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Invited Review

Watanabe rabbits with heritable hypercholesterolaemia: a model of atherosclerosis

G. Aliev and G. Burnstock

Department of Anatomy and Developmental Biology, University College London, London, UK

Summary. Many factors play important roles in the development of atherosclerotic lesions. The leading risk factor for atherosclerosis is familial hypercholesterolaemia (FH). FH is a genetic disease characterized by a deficiency of receptors for low density lipoprotein (LDL) on the plasmalemma of endothelial cells, a high level of serum LDL, and early development of atherosclerosis and skin xanthoma. Watanabe and colleagues have developed a line of rabbits with unprovoked hypercholesterolaemia, increased blood level of LDL, pronounced atherosclerosis and skin xanthoma. These Watanabe Heritable Hyperlipidaemic (WHHL) rabbits possess an inheritable mutation of one gene, similar to that in human FH. The morphogenesis of atherosclerosis in patients with FH is characterized by multifocal deposit of lipids in the stromal cells of thymus, spleen, skin, interstitial and parenchymatous cells of kidneys and the presence of some single foam cells in aorta. The manifestation of atherosclerotic lesions in WHHL rabbits increases progressively with age but the presence of atherosclerotic lesions in newborn WHHL rabbits suggest that the process may commence *in utero*. Moreover, the main mass of plasma cholesterol in WHHL rabbits is first found in LDL and to a lesser degree in lipoproteins of intermediate density. This is contrary to diet-induced atherosclerosis in rabbits where the main mass of serum cholesterol is found in very low density β-lipoproteins. Thus the distribution of cholesterol among lipoprotein fractions differs from that in WHHL rabbits. Atherosclerotic damage of arteries in WHHL rabbits goes through several stages. During the progression of intimal damage, lipid and foam cell deposits are found in the internal surface together with developing plaques and increased content of lipids in the tunica media. Calcification often follows this process. The main factors initiating atherosclerosis in WHHL rabbits are adhesion of leukocytes and platelets to endothelial cells and the accumulation of lipids in the aortic wall. The deposits of lipids in macrophages and intimal smooth muscle cells in WHHL rabbits occurs mostly at the expense of cytoplasmic neutral lipid particles with some accumulation in lysosomes. Hypertension as a risk factor increases the area of atherosclerotic damage in all arterial vessels in WHHL rabbits, particularly in the thoracic and abdominal aorta. Morphogenesis of the development of atherosclerosis in WHHL and diet-induced atherosclerosis in rabbits was similar, but differs from rats with heritable hypercholesterolaemia. Damage or loss of endothelial cells can predispose the atherosclerotic vessels to vasospasm and can leave vessels unprotected against vasoconstrictor stimuli. The development of the WHHL model has not only given insight into the mechanisms of development of familial hypercholesterolaemia but has also provided a model for assessing various therapeutic approaches for the prevention and treatment of atherosclerosis.

Key words: Watanabe heritable hyperlipidaemic rabbits, Aorta, Morphology, Endothelium, Atherosclerosis

Offprint requests to: Pof. G. Burnstock, Autonomic Neuroscience Institute, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK. FAX: 44-171-830-2949.

Introduction

Atherosclerosis is a major public health problem in Western countries. Atherosclerosis has been defined as a combination of changes of the arterial intima with the local deposit of lipids, carbohydrates, blood and its products, fibrous tissue and calcium (Gown et al., 1986; Berry, 1988).

The leading risk factor of atherosclerosis is familial hypercholesterolaemia (Buja et al., 1979, 1983; Grundy, 1986; Stamler and Shekelle, 1988; Steinberg et al., 1989; Rosenfeld et al., 1990; Ross, 1993), and studies of this have assisted in the understanding of pathogenesis of atherosclerosis (Buja et al., 1983, 1990; Goldstein et al., 1983; Mahley, 1985; Breslow, 1989; Aalto-Setala et al., 1989; Ragazzi et al., 1989, 1993, 1995; Aliev et al., 1993; Chinellato and Ragazzi, 1995).

Familial hypercholesterolaemia (FH) is a genetic disease characterized by a deficiency of receptors for low density lipoproteins (LDL) on the plasmalemma, a high level of LDL in blood plasma (the content of cholesterol is 400-1000 mg/dl or 10-25 mmol/l), as well

as the early development of atherosclerosis and skin xanthoma (Thannhauser, 1958; Brown and Godstein, 1975; Buja et al., 1979, 1990; Kita et al., 1981, 1982; Aliev et al., 1993). The absence of the gene which encodes the high affinity receptors for LDL is a genetic defect in patients with FH (Brown et al., 1974; Goldstein and Brown, 1977; Bilheimer et al., 1979; Brown and Goldstein, 1990).

The investigation of an adequate genetic model of this disease is necessary for a better understanding of the mechanisms of development of human familial hypercholesterolaemia. Watanabe and colleagues (Kondo and Watanabe, 1975; Watanabe 1980; Watanabe et al., 1985) developed a line of rabbits with unprovoked hypercholesterolaemia, increased blood levels of LDL, pronounced atherosclerosis and skin xanthoma. This line of rabbits which were named Watanabe Heritable Hyperlipidaemic (WHHL) rabbits were characterized by the inheritible mutation of one gene, similar to that in people with FH (Roberts et al., 1973; Kondo and Watanabe, 1975; Buja et al., 1979, 1983; Watanabe, 1980; Bilheimer et al., 1982; Goldstein et al., 1983; Yamamoto et al., 1986). This defective expression of LDL receptors in WHHL rabbits results in the loss of high affinity absorption and degradation of LDL (Bilheimer et al., 1982; Goldstein et al., 1983; Yamamoto et al., 1986; Buja et al., 1990). Until recently, WHHL rabbits have been the only animal model of human homozygous FH (Rosenfeld et al., 1987a,b, 1990; Rosenfeld and Ross, 1990; Chinellato and Ragazzi, 1995).

