. Estimates of labeling index for capillary endothelial cells at intervals after irradiation are shown in Table 2. No labeled cells were found on the 1st day after irradiation, and DNA synthesis might be inhibited for a short period. However, despite the use of high doses of irradiation, there was no apparent damage to endothelial cells and no increase in the rate of proliferation within the interval studied.

PROLIFERATION OF ENDOTHELIAL CELLS AT SHORT INTERVALS AFTER IRRADIATION

Materials and Methods. The right legs of 40 mice were irradiated with a single dose of either 2000 or 4000 rads. Mice were anesthetized with sodium pentobarbitol, and their right femurs were fractured by gentle digital pressure at the middiaphysis (17, 32). No attempt was made to splint or fix the fracture. Callus formed rapidly around the sites of fracture. Serial radiographs were taken, and the early callus developed into bony callus after about 3 weeks. Sufficient endothelial cells to determine labeling index could be found in regenerating callus from the 5th day after fracture, and the labeling index was estimated on Days 5, 7, 10, 13, and 16 after fracture. Counts of 100 to 500 cells were used to determine labeling index, except on Day 16 when fewer endothelial cells could be recognized. The probable labeling index on Day 3 within this range for endothelial cells (see also below), an approximate estimate of their turnover time \((T)\) may be obtained with the formula (28):

\[
L.I. = \lambda(T) / (T)
\] (A)

Here \(\lambda\) is a factor that for slowly proliferating populations has a value close to \(\log_2 2\) and \(L.I.\) is the labeling index.

For a labeling index of 0.4%, Equation A gives a turnover time of about 8 weeks; for the higher labeling index of endothelial cells lining the Haversian canals of the femur (2.4%), the corresponding value of \(T\) is about 10 days. These values must, however, be regarded as approximate as the duration of the S phase was not measured. Also, precise determination of labeling indices with values as low as those in Table 1 requires the counting of very many cells, and difficulties of recognition render this impractical.

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of cells counted</th>
<th>No. of labeled cells</th>
<th>Labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>500</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>500</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Stomach (serosa and mucosa)</td>
<td>294</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Small intestine (serosa and mucosa)</td>
<td>500</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Skin (from the leg)</td>
<td>562</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Muscle (from the leg)</td>
<td>300</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Bone (Haversian canals and other capillaries in the femur)</td>
<td>550</td>
<td>13</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Days after 2000 rads</th>
<th>Tissue</th>
<th>Labeling index (%)</th>
<th>Days after 4000 rads</th>
<th>Tissue</th>
<th>Labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Muscle</td>
<td>0</td>
<td>1</td>
<td>Muscle</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>0</td>
<td></td>
<td>Skin</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>0</td>
<td></td>
<td>Bone</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Muscle</td>
<td>0</td>
<td>2</td>
<td>Muscle</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>1</td>
<td></td>
<td>Skin</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>3</td>
<td></td>
<td>Bone</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Muscle</td>
<td>0</td>
<td>5</td>
<td>Muscle</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>0</td>
<td></td>
<td>Skin</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>3</td>
<td></td>
<td>Bone</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>Muscle</td>
<td>0</td>
<td>12</td>
<td>Muscle</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>0</td>
<td></td>
<td>Skin</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>5</td>
<td></td>
<td>Bone</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>Muscle</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Each estimate is based on counts of 100 endothelial cells.
Five mice were given injections on Day 3 and killed on Day 5 and the proportions of labeled cells estimated after 48 hr, the results were as shown in Table 3.

The proportion of labeled cells for animals killed on Day 7 at 48 hr after labeling is about twice the labeling index for cells injected at the same time (Day 5), with animals killed after 1 hr. An increase by a little less than 2 is to be expected if all labeled cells divide shortly after labeling and then become distributed around the cycle. The greater proportion of labeled endothelial cells in animals given injections of thymidine at 3 days after fracture suggests a higher rate of proliferation at that time; the 1-hr labeling index should then be about 11% (Chart 1).

