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EARLY LESIONS OF CEREBRAL ATHEROSCLEROSIS FROM INDUCED HYPERTENSION IN WATANABE HERITABLE HYPERLIPIDEMIC RABBITS

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KEY WORDS

atherosclerosis; cerebral artery; hypertension; WHHL rabbit

ABSTRACT

Watanabe heritable hyperlipidemic (WHHL) rabbits are well known to develop severe atherosclerotic lesions in extracranial arteries but not in cerebral arteries. In the present work we produced and investigated atherosclerotic lesions in cerebral arteries of WHHL rabbits by inducing hypertension. Hypertension was induced by right nephrectomy and surgically induced stenosis of the left renal artery. Six months after surgery the rabbits were killed for morphologic, immunohistochemical and transmission electron microscopic studies of extracranial and cerebral atherosclerosis. A significant rise in systolic blood pressure was evident 3 months after surgery in the hypertensive group ($p<.001$). Atherosclerotic lesions had developed areas near the vertebral-basilar arterial confluence and the circle of Willis in the hypertensive Watanabe rabbits by 6 months after surgery ($p<.001$). Atherosclerotic lesions in cerebral arteries remained less severe than in the aorta and coronary arteries, and showed qualitative morphologic differences. This is the first report of production by hypertension of experimental cerebral atherosclerosis in the WHHL rabbits, indicating that hypertension as well as hyperlipidemia was required for development of lesions in cerebral arteries. This model should be useful for studying development of cerebral atherosclerosis and may contribute to improved prevention and treatment of the human disease.

INTRODUCTION

Cerebral arteries appear to be resistant to diet-induced atherosclerosis in experimental animals, unlike the extracranial vessels.[10, 11, 36] Both hypercholesterolemia and hypertension are

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well known to be important in the pathogenesis of human atherosclerosis. The Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model of familial hypercholesterolemia, shows hyperlipidemia from birth, due to a deficiency of low-density lipoprotein receptors.[1,14,27] Aortic and coronary atherosclerosis occur at a premature age in this animal and worsen with maturation.[2,32,34] Several pathologic features observed in the WHHL rabbit resemble those seen in human familial hypercholesterolemia,[3,7] and the WHHL rabbit has served as a useful model of human atherosclerosis and familial hypercholesterolemia (FH).[4]

While animals with acquired experimental hyperlipidemia, such as that induced by feeding cholesterol, have been used to study the development and prevention of atherosclerosis, [8,15,18,19] these animals may be less suitable for such studies than spontaneously hyperlipidemic animals considering observed difference in lipid deposition in organs.[2] Therefore, the WHHL rabbit is considered a practical animal model for the study of atherosclerosis. In 1988, however, Iwabuti noted that in these animals atherosclerotic lesions were demonstrated histologically only in extracranial carotid arteries, not in intracranial arteries,[12] making the model inapplicable to cerebral atherosclerosis.

WHHL rabbits with induced renovascular hypertension represent a potentially important model for examining the relationship between hypertension and atherosclerotic vascular disease.[4] In the present study we produced renovascular hypertension in WHHL rabbits in a series of experiments intended not only to investigate the effect of hypertension on development of atherosclerotic lesions, but also to induce pathologic findings of early intracranial cerebral atherosclerosis in the hypertensive WHHL rabbits.

MATERIALS AND METHODS

Animals

A total of 22 WHHL rabbits under 10 months old that were bred at Kobe University and 7 mature adult Japanese white (JW) rabbits, from 2.2 to 2.4 kg of body weight were used for the study. All the rabbits were randomly divided into five groups. I. control group; 3-months old WHHL rabbit n=2. II. 6-month follow up control group; WHHL rabbit n=4. III. 3-month follow up post-operation of WHHL rabbit (n=6). IV. 6-month follow up post- operation of WHHL rabbit (n=10). V. 6-month follow up post-operation and without hyperlipidemia of JW rabbit (n=7). WHHL rabbits included both sexes, as no sex-related differences in degree or distribution of atherosclerosis has been reported in these rabbits.[23,24,33] During the experiment, the rabbits were housed individually in metal cages in a room maintained at constant temperature and humidity. They were fed 120g of standard rabbit chow (CR-3, Clea Japan Inc) per day. All animal experiments were conducted according to the guidelines for animal experimentation at the Kobe University School of Medicine.

