Leaky endothelial junctions occurring during cell turnover have been postulated to be a major pathway for enhanced lipoprotein transport across the vascular endothelial layer, which leads to the development of atherosclerosis. Because hypertension has been well documented as one of the major risk factors for atherosclerosis, we explored the possibility that hypertension accelerates atherogenesis by increasing the turnover of endothelial cells and hence the transendothelial macromolecular permeability. The investigations were performed on thoracic aortas of 10 male 3-4-month-old spontaneously hypertensive rats and eight male age-matched Wistar-Kyoto normotensive rats. In en face preparations of aortic specimens, mitotic endothelial cells were identified by hematoxylin nuclear staining; dying or dead endothelial cells containing cytoplasmic immunoglobulin G were detected by indirect immunoperoxidase technique; and endothelial leakage to Evans blue-albumin conjugate was visualized by fluorescence microscopy. The number of leaky foci per unit endothelial surface area in spontaneously hypertensive rats was found to be approximately three times that in Wistar-Kyoto control rats; the frequencies of both endothelial cell mitosis and death in spontaneously hypertensive rats were also approximately three times the corresponding values in Wistar-Kyoto rats. These findings indicate that hypertension in spontaneously hypertensive rats is accompanied by increased endothelial cell turnover and an attendant enhancement of permeability to macromolecules. (Hypertension 1990;16:154–161)

Risk factors for atherogenesis generally refer to conditions that have been demonstrated by epidemiological studies and statistical procedures to increase the susceptibility of an individual to the morbidity and mortality of atherosclerosis. There are several independent risk factors for the development of atherosclerosis in individuals within a population. Hypertension has been well established as one of these major risk factors. This has been demonstrated in both sexes and among various racial groups. The risk increases further if hypertension is present along with other established risk factors such as hypercholesterolemia and cigarette smoking. A number of randomized controlled trials of treating hypertension have shown that there is a significant decrease in both cardiovascular morbidity and mortality after achieving and maintaining a diastolic blood pressure less than 90 mm Hg.

Damage to endothelial cells, increased endothelial permeability, the “trap-door” effect, remodeling of the endothelial layer, and altered chemical groups on the luminal plasma membrane have been implicated as important pathogenic factors that contribute to the acceleration of atherogenesis by hypertension. Most of these studies were performed on acute, rapidly evolving forms of experimental hypertension such as deoxycorticosterone acetate–salt hypertension, aortic constriction, and renovascular hypertension. The vascular changes of gradually evolving, chronic hypertension, which is the most common form of hypertension in humans, have not been adequately studied.

In a series of recent investigations, leaky intercellular junctions occurring during endothelial mito-
sis have been demonstrated to be a significant pathway for enhanced transendothelial transport of macromolecules, including low density lipoproteins; this macromolecular leakage may lead to lipid accumulation in the intima and the development of atherosclerosis. There were also experimental investigations implicating that injured or dying endothelial cells, even though they were not associated with demonstrable areas of denudation, might become a leaky pathway for macromolecules. The present investigation was designed specifically to test whether hypertension exacerbates the development of atherosclerosis by increasing the turnover rate and hence the macromolecular permeability of endothelial cells in the aortas of spontaneously hypertensive rats (SHR), a commonly used model of human essential hypertension.

**Methods**

**Animals**

Ten male SHR, aged 3–4 months and weighing 257–333 g (mean±SD 293±25 g), were used as the experimental group. Their blood pressures ranged from 155 to 250 mm Hg with a mean (±SD) of 182±26 mm Hg. These hypertensive rats were inbred descendents of the Wistar strain originally developed by Okamoto and Aoki. Eight male, age-matched, normotensive (blood pressure 111±8 mm Hg) Wistar-Kyoto (WKY) rats, weighing 287–405 g (mean±SD 335±40 g), were used as the control group. All rats were fed on regular rat chow and provided with drinking water ad libitum.

