Aortic Endothelial Cell Replication

I. Effects of Age and Hypertension in the Rat

STEPHEN M. SCHWARTZ AND EARL P. BENDITT

SUMMARY The daily rate of cell replication in the aortic endothelium of the normal rat falls from a maximum of 13% at birth to 0.1-0.3% at age 5-6 months. This residual rate of replication largely represents the turnover rate of the endothelium in normal animals. After renal hypertension of 2-3 weeks duration, however, the rate of replication rises 10-fold to an average value of 1.6%. This increase may represent an increase in turnover; however, it probably represents, at least in part, a proliferative response to cover the expanded luminal surface of the dilated vessel.

ANTIHYPERTENSIVE drugs provide sufficient control of hypertension to avert most acute manifestations of the disease in human beings. This clinical success, however, does not prevent chronic vascular injury. Myocardial infarction and cerebrovascular disease still occur at an accelerated rate and account for premature death. This implies that humoral or hemodynamic changes in hypertension, even with moderate elevations in pressure level, interfere with the function of components of the vascular wall. For example, medial smooth muscle of the hypertensive vessel shows cell growth and connective tissue biosynthesis during the early stages of hypertension. The hyperplastic response is transient, but connective tissue synthesis continues to occur to the degree necessary to compensate for changes in wall tension. The presence of hypertension also accelerates the accumulation of smooth muscle cells to form arterial lesions in lipid-fed animals. Somewhat less is known about the effects of hypertension on the endothelium. We do know that permeability is increased. Studies using fluorescein or Evans blue-labeled serum proteins have indicated the presence of focal regions of increased permeation in arteries of animals with acute renal hypertension. Furthermore, there is evidence of transient increases in endothelial permeability following administration of hypertension-producing doses of angiotensin II. Taken together with the apparent delicate structure of the endothelium, these studies suggest that continuity of the endothelial cell layer is damaged in hypertension.

The objective of this study is to provide a quantitative measure of this injury by examining the ability of endothelium to maintain continuity in normal and hypertensive rats. This is done by examining endothelial cell replication and turnover using tritium-labeled thymidine (\(^{3}\text{H}-\text{TdR}\)) to detect replicating cells. As we will show, rats with acute renal hypertension show a 10-fold increase in replication rate as compared with normotensive controls of the same age. The significance of this increase is discussed in terms of the normal process of cell replication in young and aging rats.

Methods

ANIMALS

All experiments were performed on female Sprague-Dawley rats. Except in the aging study, rats 5-6 months of age were used. The aging study used rats ranging from newborn (less than 1 day old) to 1 year of age.

LABELING SCHEDULES

Tritium-labeled thymidine (\(^{3}\text{H}-\text{TdR}\), New England Nuclear, 6.7 Ci/mmol) was dissolved in saline and administered by ip injection. Each dose consisted of 50 /\text{Ci}/\text{g} \(^{3}\text{H}-\text{TdR}\)/100 g. The same dosage (50 /\text{Ci}/\text{g}) was used in all experiments. In all, three different dose schedules were used in the various experiments. The various dosage schedules are summarized in Table 1.

Experiment I was designed to see if labeled cells replicate. In the first group the rats received only a single dose and were killed 1 hour later. A second, comparable group treated at the same time as the first group was killed 24 hours after the same single dose.

Experiment II was designed to determine the effect of age on labeling index. The dosage schedule consisted of three doses of \(^{3}\text{H}-\text{TdR}\) (each dose: 50 /\text{Ci}/\text{g}) at 8-hour intervals. This schedule was chosen to minimize diurnal variation and to provide large enough numbers of cells to permit accurate counting. In addition, if we assume that S phase is about 8 hours long, this schedule provides an estimate of the number of cells which replicate each day.

Experiment III was designed to determine the retention or loss of labeled cells. The rats received the usual schedule of three doses but were not killed until either 24 hours or 24 days after the final dose. The group killed at 24 hours was designed to allow cells to complete the initial cell cycle begun during the labeling interval (as in experiment I). Thus, any further change in thymidine index could be attributed to further cell divisions or loss of cells from the surface.

Experiment IV was designed to assay the effect of hypertension. The same dosage schedule was used as in experiment II.
Table 1  Experimental Protocols

<table>
<thead>
<tr>
<th>Dosage Schedules</th>
<th>Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>dose 24 hours 0</td>
<td>I. Replication of labeled cells</td>
</tr>
<tr>
<td>24 hours 1h 1h</td>
<td>II. Effect of age</td>
</tr>
<tr>
<td>dose 8h 8h 24h</td>
<td>III. Retention of labeled cells</td>
</tr>
</tbody>
</table>

θ = fixation by perfusion.