Morphogenesis of atherosclerosis in patients with familial hypercholesterolaemia

The morphogenesis of atherosclerosis in patients with FH has been investigated in detail. Even in the fetus with FH there are multifocal deposit of lipids in the stromal cells of thymus, spleen, skin, interstitial and parenchymatous cells of kidneys and the presence of some single foam cells in aorta (Buja et al., 1979, 1990); the content of cholesterol in blood is 274 mg/dl or 7.2 mmol/l (compared with 31 mg/dl in the normal fetus) (Buja et al., 1990).

FH patients between 6 and 17 years of age have pronounced atherosclerosis of arteries, especially in the proximal parts of the left and right coronary arteries. All patients with FH have atherosclerotic lesions of the aorta and coronary artery. The mitral and aortal valves have, on their internal surface, a number of foam cells and moderately pronounced fibrous thickening of the walls (Buja et al., 1990). Seventy one percent show dystrophic changes of myocardium with necrosis and fibrosis, and 50% have arteriosclerosis (Buja et al., 1990). There is no damage to the veins.

Atherosclerotic lesions have a fibrous capsule and a '*nucleus*' containing a great number of intracellular and extracellular lipids including crystals of cholesterol. Often, components of the muscular layer of vascular wall are involved in this process. Deposits of lipids are found in the smooth muscle cells (SMCs) of the tunica media, in the long intimal myocytes and in macrophage-like cells. The intracellular lipids are usually seen as points of variable electron density without limited membranes or they take the form of lysosomes surrounded by membrane (Buja et al., 1990).

The following pathogenesis of atherosclerosis in patients with FH is proposed. The loss of activity of LDL receptors in such patients leads to increased levels of LDL in the plasma resulting from disturbed catabolism and over-production of lipoproteins. The cell-scavengers in various tissues are responsible for increasing levels of LDL. This process leads to accumulation of cholesterol esters in the cells (Steinberg et al., 1989; Buja et al., 1990). Deposits of the cell-scavengers laden with cholesterol, LDL and other unknown factors leads to the development of xanthomatosis and quick progression to atherosclerosis (Buja et al., 1990).

The deposit of foam cells in patients with FH may result from one of the following mechanisms: 1) excess endocytosis of LDL, independent of LDL receptors, in response to chronic hypercholesterolaemia and with further processing of LDL in lysosomes and deposition of cholesterol esters in the cytoplasm; 2) increased synthesis of cholesterol by cells and accumulation of cholesterol esters associated with the absence of LDL receptors. It has been shown that the deposits of lipids in homozygous people with FH is mostly associated with cells overfilling with plasma LDL (Goldstein and Brown, 1977; Goldstein et al., 1983; Mahley, 1985; Steinberg et al., 1989; Buja et al., 1990). Modification of LDL leads to their increased absorption by monocytes and other phagocyte cells.

Lipid exchange in WHHL rabbits

The level of total cholesterol in plasma in homozygous WHHL rabbits is 650-950 mg/dl, where 90% is concentrated in LDL (Tansawa et al., 1980). In particular, newborn WHHL rabbits have very high values of total cholesterol and triglycerides (766.5 ±119.5 and 652.2±138.1 mg/dl respectively) (Aliev et al., 1993). Similar to people with FH, these rabbits showed deficiency of LDL receptors on the membranes of cultivated fibroblasts, hepatocytes and epitheliocytes of adrenal glands (Tanzawa et al., 1980; Kita et al., 1981). The absence of any functionally activity LDL receptors leads to decreased clearance of lipoproteins (including the apoproteins B and E; apoB and apoE) (Tanzawa et al., 1980; Bilheimer et al., 1982; Rosenfeld et al., 1990) and thus to increased synthesis of LDL (Kita et al., 1982; Rosenfeld et al., 1990). This is followed by pronounced hypercholesterolaemia and hypertriglyceridaemia (Havel et al., 1982; Buja et al., 1983; Rosenfeld et al., 1990; Rosenfeld and Ross, 1990).

The main mass of cholesterol in plasma in WHHL rabbits is first found in LDL and to a lesser degree in