Surgical procedure

Rabbits were intubated for general anesthesia with halothene and oxygen using anesthesia apparatus model (FO-20S, ACOMA IKA KOGYO CO., LTD). Animals then were placed in a dorsal recumbent position and the skin was shaved and scrubbed with 0.2% iodine. Under sterile conditions a dorsolumbar paramedian incision was made. The kidney and renal artery were exposed. In a first procedure, right nephrectomy was performed. One week later the left renal artery was constricted by a silver restriction clip with a gap of 0.6 mm according to a modification of the method originally described by Goldblatt.[10)]

Blood pressures measurement and serum biochemical analyses

After surgery, systolic and diastolic blood pressure was measured from the median artery of the ear every two weeks using a Thermal Array Recorder (RAT-110, NIKON KOHDEN), connected to a Biologic Research System (SEN-6102, NIHON KOHDEN). Arterial blood samples for chemical analysis were collected before surgery and at the end of the follow-up period in each group.

Preparation of histologic sections

At the end of the follow-up period, the animals were anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg) and perfused with lactated Ringer's solution and then Bouin's fixative using a perfusion apparatus that maintained a constant pressure of 100 mm Hg. After perfusion fixation, tissue blocks were excised and immersed in Bouin's fixative for at least 24 hours. Arterial cross sections were prepared histologically from the tissue blocks as described previously[23,24)] (n=20). Tissue blocks included the aorta, coronary arteries, and vertebral arteries. Cerebral arteries were cut serially in 4-μm sectors from blocks from each segment.

Immunohistochemical examination

Serial sections from each cerebral arterial segment were stained either immunohistochemically or conventionally. Immunostaining with monoclonal mouse antibodies including anti-muscle actin HHF-35,[30)] a smooth muscle-specific antibody and monoclonal mouse anti-rabbit macrophage RAM-11,[29)] a macrophage-specific antibody was performed by a method reported previously.[29)] Each monoclonal antibody was applied to sections at a dilution of 1:1000 and immunocytochemical staining was carried out with an avidin-biotinlated enzyme complex procedure using a Vectastain ABC-PO kit (Vector Laboratories Inc.).

Electron microscopic studies

Animals used for electron microscopic study (n=11) were anesthetized with an intravenous injection of sodium phenobarbital (25 mg/kg) and perfused with 500 ml of phosphate buffer solution (PBS) and then through the carotid artery an 18 gauge needle was implanted into left

ventricle, carrying out perfusion fixation with 1000 ml of 2.5% glutaraldehyde and 2% paraformnaldehyde in 0.1 mol/L sodium cacodylate buffer at pH 7.4. The perfusion pressure was adjusted to equal the systolic blood pressure measured just before the procedure. After perfusion fixation, tissue blocks were taken from the aorta, coronary arteries, vertebral arteries, and cerebral arteries. Tissue blocks were immersed overnight at 4°C in 0.1 mol/L sodium cacodylate buffer. After postfixation with 1% osmium tetroxide buffered with 0.1 mol/L sodium cacodylate for 2 hr, dehydration in a graded ethanol series, treatment with propylene oxide, and embedding in Epon 812 (Tab Laboratories Equipment, Berkshire England), semithin Epon sections were cut on an LKB 2088 ultramicrotome (LKB-Produkter AB, Sweden) with glass knives and were stained with toluidine blue for light microscopy observation (1 μm). Ultrathin sections (700 Å) were cut on an LKB ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope; (H 7100 Hitachi, Tokyo, Japan).

Statistical analysis

Statistical significance of blood pressure and blood urea nitrogen in different groups was assessed by Student's t-test.

RESULT

Table I .Effects of one-kidney, one clip Goldbaltt hypertension of blood pressure, blood urea nitrogen and serum lipids in Watanabe Heritable Hyperlipidemic rabbits.

Group (n)	Systolic BP (mm Hg)	BUN (mg/dl)	Serum cholesterol (mg/dl)	Serum triglyceride (mg/dl)
I (n=2) 3 months old control	110±0.5	20±1	580±113	541±117
II (n=4) 6 months follow up control	112 ±0.5	19±1	407±93	391±80
III (n=6) 3 months after surgery	174±4.1*	26±3**	391±87	309±101
IV (n=10) 6 months after surgery	185±5.6*	23±1**	379±94	317±71
V (n=7) JW rabbit 6 months after surgery	167±0.9*	20±1	46±27	55±15

Values represent mean ± SEM; BP, blood pressure; BUN, blood urea nitrogen
*, $p<0.001$; **, $p<0.05$ comparing hypertensive vs. controls.