**Experimental Procedures**

Evans blue–albumin (EBA) conjugate was prepared by adding 0.5 g bovine serum albumin and 0.1 g Evans blue (E-2129, Sigma Chemical Co., St. Louis, Mo.) to 50 ml normal saline and mixing well. The experiments were performed while the rats were under pentobarbital anesthesia (30 mg/kg i.p.). The right femoral artery and left femoral vein were cannulated with 22-gauge needle catheters and PE-50 polyethylene tubing. The right carotid artery was also cannulated with a 20-gauge needle catheter and PE-90 polyethylene tubing. Blood pressures were measured directly through the carotid artery catheter connected to a Gould 2800S blood pressure recorder (Cleveland, Ohio). The needle catheter placed in the femoral vein was used for intravenous injection of macromolecular tracer; the needle catheters that were placed in the femoral artery and the femoral vein both served as the egress routes during perfusion. A total of 5 ml EBA solution was slowly injected into the left femoral vein. At 3 minutes after EBA injection, an overdose of pentobarbital was given. Shortly before the rat was killed, heparin (1,000 USP units) was injected intravenously through the femoral vein catheter to prevent intravascular blood coagulation. The carotid artery catheter was connected to a pressure reservoir and was perfused immediately with a heparinized saline solution at a pressure of 110 mm Hg until clear fluid emerged from the egress sites (approximately 10 seconds). The perfusate was then switched to 10% formaldehyde, which was perfused at the same pressure and a flow of approximately 20 ml/min for 10 minutes for preliminary perfusion fixation. After perfusion fixation, the aorta was excised between the aortic root and the diaphragm. The cane-shaped aortic specimen was removed and immersed in 10% formaldehyde for 1 hour before processing for detection of dying or dead endothelial cells.

**Methods for Detecting Dying or Dead Endothelial Cells**

The indirect immunoglobulin G (IgG) immunocytochemistry developed by Hansson and Schwartz was used as a quantitative method for identifying dying or dead endothelial cells in the aortic endothelium.

**Reagents.** The IgG antibodies used in the present study were rabbit anti-rat IgG and horseradish peroxidase–conjugated goat anti-rabbit IgG, which were purchased from Sigma. The 1-mg vial of rabbit anti-rat IgG was brought to a volume of 1 ml with distilled water. These antibodies were used at dilutions of 1:250 in phosphate buffered saline (PBS [120 mmol/l NaCl and 2.7 mmol/l KCl in 10 mmol/l phosphate buffer, pH 7.4], Sigma) containing 1% ovalbumin (Sigma).

**Preparation of aortic tissue for indirect immunocytochemistry.** After 1 hour of immersion in 10% formaldehyde, the adventitial tissue was gently removed with fine forceps under a dissecting microscope. The thoracic aorta was excised and cut open longitudinally along the ventral surface. The aortic specimen was dissected into five pieces. For en face preparation, each aortic piece was pinned with the endothelial side up onto a dental waxplate and rinsed four times for 15 minutes each with PBS. Four aortic pieces were incubated with rabbit anti-rat IgG at a dilution of 1:250 in PBS containing 1% ovalbumin for 90 minutes. One aortic piece was used as control and incubated in PBS containing rabbit IgG (Sigma) or at a dilution of 1:250 in PBS containing 1% ovalbumin for 90 minutes. After the first incubation, the aortic pieces were rinsed three times for 10 minutes each with PBS. Then, all five aortic pieces, including the control piece, were incubated for 90 minutes with horseradish peroxidase–conjugated goat anti-rabbit IgG at a dilution of 1:250 in PBS containing 1% ovalbumin. After the second incubation, the aortic pieces were rinsed three times for 10 minutes each with PBS. Horseradish peroxidase was visualized by a modification of the method described by Grahan and Karnovsky. The aortic specimens that had been treated with horseradish peroxidase–conjugated goat anti-rabbit IgG were first incubated for 15 minutes in 0.5 mg/ml diaminobenzidine in 50 mM Tris buffer, pH 7.6, and then for 3 minutes in the same solution containing 0.02% hydrogen peroxide. After repeated rinsing with PBS, the specimens were immersed in 10% formaldehyde overnight before...
hematoxylin staining and viewing under a fluorescence microscope.

Quantification of immunoglobulin G–containing dying or dead endothelial cells. After overnight immersion in 10% formaldehyde, the aortic specimens were rinsed with PBS and stained with Harris' hematoxylin for 30 seconds. IgG-containing endothelial cells (as revealed by horseradish peroxidase staining) and mitotic endothelial cells (as revealed by hematoxylin staining of mitotic nuclei) were identified and counted on en face preparations of the thoracic aorta under a fluorescence microscope equipped with a micrometer in the eyepiece. The distribution of IgG-containing endothelial cells and mitotic endothelial cells was mapped as a function of topographic locations in the aorta and correlated with the distribution of macromolecular tracer (EBA) leaky foci.