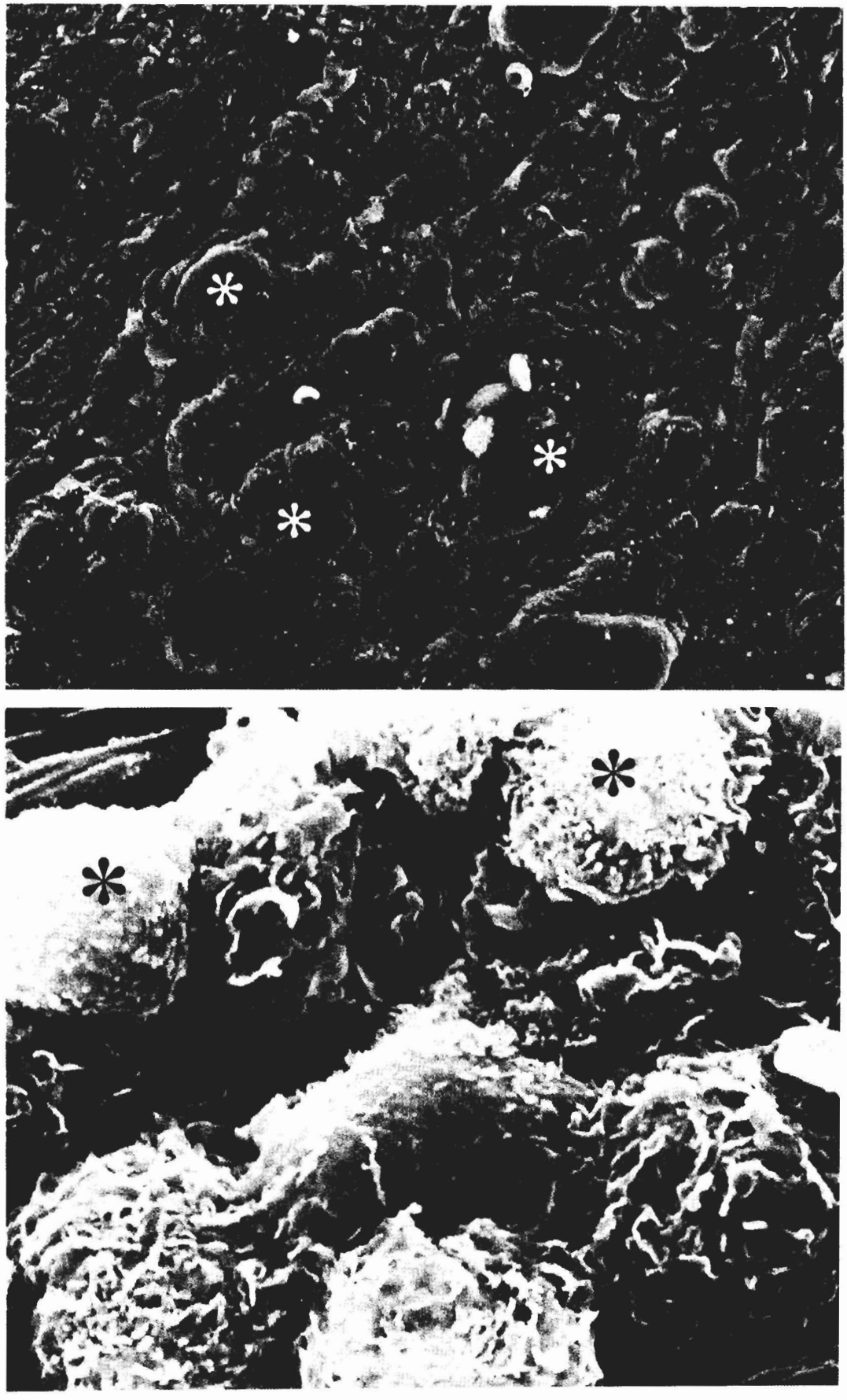

Fig. 1. Scanning electron microscopic (SEM) characteristics of the luminal surface of aortic wall in 1- and 2-year-old WHHL rabbits. **A.** Aortic arch from 1-year-old WHHL rabbit. Note atherosclerotic plaque area on the vessel surface. Reorganization of endothelial monolayer was seen (asterisks). **B.** Thoracic aorta from 2-year-old WHHL rabbit showing the column of intraluminal foam cells, having migrated through the endothelium (asterisks). A, x 600; B, x 2,000

lipoproteins of intermediate density (Watanabe, 1980). During diet-induced hypercholesterolaemia the main mass of serum cholesterol in rabbits is found in very low density β-lipoproteins (β-VLDL) (Mahley, 1979). Thus the distribution of cholesterol among lipoproteins fractions differs greatly from that in WHHL rabbits (Havel et al., 1982; Rosenfeld et al., 1990).

Age dynamics of atherosclerosis in WHHL rabbits

The manifestation of atherosclerotic lesions in WHHL rabbits increases progressively with age (Figs. 1, 2). In newborn WHHL rabbits we have shown an increased adhesion of leukocytes, probably monocytes, specific wave-like structures and fatty streaks associated mainly with blood flow branches where some areas of turbulence or stasis could occur (Aliev et al., 1991, 1993). Moreover, at the blood flow division zone, the endothelium was polygonal in shape, silver staining of cell borders was more intense and microplaques were seen (Aliev et al., 1991, 1993). Circular oriented SMCs are present in the intima of all segments of aorta of 8-day-old animals. However, in New Zealand White (NZW) rabbits these are rarely seen and often oriented in a longitudinal direction (Rosenfeld and Ross, 1990; Aliev et al., 1993). The presence of atherosclerotic lesions in newborn WHHL rabbits suggests that the process may commence *in utero* (Aliev et al., 1993). Intimal damage is found in the aorta of WHHL rabbits at the age of 3 months, where many foam cells containing a large number of lipid droplets are seen (Buja et al., 1983, 1990). According to the data of Van Niekerk and co-workers, (Van Niekerk et al., 1984a,b) there are no sudan-positive areas in the aorta in 14-week-old rabbits. Atherosclerotic lesions (mainly fatty streaks) are found in all segments of aorta in 6-month-old animals, but are not present in the coronary arteries (Watanabe et al., 1985; Hatanaka et al., 1987; Clozel et al., 1988). A few foam cells have ruptured plasmalemma. The foam cells contain mostly cholesterol esters, while deposits of phospholipids, myelin-like structures and multi-lamellar bodies are present in the "ruptured" cells (Fig. 3).

In 40 week-old WHHL rabbits, the majority of the intimal surface of the aortic arch is covered with atherosclerotic plaques, with less covering in the thoracic aorta (60 and 20%, respectively; Van Niekerk et al., 1984a,b). At the age of 15 months fatty streaks containing "*visible foam cells*" are adjacent to sites of damage and some of these cells show ruptured cellular plasmalemma. Some regions often show necrosis. A positive reaction for β-galactosidase (a marker of lysosomal enzymes) is seen in *viable* foam cells, but decreases in ruptured cells and is negative in regions with large deposits of crystalline cholesterol. Although the viable foam cells contain mostly cholesterol esters, the ruptured foam cells have large amounts of phospholipids. Sphingomyelin has gradually accumulated in the ruptured foam cells. Phospholipids prevail in some necrotic regions (Van Niekerk et al., 1984a,b).

Injuries which are projected to the vessel lumen are described in WHHL rabbits of 6 and 15 months of age. These injuries have the features of developed atherosclerotic plaques or atheroma, which are characterized by the presence of a central nucleus of extracellular lipids and necrotic tissue, a peripheral region with foam cells filled with lipids and a superficial capsule containing 'long' cells with a variable deposits of lipid (Figs. 2, 3) (Buja et al., 1990). Among cells of the plaque was found not only macrophage-containing lipid droplets and foam cells full of lipids, but also SMCs with lipid inclusions (Fig. 3A). Animals of 2-years-old have pronounced atherosclerotic damage, particularly in the arterial vessels (Paro et al., 1991, 1992; Ragazzi et al., 1995), including the femoral artery and the limb microcirculation (Cirillo et al., 1992).