Fixation Procedures

The procedures for fixation by perfusion with formaldehyde-glutaraldehyde solution at physiological pressures and flow rates were as previously described. Under ether anesthesia, a catheter was inserted in the carotid artery. A flowmeter was used to estimate the pressure drop between the perfusion bottle and the catheter tip. This provided an accurate estimate of intravascular pressure. For all rats, including those with hypertension, vessels were fixed at an intraaortic pressure of 90-100 mm Hg and at flow rates of 20-40 ml/min over an interval of 5-10 minutes. The hypertensive rats were fixed at "normal" pressures because of variability in pressures measured in anesthetized rats at time of sacrifice. The procedures for newborn and 2-week-old rats were similar except that the catheter was inserted in the left ventricle.

Tissue Samples

After removal of the vessel, all aortas except those of newborn and 2-week-old rats were divided into five pieces: A, B and C represented three equal segments of thoracic aorta; D represented the upper portion of the abdominal aorta and E represented the lower portion of the abdominal aorta, including the trifurcation. Aortas of newborn rats were redivided into equal upper and lower segments.

Measurement of Vessel Area and Cell Density

Vessels used for measurement of aortic surface area were removed from the rat as described above, except that the aortas were not divided into segments. The aorta was cut just below the subclavian artery and just above trifurcation. This single, large piece was pinned out flat on a Teflon sheet without distending the vessel beyond its fixed dimensions. Measurements of length and width were made directly on the vessel at several points and transferred to graph paper. Area was measured from the drawings on the graph paper.

Cell density (CD), the average number of cells per unit area, was obtained from enface preparations as described below under Collection of Data.

Tissue Preparation

Our procedure for preparation of endothelium for autoradiography has also been described in detail. In brief, it consists of three steps: (1) Removal of endothelium: The aorta is opened along the dorsal margin and pinned out flat. The tissue is dehydrated and the endothelium is embedded in a layer of collodion. The collodion film is stripped away from the aorta and removes the endothelial layer as a sheet. (2) Exposure of luminal surface of endothelial cells: At this point, the preparation consists of endothelial cells with their luminal surface embedded in collodion. In order to remove the collodion, we first fix the exposed subendothelium to a glass slide using a layer of gelatin which is then polymerized with formaldehyde. Once the subendothelium is fixed to the slide, the collodion is removed by dissolving it in 1:1 ether/ethanol. (3) Autoradiography: This leaves a sheet of endothelium with the luminal surface directly exposed and available for coating with emulsion for autoradiography. The cells are preserved in their original distribution and most or all cells are included. The preparations are subsequently coated with Kodak NTB-2 emulsion and exposed for 2 weeks. The preparations are then developed, stained with hematoxylin and mounted under coverslips.

Collection of Data

Segments from which extensive portions of the endothelium had not been removed by the collodion layer, or in which the endothelium was obscured by folding, were
discarded. This may produce some bias in some calculations, since most frequently the discarded portions represented D or E segments. For this reason, data is presented for individual segments in Figure 5. Approximately 75% of the tissue samples prepared in this way were suitable for counting. Cell counts were made using a square-shaped reticle in a 10× eyepiece with a 40× objective. The square field was systematically moved over the entire specimen to count the total number of labeled cells (N*) and the total number of fields (Nf). Then five horizontal rows were chosen at different levels along the length of the specimen. The total number of cells in the five rows was divided by the number of fields to obtain an average cell density (CD) for the entire specimen. Finally, the total number of cells in the specimen (N) was determined as the product of CD and the total number of fields, N = CD × Nf. The thymidine index was calculated as the fraction of cells labeled N*, divided by the total number of cells, N, i.e., thymidine index = N*/N. Statistical comparisons between groups of rats were made by the Student's t-test.

SURGICAL PROCEDURE, HYPERTENSIVE RATS

"Normotensive" rats were selected for this experiment by measuring the pressure (see below) at least three times over the week prior to surgery. Only those with pressures consistently below 110 mm Hg were used. Typical rats showed normal pressures of 90–100 mm Hg. The surgical procedure began with intraperitoneal anesthesia with pentobarbital (dose, 30 mg/kg). The renal artery was exposed through a dorsal incision and the artery was dissected free. A figure-8 suture, 000 braided silk, was placed about the vessel snugly but without totally occluding flow. The muscle layers and skin were closed with 000 monofilament nylon. Sham-operated rats were treated in the same manner; this included dissection of the artery, but no suture was placed about the vessel. Operated rats were defined as hypertensive once the pressure remained over 130 mmHg for 2 weeks. (2) At the time of death, blood pressure was measured directly via an indwelling catheter over a period of 2 weeks. (3) Again, the pressure was measured twice weekly. The pressure of normotensive, sham, or unoperated control rats was the mean of three consistent measurements. Measurements were made biweekly.