Blood pressure

Systolic blood pressure had risen by the third month after surgery and continued to be elevated until killing of animals months after surgery in both WHHL postoperative and JW groups (Table Ⅰ).

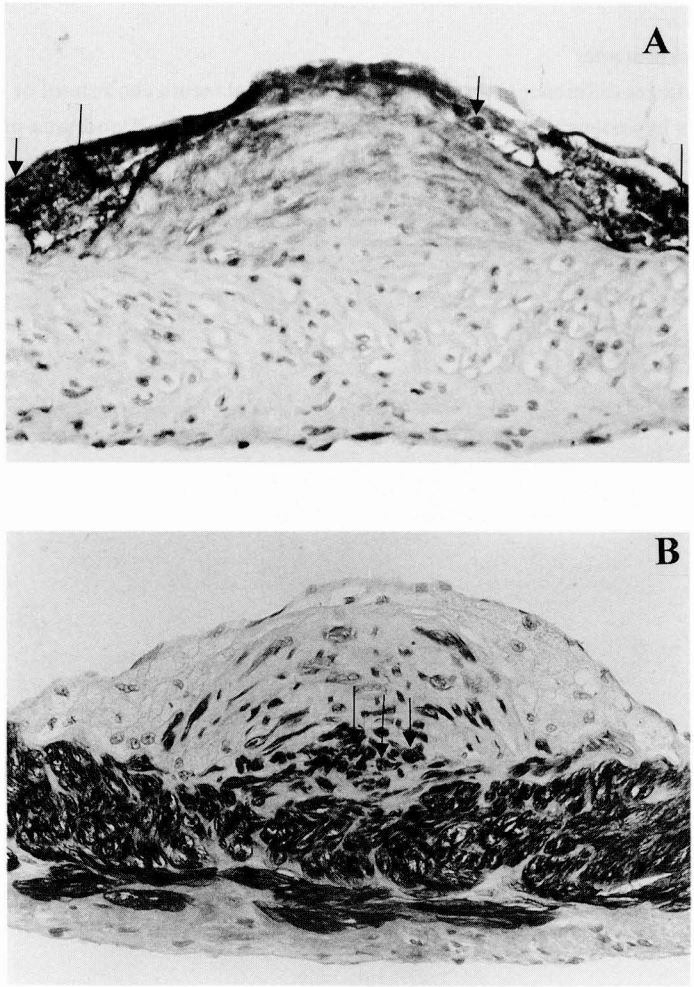

Fig.1. Photomicrographs of early cerebral arterial atherosclerotic lesions in a WHHL rabbit after 6 months of induced hypertension. Sections show the distribution of macrophages

(large arrows) and smooth muscle cells (large arrowheads). Immunohistochemical staining was performed with the RAM-11 monoclonal antibody, which is specific for macrophage (A), and monoclonal antibody HHF-35, which is specific for smooth muscle cells (B). Macrophages are round and often are aligned in the superficial portion of the intimal lesion (A), while smooth muscle cells are elongated or stellate and located predominantly in the intermediate and deep portions of the intimal lesion (B). Bars=25μm.

Serum biochemical studies

No significant differences were present in either total serum cholesterol or triglyceride levels between hypertensive and normotensive groups (Table Ⅰ). Blood urea nitrogen was