The relative area of the luminal surface of arterial vessels with atherosclerotic damage is increased with age (1-month-old animals, 2%; 3-month-old animals, 5%; 6-month-old animals, 48%; 12-month-old-animals, about 70%). Moreover, the intimal/medial index increases with age of WHHL rabbits, particularly on zones of haemodynamic tension. At 3-months of age animals have leukocytes adhering to the surface of endothelium which is predisposed to atherosclerotic lesions. WHHL rabbits of 6-months of age have greater damage in the area of atherosclerotic lesions (Butler et al., 1987; Buja et al., 1990; Ragazzi et al., 1993). In the aortic arch of 3-5 month-old animals, 11% of the vessel surface has damage due to atherosclerosis, whilst 6-9 month-old animals have 28% and 12-14 month-old animals have 54% damage (Kolodgie et al., 1990).

At autopsy, minor atherosclerotic lesions were found in 14% of 1-4 month-old rabbits. Moderate and more severe atherosclerotic damage found in 100% of rabbits at the age of 5-35 months (Watanabe, 1980). Watanabe and co-workers (Watanabe et al., 1985) demonstrated that at the age of 5-36 months, 30-35% of rabbits had atherosclerotic lesions of coronary arteries. Plaques were found usually at the opening of the left coronary artery and rarely in the anterior interventricular branch.

Morphogenesis of damage

Atherosclerotic damage of arteries in WHHL rabbits goes through several stages. The fatty streak is the earliest form of lesion which is seen under the microscope; it is slightly projected into the vessel lumen and is characterized by a high content of cholesterol esters, mostly in foam cells, as well as by necrosis of some individual cells. The development of fatty streaks in WHHL and in cholesterol-fed rabbits occurs by; 1) continuous migration of monocytes into the subendothelial layer and formation of a layers of foam cells and later into the adjacent SMC layer; 2) increased size of foam cells; 3) accumulation of components of the extracellular matrix; 4) penetration and deposit of components of the blood plasma in the subendothelial layer (Rosenfeld et al., 1987a,b; Rosenfeld and Ross,

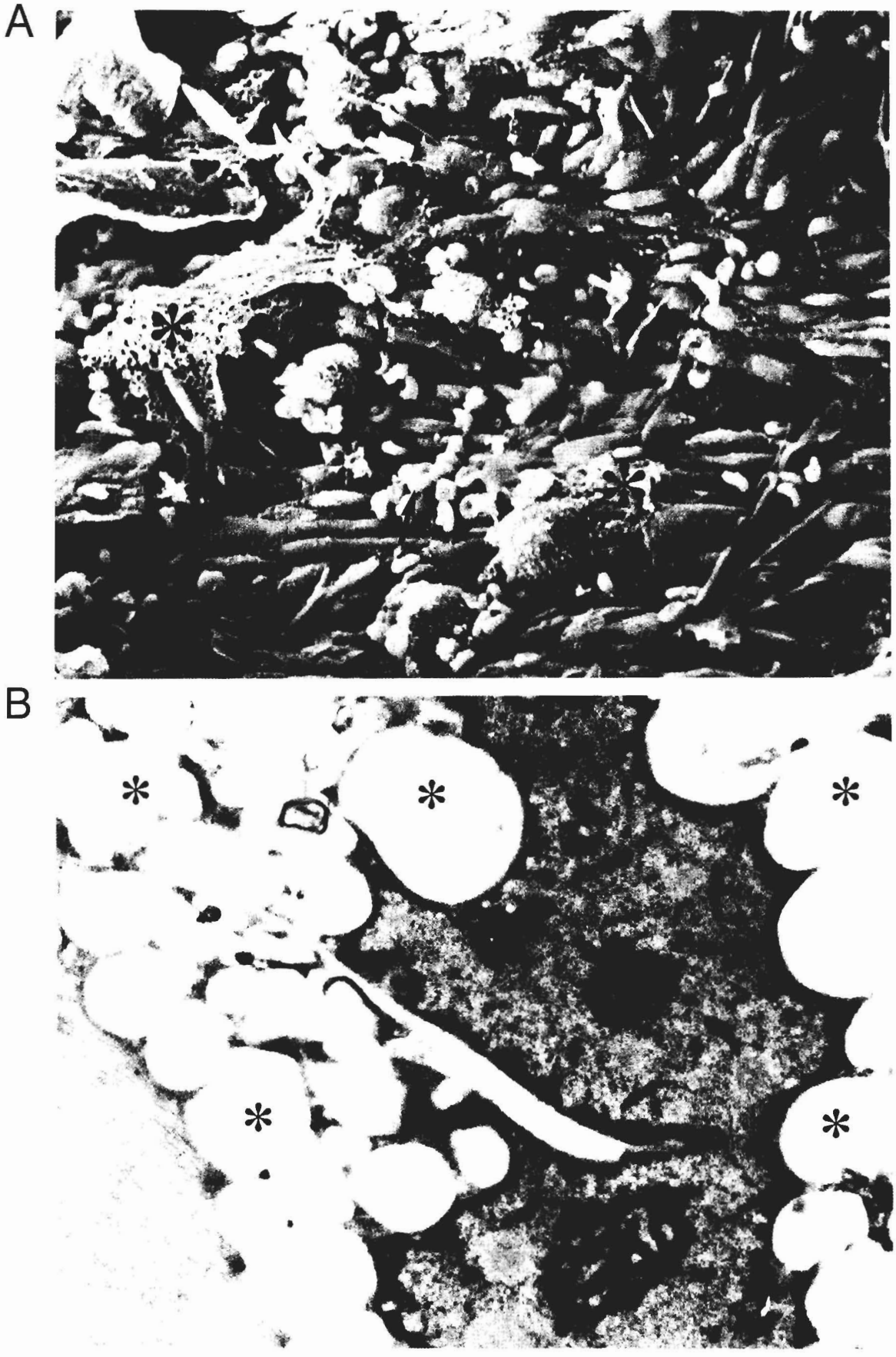

Fig. 2. SEM and TEM characteristics of the thoracic aorta from 1- and 2-year-old WHHL rabbits. **A.** Luminal surface of thoracic aorta showing the presence of microthrombus (asterisks). Note some blood cells which have migrated through the endothelium (arrows). **B.** Thin section shows the presence of large number of lipid droplets in the cytoplasm of subendothelial foam cells (asterisks) in the thoracic aorta of a 2-year-old WHHL rabbit. A, x 600; B, x 8,000