MEASUREMENT OF PRESSURE

Blood pressure was measured by two techniques. (1) Prior to death, pressures were measured by a tail-cuff method using a piezoelectric pressure transducer to detect pulsations distal to the cuff. The rats were lightly anesthetized with ether and tail temperature was maintained with an electric heating pad. After the surgical procedure, pressures in hypertensive, sham, or unoperated control rats were measured at least twice weekly. The pressure of normal rats was the mean of three consistent measurements over a period of 2 weeks. (2) At the time of death, pressure was measured directly via an indwelling catheter in the carotid artery. This procedure proved less reliable than the method described above. In no cases was the pressure higher than the value obtained by the indirect method; frequently, however, it was lower. The difference in the two measurements was attributed to variations in depth of anesthesia.

<table>
<thead>
<tr>
<th>Hours</th>
<th>Thymidine index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.07% ± 0.04</td>
</tr>
<tr>
<td>24</td>
<td>0.17% ± 0.02</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE.

* Based on average of all segments in each rat.
the number of cells per unit area, the "cell density," in rats of different ages. If cell density were constant in all portions of the aorta, then the total number of cells could be determined as the product of area and cell density. Unfortunately, we found that cell density varies as a function of position along the length of the aorta as is seen in Figure 3. As a result, it is difficult to estimate the absolute number of endothelial cells per vessel. However, cell density distributions at 6 and 12 months are quite similar, both terms of the number of cells per unit area and the distribution along the aortic length. During the same time, the area of aortic surface increases, on the average, by 14% (area in mm² ± 1 se: 6 months, 378 ± 39; 12 months, 431 ± 31). Thus the rate of growth in total cell number, on the average, is approximately 14%. Similarly, between 1 and 2 years there is, if anything, a decrease in cell density while the average increase in area is 9% (area in mm² ± 1 se: 24 months, 470 ± 23). Although the variance of the area measurements is quite high, there appears to be little growth in total cell number between age 6 months and 1 year. In the second year of life, with increasing area and decreasing cell density, it is conceivable that the endothelial cell population is constant in number and is being diluted in density by an age-associated increase in aortic surface area. In contrast, total body weight of these rats continued to increase between 6 months and 2 years of age (weight in grams ± 1 se: 6 months, 293 ± 12; 12 months, 322 ± 13; 24 months, 371 ± 18).

RETENTION OF LABELED CELLS

The purpose of this experiment was to determine the fate of labeled cells. The usual schedule of three doses was given to 12 rats to label all cells entering S phase over a 24-hour interval. Six rats were sacrificed 24 hours after the final dose, because experiment I had demonstrated that labeled cells would have completed an initial cell cycle by this time. Thus any changes in thymidine index after this time would be due either to loss of cells from the surface or to further cell division. The remaining six rats were allowed to survive for an additional 24 days before they were killed. As can be seen in Table 5, the thymidine index at 24 days is only 1/3 of the value found at 24 hours after labeling.

The first conclusion drawn from this experiment is that something has happened to the labeled cells. Either they have been lost from the surface or they have divided sufficiently rapidly to allow dilution of label below the minimum required for autoradiography. It is also evident that the process producing disappearance of cells must be nonrandom. For example, we know that the total number of cells is either constant or increasing. Therefore the average rate of cell loss, i.e. turnover, can be no greater

![Figure 1: Proliferation vs. turnover. The progeny of endothelial cell replication may have one of three fates: As shown in A, the progeny may be retained in the population without loss of other cells. This results in proliferation, that is, an increase in total cell number. As shown in B, the progeny may be retained without loss of progeny but with a reciprocal loss of cells elsewhere on the surface producing a form of "turnover" similar to cell replacement in gut epithelium. Finally as shown in C, the progeny may be produced in "high turnover" areas. In this model the progeny themselves are more likely to be lost from the surface than are cells from other areas with low turnover.](image1.png)

![Figure 2: Effect of age on endothelial cell replication. Data shown mean ± se for female rats between 24 hours and 12 months after birth. Numbers of rats at each age include: newborn, 5; 2 weeks old, 9; 4 weeks old, 9; 3 months old, 6; 5 months old, 6; 5½ months old, 4; 6 months old, 7; 1 year old, 6.](image2.png)

**Table 3** Thymidine Index as Function of Age

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Number of rats</th>
<th>Percent thymidine index</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–6</td>
<td>16</td>
<td>0.15 ± 0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± se. Thymidine index is based on the average for all segments of aorta in each rat.