Criteria for Identifying Mitotic Figures and Immunoglobulin G–Containing Dead Endothelial Cells

With hematoxylin staining, different stages of endothelial cell division in the mitotic phase of the cell cycle in en face preparations of the aorta were identified according to the following morphological criteria.20 Cells with condensed chromosomes and an intact nuclear envelope were defined as being in prophase. Cells with chromosomes aligned at a metaphase plate halfway between the poles and without a nuclear envelope were defined as being in metaphase. Cells with separated chromatids being pulled toward the poles were defined as being in anaphase. Cells with chromatids clustered at each pole, a contractile ring creating cleavage furrow, and a reformed nuclear envelope were defined as being in telophase. Cells with brown-stained cytoplasm and normal-shaped or pyknotic nuclei were defined as IgG-containing dying or dead endothelial cells.

Fluorescence Microscopy

A Nikon epifluorescence microscope (Microphot-FX, Nikon Corp., Tokyo, Japan) equipped with a micrometer in the eyepiece was used for en face observation. All mitotic and dying (IgG-positive) cells, as well as EBA leaky foci, were registered by using one visual field confined by the micrometer (0.5 mm × 0.5 mm) at ×200 magnification as the surface area unit. Then the entire endothelial surface, including the branching region (an area of 2.5 mm × 2.5 mm with the orifice in the center), of each aortic segment was carpet-scanned under the fluorescence microscope. The numbers of EBA leaky foci, mitotic endothelial cells, and IgG-containing dying or dead endothelial cells were counted. The EBA fluorescence was detected with a combination of an excitation filter at 510–560 nm, a dichroic mirror at 580 nm, and a barrier filter at 590 nm. Color slides were made with Kodak Ektachrome P800/1600 professional color reversal films for photomicrographs.

Results

The major aim of this investigation was to study whether endothelial macromolecular permeability and turnover rate increase concordantly in SHR. We used the frequencies of cell mitosis and cell death to represent the degree of cell turnover instead of tritiated-thymidine uptake autoradiography, which has been commonly used as an index of cell turnover, because it has been shown that leakage only occurs in specific periods in the cell turnover cycle,12 such as the mitotic phase and cell death.

To confine the leaky foci to small spots, we allowed EBA to circulate for only 3 minutes; the results
Figure 2. Fluorescence photomicrograph showing same field as in Figure 1. Note endothelial leakage to Evans blue-albumin that corresponds to the two mitotic cells.

showed that fluorescent leaky spots correlated well with endothelial cell mitosis (Figures 1 and 2), similar to previous findings in which 5 minutes was allowed for EBA circulation time.\textsuperscript{11} Transverse cryosection of the thoracic aorta demonstrated the deposition of fluorescent EBA molecules in the subendothelial space with marked lateral spread and moderate radial diffusion (Figure 3).

Dying cells, usually appearing in clusters, also showed considerable correlation with leakage (Figures 4 and 5); however, whether leakage was through permeabilized cell membrane or altered cellular junctions cannot be concluded.

A comparison of the findings of SHR and WKY rats showed EBA leaky foci per unit endothelial surface area 2.8-fold larger in SHR (7.46/mm\textsuperscript{2}) than in WKY (2.70/mm\textsuperscript{2}) rats (Figure 6). This increase of leakage could be attributed at least partially to a higher endothelial cell turnover rate in SHR, whose average mitotic frequency was 2.9 times (0.047\% versus 0.016\%; Figure 7) and cell death frequency was 3.2 times (0.241\% versus 0.076\%; Figure 8) the corresponding values in WKY rats. All these differences showed statistical significance as analyzed with Student's \( t \) test.

Comparison between branching and nonbranching regions showed significant increases of EBA leakage
and endothelial cell turnover (mitosis and death) in the branching regions ($p<0.05$ by paired $t$ test).

Discussion

It is well known that the development of atherosclerotic lesions in both human beings and experimental animals is accelerated by hypertension and several other major risk factors. However, the mechanisms by which hypertension exacerbates atherosclerosis remain unclear.