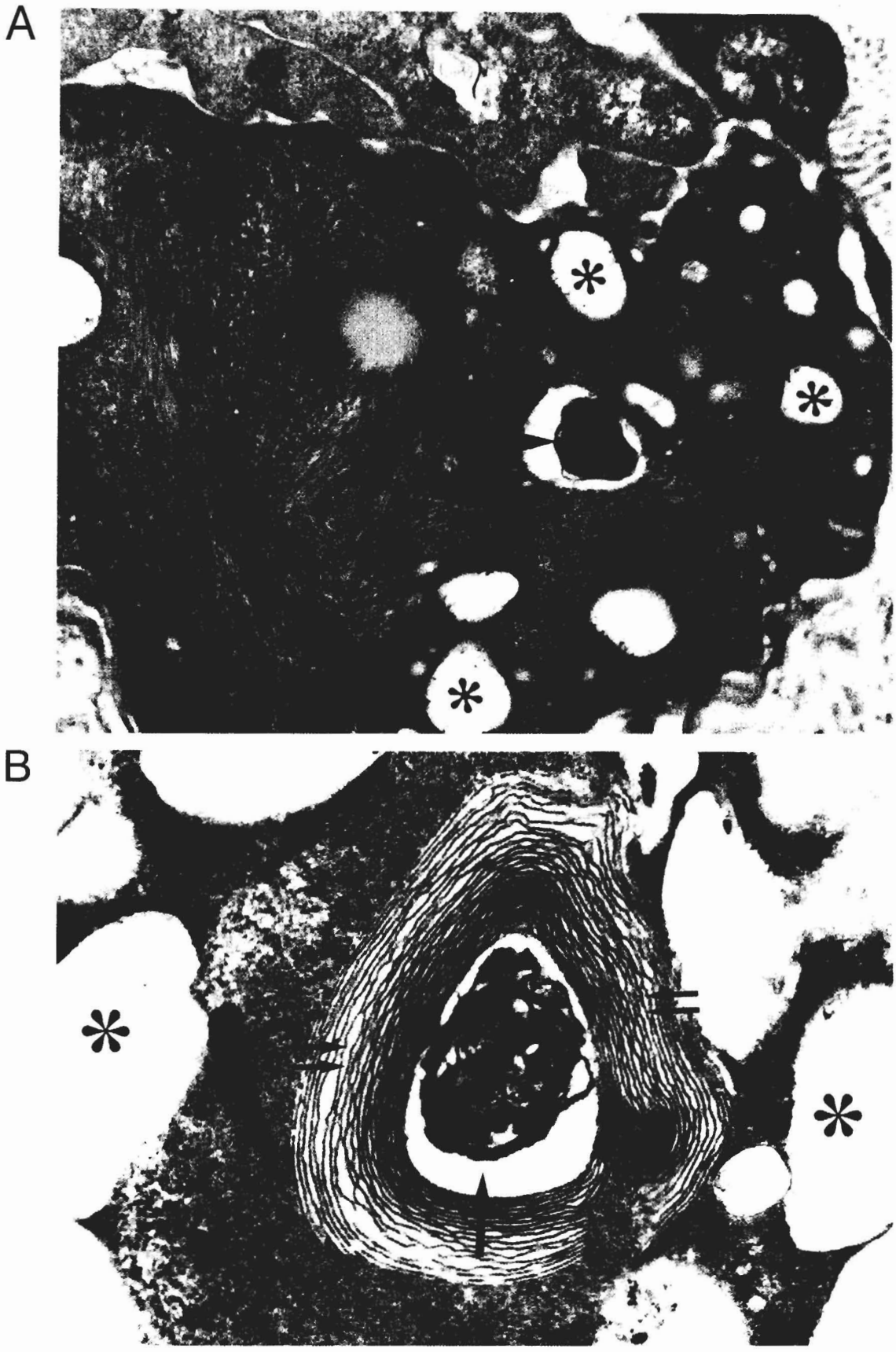

Fig. 3. Ultrastructural features of aortic intima in 2-year-old WHHL rabbits. **A.** Thin sections from aortic arch showing myelin-like structure (arrow) and lipid droplets (asterisks) in the cytoplasm of one subendothelial SMC. **B.** Note the presence of lipid drops in the surrounding area (asterisks) and in the cytoplasmic matrix (arrow) of subendothelial foam cells. Myelin-like structures were seen in the cytoplasmic matrix, and mostly consisted of concentric forms of a fibrillar structure (double arrows). Thin sections were stained with uranyl acetate and Reynold's lead citrate. A, x 17,000; B, x 20,600

1990).

Hypertrophy of the foam cells which are under the endothelium leads to rupture of the endothelium and release of foam cells into the blood. The mechanism by which this occurs is not clear although Rosenfeld and co-workers (Rosenfeld et al., 1987b) have suggested that it may be mechanical extension with chemical factors discharged by the foam cells, thereby initiating reduction.

Damage of endothelial cells (ECs) may play an important role in progression of fatty streaks to proliferative damage (Ross, 1986, 1993; Rosenfeld et al., 1987b). It has been shown that some sites of endothelial proliferation with adjacent platelets develop into areas of proliferative damage (Rosenfeld et al., 1987b). Injuries of ECs together with development of fatty streaks leads to increased migration of the components of the blood into the vascular wall (Rosenfeld, et al., 1987b).