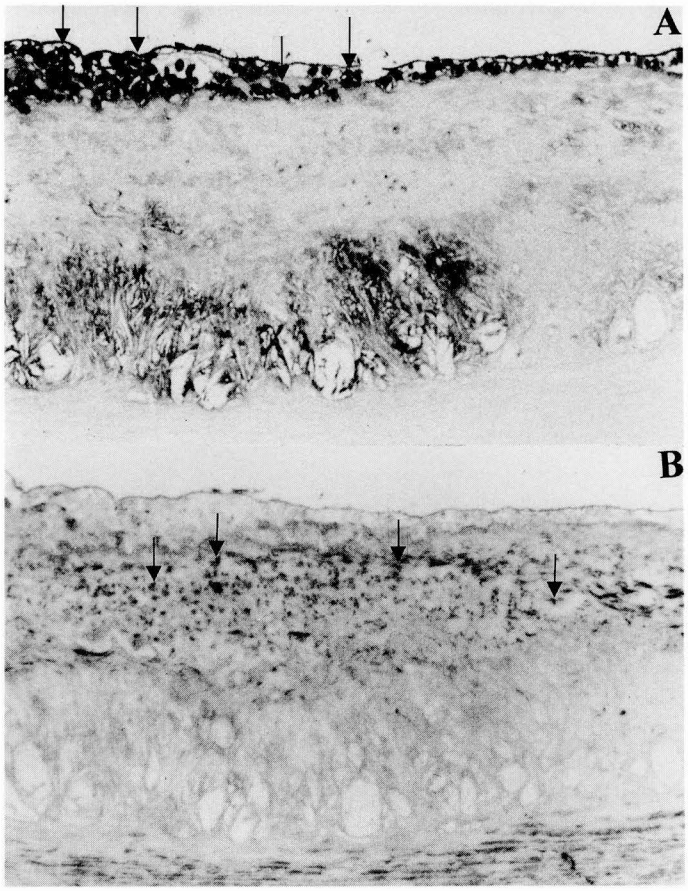

Fig. 2. Photomicrographs of aortic atherosclerotic lesions from a WHHL rabbit after 6 months of induced hypertension. Symbols and staining are the same as in Figure 1. Both macrophages and smooth muscle cells are observed throughout the intimal lesion. Bars=25μm.

increased somewhat in both hypertensive groups compared with normotensive WHHL rabbits (Table Ⅰ).

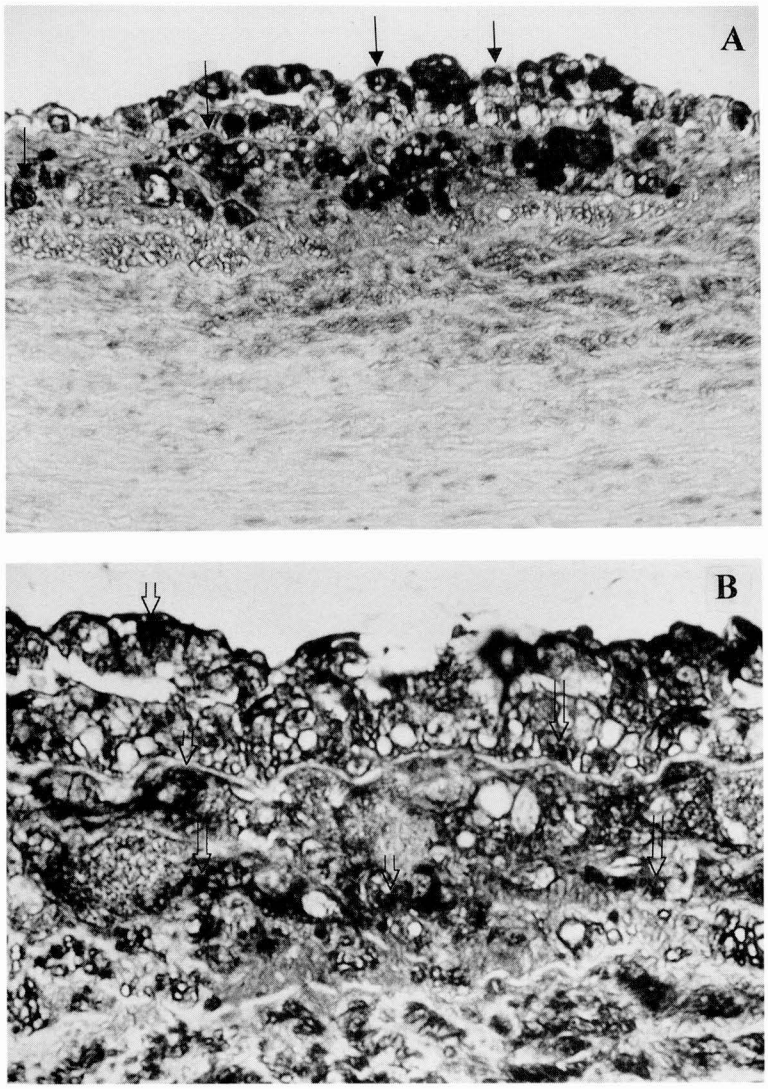

Fig. 3. Photomicrographs of an early aortic atherosclerotic lesions from a 3-months old control WHHL rabbit. Symbols and staining are as in Figure 1. Macrophages are scattered in the superficial portion of the intimal lesion, while smooth muscle cells are observed throughout the intimal lesion. Bars=25μm.