Increases in transendothelial permeability in the arteries have been reported in hypertensive animals, and it has been suggested that hypertension may act directly by increasing the filtration pressure or indirectly by altering permeability characteristics of the aortic endothelium and that either of these actions may lead to an enhancement of plasma lipid entry into the intima. By studying the effect of blood pressure levels on transendothelial macromolecular transport, Hütten et al demonstrated that the passage of protein tracers (horseradish peroxidase and ferritin) through rat arterial endothelium is influenced by sudden changes of arterial blood pressure. Studies by Bretherton et al also indicated that hypertension increases the entry of low density lipoprotein into the aortic intima in rabbits. Since the reversal of hypertension did not result in an immediate reduction in the rate of low density lipoprotein entry, they concluded that the increased low density lipoprotein entry in hypertension resulted from an...
effect on vessel wall permeability rather than a direct effect of increased filtration pressure. Although these previous studies have implicated an enhancement of endothelial permeability in hypertension, the mechanism of this effect has not been established at the cellular level. In view of our recent findings\textsuperscript{10-14} that increased endothelial cell mitosis and the associated transiently leaky junctions can cause an enhancement of endothelial permeability to macromolecules in normal rat aorta, the present experiments were designed to test whether such a mechanism might be operative in SHR.

The results of the present study showed a significantly higher number density of leaky foci in the thoracic aorta in SHR than in WKY control rats (7.46/mm\textsuperscript{2} versus 2.70/mm\textsuperscript{2}). This enhancement in endothelial macromolecular permeability was found to be accompanied by greater frequencies of endothelial cell mitosis (0.047\% versus 0.016\%) as well as endothelial cell death (0.241\% versus 0.076\%) in SHR than in WKY rats. These findings suggest that hypertension correlates with increased endothelial cell turnover, which may produce more leaky endothelial junctions and thus lead to an enhanced endothelial permeability to macromolecules.

Majack and Bhalla\textsuperscript{23} studied aortic endothelial permeability in SHR and WKY rats by using colloidal carbon as a tracer. Although they found morphological defects in the endothelium that suggested an enhanced permeability, there were no differences in permeability between SHR and WKY rats. In a previous study\textsuperscript{12} on the permeability characteristics of normal aortic endothelium to different tracers, we deduced that the dimension of the leaky junction accompanying endothelial cell turnover undergoes dynamic changes with a peak widening to approximately 30 nm. This would explain the increase in permeability observed in the present study with albu-
min (~6 nm) but not in experiments by Majack and Bhalla on colloidal carbon particles (25–50 nm).

In the present study, we did not find a tendency of codistribution of dead and mitotic cells, so we do not know whether the increase of endothelial replication is due to the local stimulation produced by dying or dead endothelial cells or due to the direct influence of hypertension.

Clinical and postmortem studies indicate that it is often the regions of arterial branching and sharp curvature that have the greatest predilection for the development of atherosclerosis. These are also the regions that show flow disturbance with shear stress variation and correspond to the "blue area" in animal experiments. Our study also demonstrated a significant increase of EBA leaky foci in the branching regions of aorta, where higher frequencies of endothelial cell mitosis and death were simultaneously noted. This finding could serve as a pathogenetic basis for the higher propensity for atheromatous lesions in the branching areas.

By examining permeability and the histological and ultrastructural aspects of the intima, Hadijsky and Peyr showed that the blue areas, as demarcated by the "blue area" in animal experiments. Our study also demonstrated a significant increase of EBA leaky foci in the branching regions of aorta, where higher frequencies of endothelial cell mitosis and death were simultaneously noted. This finding could serve as a pathogenetic basis for the higher propensity for atheromatous lesions in the branching areas.

Our studies were performed with the use of albumin as a tracer. Although our previous investigations on normal rats showed that endothelial cell turnover had similar effects on albumin and low density lipoprotein, further studies using low density lipoprotein as the tracer are needed to obtain direct evidence that the enhanced endothelial cell turnover in SHR may contribute to atherogenesis by increasing low density lipoprotein permeability. Atherogenesis is a complex process involving many factors in addition to macromolecular permeability through the endothelial layer. At high blood pressures, the aortic media is compressed and offers a greater resistance to convective flux and, presumably, to passage of macromolecules carried by solvent drag from the intima through the media. Such a mechanism, coupled with an enhanced endothelial permeability, might contribute to the accumulation of macromolecules such as lipoproteins in the intima layer.

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References


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