After maturation of fatty streaks, macrophages occupy the subendothelium. Alteration of SMCs and macrophages then follows (Rosenfeld et al., 1987b). At the early stage of its development the atherosclerotic plaque is characterized by the presence of a necrotic centre containing crystals of cholesterol. The progression of the "*early*" plaque is similar to fatty streaks. Endothelium covering the developed atherosclerotic damage usually does not shows any visible changes (Fig. 1A) although sometimes microthrombi are seen on the vessel surface (Fig. 2A).

During the progression of intimal damage lipid and foam cell deposits are found in the internal surface together with developing plaques and increased content of lipids in the tunica media. Calcification often follows this process (Buja et al., 1990). The concentration of esters and free cholesterol increases in the arterial wall (Ross, 1986, 1993; Rosenfeld et al., 1990). In other organs, lipid deposits are occasionally observed.

Cellular mechanisms of morphogenesis of atherosclerosis in WHHL rabbits

The *initiating* mechanism of atherogenesis in WHHL rabbits is adhesion of leukocytes and platelets to ECs. The platelets that adhere to ECs produce platelet derived growth factor (PDGF), which is a potent mitogen (Bowen-Pope and Ross, 1982; Rosenfeld et al., 1987b; Ross, 1993) and a chemoattractant (Grotendorst et al., 1982) for SMCs and monocytes (Deuel et al., 1982). PDGF also stimulates further migration of macrophages into the subendothelial layer and proliferation of SMCs. Later, macrophages and ECs can synthesize and discharge the growth factor themselves (Glenn and Ross, 1981; Di Corleto et al., 1983; Shimokado et al., 1985; for detail see: Ross, 1993) and under certain conditions can produce a PDGF-like factor. The injury of ECs in hypercholesterolaemia can stimulate release of growth factors by ECs and macrophages. The formation of the vesicular lipid structures due to increased entry of LDL into the subendothelial layer (Simionescu and Simionescu, 1993) attracts new macrophages and SMCs, cutting off the cycle (Vijayagopal et al., 1985; Rosenfeld et al., 1987b). Usually the process of adhesion of leukocytes to endothelium and the deposit of lipids progress in parallel. It has also been shown that the accumulation of lipids in regions of high risk of atherosclerosis can take place even when the number of adhered leukocytes is low and this suggests that some other factors may also be involved in the process (Buja et al., 1990).

When lipids in the form of native lipoproteins are absorbed by macrophages they can stimulate the secretion of a material which can function as a chemoattractant for other macrophages (Fogelman et al., 1980; Camejo, 1982; Rosenfeld et al., 1990). Modified lipoprotein can be absorbed by receptors, scavenger macrophages or ECs (Vijayagopal et al., 1985; Simionescu et al., 1986; Simionescu and Simionescu 1993). The macrophages can also oxidize lipoproteins (Ross, 1986, 1993) and form superoxide anions which are toxic for neighbouring cells including ECs (Ross, 1986, 1993; Rubanyi, 1988, 1993). The lesions of ECs and SMCs caused by the macrophages can in their turn stimulate these cells to produce and secrete growth factors (Deuel et al., 1982; Grotendorst et al., 1982). Activated macrophages are able to release growth factors (Glenn and Ross, 1981), including molecules of PDGF (Shimokado et al., 1985). If this occurs then the growth factors secreted in the arterial wall may be involved in the process of stimulation of migration and proliferation of SMC and this initiates the process of atherogenesis (Walker et al., 1986a).

The deposit of lipids in macrophages and intimal SMC in WHHL rabbits occurs mostly at the expense of cytoplasmic neutral lipid particles with some accumulation in lysosomes (Buja et al., 1990). The same process occurs in people with FH. The accumulation of components of extracellular matrix in WHHL rabbits and rabbits fed on a cholesterol-rich diet leads to further deposits of elastin and collagen (Rosenfeld et al., 1987b). The deposit of proteoglycans occurs simultaneously. It seems that crystals of cholesterol are produced in the necrotic regions rich with phospholipids. It seems likely that during development of atherosclerosis foam cells produce large amounts of phospholipids. In the same manner, cholesterol esters are broken down by lysosomal enzymes, releasing free cholesterol. In necrotic regions, phospholipids are broken down by phospholipases, again releasing free cholesterol. The increased amount of crystalline cholesterol replaces cholesterol esters, phospholipids and lysosomal ferments (see: Brown and Goldstein, 1990).

It has been shown that macrophages in atheromas in WHHL rabbits arise from blood monocytes and intimal SMCs originate from the tunica medium of the vascular wall (Buja et al., 1990). Proliferating cells of the plaque are present at all stages of development and many of these are foam cells (McMillan and Duff, 1948; Spraragen et al., 1962; McMillan and Stary, 1968; Stary

and McMillan, 1970; Cavallero et al., 1971; Walker et al., 1986b; Rosenfeld and Ross, 1990, Ross, 1993; Ragazzi et al., 1995). MacMillan and Duff (1948) were the first to describe mitotic features in the foam cells in plaque regions of rabbits. This was confirmed later with further experiments on rabbits (Spraragen et al., 1962; McMillan and Stary 1968; Stary and McMillan, 1970; Cavallero et al., 1971), pigs (Florentin et al., 1969) and monkeys macaque-rhesus (Stary, 1974).

Hypercholesterolaemia is characterized by production of foam cells, and perforation into the sub-endothelium layer on exposure of the foam cells to the blood flow. This has been described in non-human primates (Faggiotto et al., 1984; Faggiotto and Ross, 1984) and pigs (Gerrity 1981a,b; Gerrity et al., 1985). The rupture of the endothelium leads to increased entry of blood components into the vascular wall. Even erythrocytes are found within the wall (Rosenfeld et al., 1987b). Deposits of intact lipoproteins are found in the subendothelial layer, and modifications often occur (Fogelman et al., 1980) leading to lipoprotein complexes. These complexes may contain proteins of the extracellular matrix (Simionescu et al., 1986; Rosenfeld et al., 1987a, 1990; Simionescu and Simionescu, 1993) and aggregation of lipoproteins with the formation of vesicular structures (Simionescu and Simionescu, 1993); such structures are typically seen in the extracellular matrix during development of fatty streaks (Simionescu et al., 1986; Rosenfeld et al., 1987a; Simionescu and Simionescu, 1993).