Histopathology and immunohistochemistry

Cerebral arteries: Only hypertensive WHHL rabbits 6 months after surgery showed

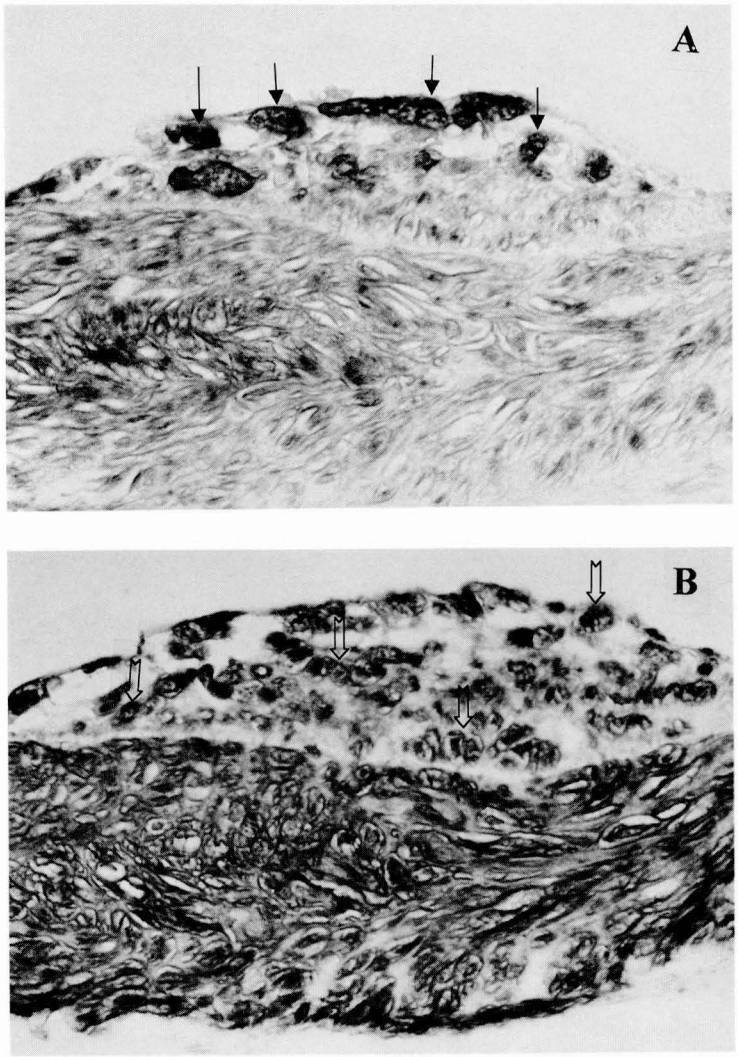

Fig. 4. Photomicrographs of an early coronary arterial atherosclerotic lesion from a 3-months old control WHHL rabbit. Symbols and staining are the same as in Figure 1. Macrophages are scattered in the superficial portion of the intimal lesion and smooth muscle cells are observed throughout the intimal lesion. Bars=25μm.

morphologic or immunohistochemical evidence of early atherosclerotic changes in the vertebral basilar arteries confluence and portions of the circle of Willis (the basilar artery-posterior cerebral artery junction, the posterior cerebral artery-posterior communicating artery junction, and the internal carotid artery-posterior communications artery junction) (n=10). By light microscopy, localized intimal lesions in cerebral arteries showed thickening composed mainly of accumulations of macrophages and smooth muscle cells. Smooth muscle cells were elongated or stellate in shape and located predominantly in deeper portions of the intimal lesion. Macrophages were round and often were seen in a linear array in the superficial portion of the intimal lesion in WHHL rabbits with renal hypertension 6 months after surgery (Fig.1).