The results of the double-labeling experiment were as follows: number of cells counted, 542; number of cells labeled with both isotopes (N), 46; number of cells labeled with 3H alone (n1), 7; number of cells labeled with 14C alone (n2), 8. The probable duration of DNA synthesis may then be calculated from the formulae:

\[
Ts = \frac{(N+n_1) + (n_2)}{(n_1 + n_2)} \text{ hr}
\]

The best estimate is obtained by combining the 2 expressions to give:

\[
Ts = \frac{(2N + n_1 + n_2)}{(n_1 + n_2)} = 7.1 \text{ hr}
\]

The standard error of the mean estimate of \( Ts \) is approximately 2 hr. The estimated duration of DNA synthesis is 7.1 hr.

The standard error of the mean estimate of the turnover time of capillary endothelial cells, a repeated thymidine-labeling experiment was started on the 5th day after fracture. Thirty mice were given repeated injections of 25 μCi TdR-3H at 6-hr intervals, and 3 mice were killed 1 hr after each injection. Autoradiographs were prepared, and the proportions of labeled cells were determined from randomized sections.

**Results.** The relation between the labeling index of capillary endothelial cells in the regenerating callus and time after fracture is shown in Chart 1. The mean labeling index decreased from a mean value of about 9% on Day 5 to zero on Day 16. When animals were given injections of TdR-3H on Days 3 and 5, and the proportions of labeled cells estimated after 48 hr, the results were as shown in Table 3.

The proportion of labeled cells for animals killed on Day 7 at 48 hr after labeling is about twice the labeling index for cells injected at the same time (Day 5), with animals killed after 1 hr. An increase by a little less than 2 is to be expected if all labeled cells divide shortly after labeling and then become distributed around the cycle. The greater proportion of labeled endothelial cells in animals given injections of thymidine at 3 days after fracture suggests a higher rate of proliferation at that time; the 1-hr labeling index should then be about 11% (Chart 1).

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\]

The standard error of the mean estimate of \( Ts \) is approximately 2 hr. The estimated duration of DNA synthesis is 7.1 hr.

### Table 3

**Proportions of labeled cells in animals given injections of TdR-3H on Days 3 and 5**

For further details, see the text.

<table>
<thead>
<tr>
<th></th>
<th>Total cells counted</th>
<th>Total positive cells</th>
<th>% labeled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five mice given injections on Day 3 and killed on Day 5</td>
<td>276</td>
<td>62</td>
<td>22.5 ± 2.5°</td>
</tr>
<tr>
<td>Five mice given injections on Day 5 and killed on Day 7</td>
<td>346</td>
<td>64</td>
<td>18.5 ± 2.1</td>
</tr>
</tbody>
</table>

° Mean ± S.E.

*From equation C* 

Thus 

\[
\log_e(TS - 1) = \log_2 - \log_2(\frac{n_1}{N}) + (\frac{n_2}{N})
\]

Differentiating 

\[
\frac{\Delta TS}{TS - 1} = \frac{\Delta(n_1/N) + \Delta(n_2/N)}{(n_1/N) + (n_2/N)}
\]

Binomial estimates for the standard errors of \( n_1/N \) and \( n_2/N \), ie.: 

\[
\Delta(n_1/N) = (\sqrt{n_1} / (N - n_1))(N^{1/2})
\]

\[
\Delta(n_2/N) = (\sqrt{n_2} / (N - n_2))(N^{1/2})
\]

\[
give \Delta TS = 2.0 \text{ hr.}
\]
The present estimates of thymidine-labeling index for capillary endothelial cells in unstimulated mouse tissue were mostly in the range 0 to 0.4%, and values of turnover time are probably about 2 months or more. Our mice, weighing 25 to 30 g, grew slowly, but mainly by accumulation of fat. However, estimates of the distance between tattoo marks on the legs have demonstrated slight growth of bones in mice of
this size (S. Hayashi, unpublished information); this growth may be reflected by the higher labeling index (and presumably, therefore, shorter turnover time) observed for endothelial cells in the femur. In Table 4 we have listed published estimates of thymidine-labeling index for endothelial cells in a variety of species; most of the values obtained in mature animals are less than 1%, confirming that unstimulated endothelium generally has a slow turnover.