A number of lipid droplets with and without membranes are found in the foam cells. These have sharp "onion-like" features with concentric lamella structures surrounded with filaments of 10 nm (Fig. 3B). Droplets surrounded with limited membranes probably correspond to lysosomes full of lipids. The accumulation of lipids in the extracellular matrix occurs as vesicular structures among collagen fibres. The structure of these lipids is similar to the lipids of lysosomes in the foam cells (Amanuma et al., 1986). Exposure of the foam cells to blood flow leads to thrombus deposits (Fig. 2A). Degranulation of platelets in the areas of damaged endothelium can accelerate the penetration of growth factors, including PDGF, into the fatty streaks (Rosenfeld et al., 1987b).

Haemodynamic factors in the development of atherosclerotic lesions in aorta of WHHL rabbits

The past two decades have seen fluid mechanics develop from being viewed by medical and biological investigators as merely an interesting aspect of atherosclerosis to being appreciated as a key factor, not only in understanding the aetiology of the disease but also in serving as a regulator and modifier of cellular biology in both normal and diseased arteries (Chobanian 1983, 1990; Lichtenstein and Chobanian, 1990). Blood vessels must be able to sense shear stress/tension to be able to respond to changes in haemodynamic forces (Chobanian, 1983, 1990).

Haemodynamic factors play an important role in the development of atheromas in WHHL rabbits (Chobanian, 1990). The area of atherosclerotic lesions of vessels is much greater in the ascending aorta and aortic arch, than in the distal regions. Atherosclerotic damage in the thoracic aorta has a V-like form and is situated more distal to the intercostal arteries (Chobanian et al., 1986, 1989; Ragazzi et al., 1993, 1995), where the haemodynamic influence of blood flow is much less.

The development of atherosclerosis may be related to the physiological response of arteries to wall shear on a localized basis (Chobanian, 1990). The deficit in LDL receptors in ECs and the large amount of circulating LDL in WHHL rabbits suggests that the intimal thickening may be pathological i.e. an atherosclerotic plaque may develop. The adhesion of leukocytes, mainly monocytes, may also occur (Rosenfeld and Ross, 1990; Aliev et al., 1991, 1993).

Hypertension as an atherogenesis factor

The means by which hypertension induces lesions of atherosclerosis is poorly understood (Chobanian 1983, 1990). The high intravascular pressure appears as one of the most important risk factors in the development of atherosclerotic plaques (Chobanian et al., 1986, 1989). The morphological changes in the vessel wall in animal models of both hypertension and atherosclerosis (animals fed on a high cholesterol diet) are similar in many aspects; the penetration of monocytes into the vascular wall is increased, the structure of the endothelium is changed, the migration and proliferation of SMCs are stimulated, adhesion of leukocytes to the EC surface is increased and monocytes or macrophages and extracellular components accumulate in the intima (Weiner et al., 1969; Schwartz and Benditt, 1977; Brecher et al., 1978; Haudenschild et al., 1981, 1985; Clowes and Schwartz, 1985; Chobanian et al., 1986, 1989; Lichtenstein and Chobanian, 1990). However, hypertension alone does not lead to deposition of lipids in the vascular wall (Chobanian et al., 1986, 1989).

The frequency and manifestation of atherosclerotic damage increases in animals with hypertension and with increased blood levels of cholesterol (Dill and Isenhour, 1942; McGill et al., 1985; Chobanian et al., 1989). In hypertension, the manifestation of the fatty streaks and fibrous plaques and the degree of arterial stenosis increases in many arterial vessels, including coronary, cerebral and limb vessels (Wilkins et al., 1959; Robertson and Strong, 1968; Chobanian et al., 1989). This has been recognized using the model of arterial hypertension in Watanabe rabbits which showed that the development of atherosclerotic lesions intensifies greatly under the condition of chronic increase of arterial pressure (Chobanian et al., 1989).

Hypertension increases penetration of lipids into ECs and also accelerates accumulation of lipids in the aortic intima. Hypertension of 2-2.5 months duration is

sufficient to stimulate atherosclerosis in WHHL rabbits. Increased duration of hypertension only increases the depth of damage, but not the area of atherosclerotic lesions (Chobanian et al., 1989).

Hypertension increases the area of atherosclerotic damage in all arterial vessels in Watanabe rabbits (Chobanian et al., 1989), particularly in the thoracic and abdominal aorta. The vessel content of free and esterified cholesterol is also increased. The difference between hypertensive and normotensive Watanabe rabbits in their ascending aorta and aortic arch is less marked, because the process of development of atherosclerosis is progresses independently of hypertension (Chobanian et al., 1989). At the same time the atherosclerotic damages in Watanabe rabbits with hypertension is more diffuse and their origin is not always defined. It is possible that haemodynamic stress may play a role in the alteration of the endothelium (Karino and Goldsmith, 1985; Karino, 1986; Chobanian et al., 1989).

In experimental hypertension, Chobanian and coworkers (1986) have demonstrated endothelial dysfunction with monocyte adhesion and transendothelial migration of the adherent cells. The monocytes become macrophages after they enter the intima even though little or no lipid accumulates under these conditions. The combined effects of hyperlipidaemia and hypertension in rabbits can lead to marked enhancement of atherosclerotic lesion. Thus, the common theme of endothelial dysfunction, inflammation, and a fibroproliferative response occurs in hypertension as well as in hyperlipidaemia (Ross, 1993).

Differences in morphogenesis of atherosclerosis in WHHL rabbits and rabbits with diet-induced hypercholesterolaemia

A number of reports have described the intimal changes that precede the formation of fatty streaks in animals and in humans (for review see: Ross, 1993).