**Table 4** Blood Pressure as Function of Age

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Number of rats</th>
<th>Pressure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–6</td>
<td>26</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>110 ± 4</td>
</tr>
</tbody>
</table>

* Determined by tail-cuff method; mean ± se.
that way diluting the label below the minimum required for counting. Might be explained by their dividing several times over the 3 weeks and in replications of the initially labeled cells. That is, the loss of labeled cells calculate compound interest. Note that this equation would also describe the rate of loss of labeled cells if the label itself were lost due to serial sections. Assume no cell loss; this would imply that the progeny of the originally labeled cells have added 5% to the total number of aortic endothelial cells over a 3-week interval. This is far in excess of the apparent rate of growth of the aortic endothelium. Thus it is not likely that proliferating cells could replicate enough times to produce the apparent loss of labeled cells.

In summary, the loss of labeled cells must result from cell turnover. This could, however, occur in two ways. The replicating cells could be located in areas of the surface or subpopulations of the aortic endothelium which are subject to a high rate of cell loss (Fig. 1B). Alternatively, the dividing cells could belong to germinal centers, similar to the intestinal crypts, that is, areas of cell growth which provide new cells to replace cells lost elsewhere in the endothelial surface (Fig. 1C).

EFFECTS OF HYPERTENSION

The purpose of this experiment was to obtain an estimate of the daily replication rate in rats with acute sustained hypertension. Rats 5-6 months old, were used in order to minimize the contribution of cell replication to growth (experiment II). The rats were killed within 2 weeks after their blood pressure exceeded a value of 130 mm Hg. Since this usually required about 2 weeks after surgery, the total interval ranged from 4-6 weeks.

Two groups of hypertensive rats were studied. The initial group consisted of 12 age-matched rats and included 4 operated rats, 4 sham-operated rats and 4 unoperated controls. The 4 operated rats showed an increase in thymidine index compared to either unoperated controls or sham-operated rats (Table 6). The second group of rats consisted of a series of 20 additional operated hypertensive rats without paired controls. This larger group was added to the rats in Table 6 to provide more extensive data for comparison with data from normal rats of the same age (experiment 2). As can be seen in Figure 4, hypertensive rats of ages between 5 and 6 months showed a 5- to 10-fold increase in replication rate compared with age matched controls.

Since the observations included cells from the entire aortic surface, data from all hypertensive rats and from controls aged 5-6 months were used also to examine the effect of location on the aortic surface on the rate of
TABLE 6  Hypertensive Rats vs. Sham and Unoperated Controls

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Blood pressure*</th>
<th>Thymidine index (%)</th>
<th>Blood pressure</th>
<th>Thymidine index</th>
<th>Blood pressure</th>
<th>Thymidine index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>145</td>
<td>1.8</td>
<td>100</td>
<td>0.05</td>
<td>100</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>165</td>
<td>2.4</td>
<td>120</td>
<td>0.07</td>
<td>100</td>
<td>0.11</td>
</tr>
<tr>
<td>3</td>
<td>190</td>
<td>1.1</td>
<td>100</td>
<td>0.04</td>
<td>92</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>110†</td>
<td>0.3</td>
<td>90</td>
<td>0.08</td>
<td>100</td>
<td>0.10</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.4</td>
<td></td>
<td>0.06</td>
<td></td>
<td>0.14</td>
</tr>
</tbody>
</table>

* By tail-cuff method.
† Daily replication rate, average of all segments of the aorta in each rat.
‡ Rat 4 did not meet our experimental criteria for hypertension as an elevation to >130 mm Hg. However, this rat was included in the evaluation of data to preserve the pairing with unoperated and sham-operated controls.

endothelial cell replication in normal and hypertensive rats (Fig. 5). The hypertensive rats showed an elevated rate of replication in all segments examined. The usual regional variations seen in nonhypertensive controls, however, were maintained. Thus in both hypertensive and normotensive rats, the replication rate was lower in the abdominal segments of aorta (D and E) than in the thoracic segments. It is interesting to note that the area of the trifurcation (E) showed a lower replication rate than other segments. This was true both for hypertensive and normal rats, although again the replication rate was higher in the endothelium of hypertensive rats.