It has been proposed by many authors that capillary damage might be responsible for some of the dose-limiting late effects observed in normal tissues after radiotherapy. Changes in capillary function, such as permeability or blood flow, are often observed shortly after irradiation (6, 15, 18), but the lysis of capillary endothelial cells is seldom seen. However, Phillips (21, 22) has reported that rats irradiated with 1600 rads or more, and protected from acute death by hypoxia, died at 3 to 6 months from acute radiation pneumonitis, with complete loss of endothelium from the alveolar capillaries. Strong correlations between visible capillary damage and late radiation necrosis of brain (19) or late myocardial fibrosis (10) have also been observed.

From the present data and from values of labeling index listed in Table 4, it appears that the turnover time of unstimulated capillary endothelium is typically a few months. In some tissues an increased rate of proliferation has been observed at short intervals after irradiation (2), but we found no significant changes in labeling index up to 2 to 3 weeks. Other authors (10, 16) have also found no tendency for increased proliferation of endothelial cells at least up to 40 days after irradiation. The interval between irradiation and observation of acute capillary damage might therefore reflect the time for a critical number of damaged endothelial cells to enter mitosis and then become pycnotic. This effect is probably cumulative, with other endothelial cells being stimulated to divide in an attempt to replace those that have died at mitosis. Thus, Fajardo and Stewart (10) have observed some increase in thymidine-labeling index at 40 to 70 days after 2000 rads irradiation to rabbit hearts, and this increased proliferative activity is followed by the appearance of myocardial fibrosis. Cumulative pycnosis of capillary endothelial cells could lead to complete breakdown of the capillary circulation, and nutritional failure might then lead to the delayed radiation necrosis of parenchymal cells that has been observed in many tissues.

The maximum labeling index of capillary endothelial cells in response to the mechanical injury of fracture of the femur (about 11%) was the same as the labeling index observed previously in a transplanted mouse mammary tumor growing in the same C3H strain of mouse (30); the corresponding value of turnover time was about 50 hr. In the tumor, well-nourished carcinoma cells had a much higher rate of proliferation and slowing of tumor growth resulted from nutritional failure as the separation between neighboring capillaries increased (30). In the regenerating callus, the periendothelial cells have likewise been reported to have a higher rate of proliferation than endothelial cells (32). Thus the proliferation of capillary endothelial cells may be rate limiting for both tumor growth and healing of fracture.

The relation between labeling index and time after fracture (Chart 1) suggests that the proliferation of endothelial cells might be dependent on the release of a stimulatory factor (or repression of an inhibitor) which then decays as healing takes place. Continuous proliferation of endothelium in the tumor at a rate equal to the maximal response to injury would be consistent with the continuous release of an angiogenesis factor by tumor cells. There is now convincing evidence for a stimulatory angiogenesis factor released by tumor cells. In experiments where tumor pieces were implanted in Millipore filters, vascularization in surrounding normal tissue was observed (12, 14); implantation of normal tissue within such filters gave negative results (12). The stimulatory factor was recently isolated from several tumors and was found to be a soluble molecule the important component of which was an RNA-protein complex (12). However, if the same factor is responsible for angiogenesis in response to injury, it must also be present in normal tissue, or its production must be stimulated by the injury. The lack of an acute proliferative response to damage by radiation suggests that the release of an angiogenesis factor might follow the expression rather than the induction of vascular damage. With fracture of the femur the expression of damage is immediate and acute, but after irradiation it appears to be delayed and chronic.

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