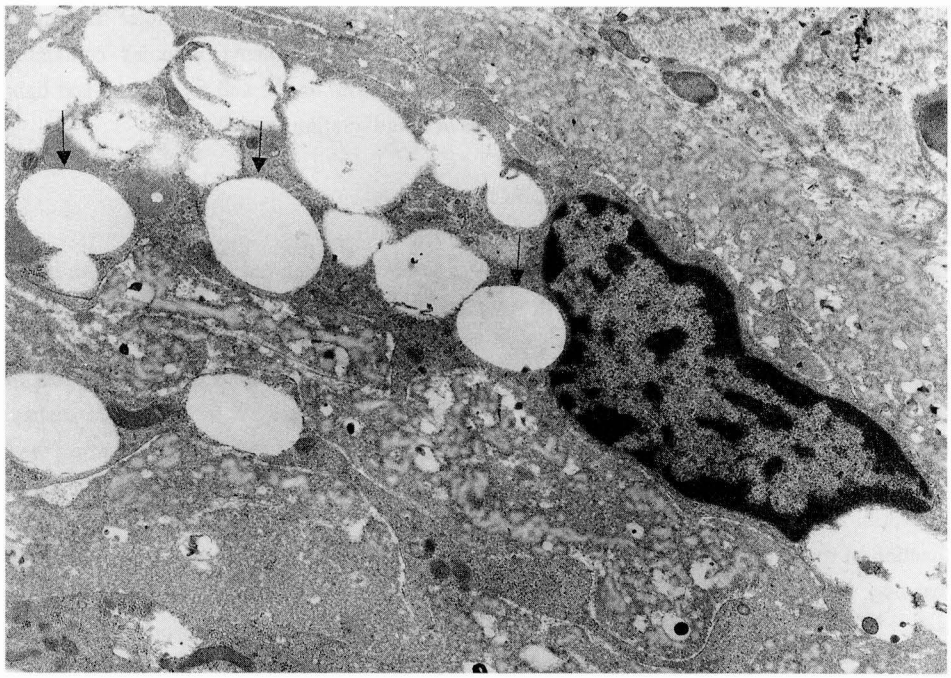

Fig. 5. Transmission electron micrograph of atherosclerotic cerebral arteries from a WHHL rabbit after 6 months of induced hypertension. Lipid droplets are seen in a modified smooth muscle cell within the intima (× 12 000).

Aorta and coronary arteries: Atherosclerotic lesions were not found in the aorta, coronary arteries, or cerebral arteries of JW rabbits with induced hypertension 6 months after surgery. Both

WHHL groups with hypertension (groups III and IV) and without hypertension (groups I and II) showed atherosclerotic changes in the aorta and the coronary arteries, where atherosclerosis was evident mainly as thickening and fibrosis. In WHHL rabbits with renovascular hypertension 6 months after surgery, smooth muscle cells were observed throughout the intima, and macrophages also were seen in throughout the superficial portion of the intimal lesion (Fig.2). On the other hand, in 3-month old WHHL rabbits, early aortic and coronary atherosclerotic lesions both showed smooth muscle cells were observed conglomerated throughout the intima but macrophages were restricted to the superficial portion of the intimal lesion (Figs. 3,4).

Electron microscopy

In addition to smooth muscle cells as indicated by the presence of dense bodies, microfilament bundles, and a basement membrane, transmission electron microscopy revealed isolated foamy cells within the intima of the vessels characterized by such cellular features as enlarged cytoplasm filled with clear lipid droplets, membrane-bound vacuoles containing membranous debris, myelin figures, microvilli protruding from on the cell membrane, and tight intercellular attachments with complicated patterns of interdigitation (Fig. 5).

DISCUSSION

Atherosclerosis generally is known to be less severe in human cerebral arteries than in the aorta and coronary arteries.[22, 37] In addition, cerebral arteries have been considered relatively resistant to cholesterol-induced atherosclerosis in experimental animals.[16, 35] These findings are consistent with pathologic studies in humans showing that atherosclerotic lesions first appear in the aorta, later in the coronary arteries, and last in the cerebral arteries.[26] In the present studies, the atherosclerotic lesions appearing within 3 months in normotensive WHHL groups and hypertensive WHHL groups were found only in the aortic and coronary arteries. Differences in susceptibility of the cerebral arteries and other vessels to atherosclerosis may involve a barrier function of the intima. The cerebral vessels have been shown to have tight endothelial junctions that may constitute a barrier preventing passage of circulating macromolecules into the vessel well.[13, 16,20] Absent or slow of transendothelial transport by pinocytosis may also contribute to a permeability barrier.[20] A thick subendothelial basement membrane and the internal elastic membrane of cerebral arteries also may tend to reduce vascular permeability. The importance of vascular permeability in influencing susceptibility to atherosclerosis previously has been examined in the rabbit;[16] in these studies, cholesterol feeding was found to enhance permeability of coronary and aortic vessels without altering the permeability of the cerebral arteries. Prolonged feeding of the atherogenic diet resulted in atherosclerosis of the coronary and aortic vessels but not of the cerebral arteries.