The presence of a relatively low rate of replication near...
FOCAL REGIONS OF ENDOTHELIAL CELL TURNOVER

We attempted to estimate the amount of turnover more directly in experiment III. In this experiment rats were killed 24 hours and 24 days after labeling. The number of labeled cells decreased by 1/3 over the 3-week interval. Unless "H-TdR is toxic to the labeled cells, these data support the idea that the daily replication rate largely represents cell turnover. Moreover, as presented in Results, the data imply that the replicating cells occur as clusters of aortic endothelial cells which are subject to high rates of cell turnover. This is consistent with other data from our laboratory based on maps of the distribution of labeled cells on the aortic surface.9,14 These maps showed focal areas where as many as 10-50% of the cells were labeled, while other areas showed few or no labeled cells. It is reasonable to postulate that the pool of rapidly turning over cells which was demonstrated in this study corresponds to the clusters of labeled cells demonstrated in the maps. In summary, the normal aortic endothelium of the adult rat contains focal areas of increased cell turnover.

EFFECTS OF HYPERTENSION

The principal effect reported here is a 10-fold increase in the replication rate of endothelium in hypertensive rats. One interpretation of this observation is that, in some way, hypertension causes endothelial cell damage and that this "damage" is reflected by an increased rate of cell turnover. If this is true, then the mean life span of endothelium has gone from 1-3 years to only 100 days (life span = reciprocal of replication rate, assuming no cell proliferation). Moreover, if this increased turnover occurs in clusters, as described above, there should be focal areas where the mean life span is extremely short. This is important since a rapidly turning over endothelium may be unable to maintain itself as a continuous cell layer. Furthermore, even in the presence of a "continuous" endothelium, regenerated cells may be abnormal in structure and function. Our ultrastructural studies have shown defects in the intracellular junctions of regenerated endothelium as late as 6 months after mechanical denudation.15 Similar physiological studies show increased permeability to plasma proteins even after continuity of injured endothelium has been reestablished.16 Thus an increase in turnover would be expected to have important consequences for endothelial function.

Increased turnover, however, is not the only possible interpretation of increased replication. The change in replication could equally represent a purely proliferative response. That is, an increase in the number of endothelial cells could occur because of proliferation in compensation for stretching of the wall and the resulting increase in surface area. Similar changes have been described for medial smooth muscle. Wolinsky has shown an increase of 25% in aortic diameter of Goldblatt hypertensive rats 2.5 months after surgery. During this acute phase, smooth muscle cells undergo proliferation and hypertrophy. By 16 months after surgery, however, the blood pressure stabili-
lizes at a new level and smooth muscle cell growth ceases. The result is an increase in wall thickness in proportion to elevated pressure and dilation of the vessel. The increased thickness is adaptive, producing a value for wall stress no different from that of control animals. Once stress has returned to normal, cell growth and connective tissue production also return to normal. Similarly, a 25% dilatation of the aortic diameter will produce a 25% increase in the surface area which must be covered by endothelium. By analogy to the behavior of smooth muscle cells, an increased replication rate in the endothelium then may represent a transient hyperplastic response required to reestablish a normal number of cells per unit surface area.

Finally, if the increase in cell replication is due to endothelial injury, it is important to consider possible eliciting factor(s). An increase in turnover might result directly from elevated pressure. However, it is hard to imagine that the endothelial cell is damaged by the relatively small changes in absolute levels of pressure seen in this experiment. Endothelial cells sit inside a rigid tube formed by the elastic layers of the tunica media. It is likely that most of the gradient of pressure drop across the wall occurs in the media rather than across the fragile endothelium. This implies that if hemodynamic parameters are important, parameters other than the absolute pressure level are more likely to cause injury. On the other hand, there is evidence that components of the renin-angiotensin system may cause vascular injury. Elevated levels of renin have been implicated in accelerated atherosclerosis in man and renin or angiotensin can induce increased endothelial permeability in small arteries and the aorta. Moreover, renin is elevated in the early stages of renal hypertension, although it falls to normal in later stages. Thus it is possible that the increased replication seen here represents a response to the transient elevation of renin secretion. If this is so, then, as in the case of increased proliferation in response to dilation, one would expect to find a normal rate of replication in operated animals with chronic, stable hypertension. Experiments to test these possibilities are now under way.

References