Our observations, in accord with many earlier studies, demonstrated that hypertension

markedly accelerates cerebral atherosclerosis, while hyperlipidemia in the absence of hypertension has a weak influence on intracranial cerebral vessels but a strong affect on extracranial vessels. Several studies of human autopsy cases and animal experiments have suggested that hypertension, together with hypercholesterolemia, accelerates cerebral atherosclerosis.[9, 16,31] Increased arterial permeability may arise from distention of the arterial lumen and endothelial damage induced by hypertension.[6] In the presence of hyperlipoproteinemia, as seen with the WHHL rabbit, increased intimal permeability induced by hypertension could lead to more rapid entry of lipoproteins into the intima. On the other hand, angiospasm produced by high blood pressure, ischemia in peripheral arteries resulting from angiospasm, or a renal factor might contribute to cerebral atherosclerosis. In this study, we observed in WHHL rabbits with renal hypertension that the lesions induced involved mainly bifurcations and confluence sites in cerebral vessels, closely resembling patterns described in humans.[25, 28] The localization of lesions suggests that such local hemodynamic factors as shear stress, nonlaminar flow, and turbulence may contribute importantly to the development of cerebral atherosclerosis.[5] These factors, together with increased tangential stress on the vessels caused by elevated blood pressure, might account for the increased frequency and severity of cerebral atherosclerosis in hypertension. These findings suggest that hypertension may contribute to risk of cerebrovascular disease by accelerating the course of cerebral atherosclerosis, and accordingly control of blood pressure may reduce stroke occurrence.

In our studies, the early lesion in cerebral arteries observed immunohistochemically and by conventional light microscopy was mainly an accumulation of smooth muscle cells and macrophages. Transmission electron microscopy demonstrated monocyte/macrophages and smooth muscle cells, and an accumulation of lipid-laden foam cells on the endothelial cell surface. Smooth muscle cells, monocyte-derived macrophages, and mast cells have been identified in human atherosclerotic lesions. Smooth muscle cells, the predominant cell in most lesions, are present in diffuse intimal thickening, fatty streaks, and plaques, and are thought to be derived from medial smooth muscle cells that have migrated into the intima. Smooth muscle cells contribute to atherosclerotic lesions by intracellular lipid storage and connective tissue synthesis. Subendothelial migration of monocyte/macrophages is also another important event in the pathogenesis of atherosclerosis. Hypercholesterolemia may induce the macrophage to phagocytize excess lipids and become foam cells. Pathways involving these two major cell types appear to lead to the more advanced lesions. It is suggest that the distribution of smooth muscle cells in early lesions in cerebral arteries involved mainly the deeper portion of intimal lesions.[8] This localization differs from early aortic and coronary artery atherosclerotic lesions, in which smooth muscle cells are observed throughout the intima. The difference suggests that smooth muscle cell proliferation in the media and migration from the media into the intima are less prominent in cerebral arteries than in aortic and coronary arterial lesions. Stimuli promoting migration and proliferation of smooth muscle cells from the media into the intima mainly include platelet-

derived growth factor (PDGF) and low-density lipoprotein (LDL).[21] Several potential explanations could account for the suppression of smooth muscle cell migration in cerebral arteries. First, the structural character of cerebral arteries, with a thick subendothelial basement membrane and internal elastic membrane, may impede migration and proliferation of smooth muscle cells. Furthermore, diffusion of LDL, PDGF, and other stimulatory factors, as well as the migration of monocyte/macrophages, may be restricted by the barrier function of the intima with consequent reduction of stimulation of smooth muscle cells to proliferate and migrate. Second, alterations of PDGF, LDL, or other etiologic factors including receptor numbers or affinity may occur in smooth muscle cells.[17] Future studies are needed to elucidate the mechanisms suppressing migration and proliferation of smooth muscle cells and monocyte/macrophages in the intima.

CONCLUSIONS

This is the first report of cerebral atherosclerosis produced by inducing hypertension in WHHL rabbits. In this model hypertension with hyperlipidemia significantly affected cerebral arteries. The distribution of the lesion components in cerebral atherosclerosis differed from that in early aortic and coronary arterial atherosclerosis. This model may be useful for studying development of cerebral atherosclerosis and possibly its prevention and treatment.

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