The cellular interaction which take place during the formation of fatty streaks in diet-induced atherosclerosis of non-human primates (Faggiotto et al., 1984; Faggiotto and Ross, 1984), pigs (Thomas et al., 1979; Gerrity, 1981a,b; Gerrity et al., 1985), rabbits (Simionescu et al., 1986; Simionescu and Simionescu, 1993), rats (Joris et al., 1983, Chinellato et al., 1994a,b) and pigeons (Jerome and Lewis, 1984, 1985) has been well documented. Several authors (Simionescu et al., 1986; Mori et al., 1989; Simionescu and Simionescu, 1993), using electron microscopy, have detected subtle changes in lesion-prone areas of the aortic arch of rabbits in the first 2 weeks of diet-induced hypercholesterolaemia, before monocytes enter the artery and become foam cells. These changes are characterized by a progressive accumulation of small unilamellar and multilamellar vesicles within the extracellular matrix, in the region between the endothelium and the internal elastic lamina. These vesicles contain large amounts of free cholesterol and are described as extracellular liposomes and are thought to represent particles that some investigators have described as cell debris or precipitates (see: Ross, 1993).

Monocytes become macrophages which develop in to foam cells and then, in addition, SMCs begin to migrate towards the subendothelium. During the late phases, SMCs also play an important and particular role in the formation of fatty streaks.

Modification of feeding conditions by reducing dietary cholesterol and fat and increasing periods of feeding leads to damage which is similar to other human atherosclerotic lesions (Vesselinovitch, 1979, 1988; Buja et al., 1983, 1990).

In both WHHL rabbits and rabbits fed on a high cholesterol diet, a reduction in circulating high density lipoproteins (HDL) is noted; and the process of reverse transport of cholesterol from cells and its removal is disturbed (Rosenfeld et al., 1990; Rosenfeld and Ross, 1990).

The progression of atherosclerosis in WHHL rabbits includes the formation of atheromatous plaques to a greater extent with the formation of foam cells, although there are not any manifested lipid deposits in the macrophages of spleen, liver, or in lymphatic nodes. When rabbits are fed on a high cholesterol diet, damage of arterial vessels is usually of the "foam cell" type with the simultaneous deposit of lipids in the macrophages of liver, spleen and lymphatic nodes as well as in the red bone marrow. In this case there are a number of SMCs with lipid deposits although many more cells have features of macrophages (Figs. 2B, 3A) (Imai et al., 1966; Scott et al., 1971).

In WHHL rabbits and rabbits which are fed on a cholesterol diet, atherosclerotic plaques develop with increased adhesion of monocytes (Fig. 1B). Using immunocytochemistry, migration into the subendothelial layer has been observed, resulting in the formation of only one layer of intimal foam cells which again form macrophages (Tsukada et al., 1987; Rosenfeld et al., 1987a,b; Rosenfeld and Ross, 1990). These data suggest that functional activity of LDL receptors and the difference in the distribution of cholesterol in WHHL rabbits and in rabbits fed on a cholesterol diet, does not influence initiation of morphogenesis of atherosclerotic damage in either model (Rosenfeld et al., 1987b; Tsukada et al., 1987).

The level of atherosclerotic lesions including the extent of damage is well correlated with the intima and intimal/medial indices (Rosenfeld et al., 1987b; Rosenfeld and Ross, 1990). Considerable incorporation of thymidine into cells of the plaque is observed mostly in the superficial layer and in the lateral parts of the atheromatous plaques. About 12% of labelled cells are foam cells which are usually localized just under the endothelium and along the lateral part of the plaque (Rosenfeld and Ross 1990). This has been confirmed by many authors (Spraragen et al., 1962; McMillan and Stary, 1968; Cavallero et al., 1971). A small population of labelled foam cells is localized in the "necrotic

nucleus" of the manifested atheroma. Plaques of the same size have the same index in all segments of aorta irrespective of the level. Using immunocytochemistry, it has been shown in both WHHL rabbits and NZW rabbits on high cholesterol diet with atheromatous lesions that 30% of the labelled cells are macrophages and 45% are SMCs (Tsukada et al., 1987; Rosenfeld and Ross, 1990).

ECs in WHHL rabbits contain more lipids than cholesterol-fed NZW rabbits (Rosenfeld et al., 1987a, 1990). There is a large difference between cholesterol- and fat-fed NZW and WHHL rabbits in the pathobiology of atherogenesis. Normal rabbits fed a cholesterol diet show the following features: 1) a high level of β-migrating very low density lipoproteins (β-VLDL) but no LDL development; 2) damage is mainly confined to fatty streaks and contains a large number of foam cells of macrophage origin and a very small number of SMCs in the intima; 3) a massive deposit of lipids is noticeable in extravasal macrophages (in the reticulo-endothelial system); 4) the involvement of small vessels in the process is expressed well (Rosenfeld et al., 1990).

Normal tissue macrophages possess receptors which bind β-VLDL, but not LDL, and in this way help to carry out selective absorption of β-VLDL particles by macrophages, resulting in the deposit of cholesterol esters (Goldstein et al., 1983; Mahley, 1985; Steinberg et al., 1989). In spite of this metabolic peculiarity, morphological investigation shows that the key components involved in the initiation of damage and its progression are the same in WHHL rabbits and NZW rabbits fed on either a cholesterol or a fat diet (Rosenfeld et al., 1987a,b). After 8 weeks of development, the structure of plaques in WHHL rabbits and cholesterol-fed NZW rabbits is similar (Rosenfeld et al., 1987b). The main components in both cases are macrophages and foam cells or derivatives of these cells (Rosenfeld et al., 1987b).

Functional morphology of the aortic lesions in rat with heritable hypercholesterolaemia (Yoshida Pittsburgh Heritable Hyperlipidaemic rats)

The association between hypercholesterolaemia and atherosclerotic lesions in humans has been widely demonstrated (Goldstein and Brown, 1977; Steinberg et al., 1989; Nordoy and Goodnight, 1990). WHHL rabbits are characterized by a genetic lack of functional LDL receptors and develop severe atherosclerosis, as in FH hyperlipidaemic patients. The best characterized model is the Watanabe rabbit, a model of homozygous and heterozygous type IIa hypercholesterolaemia, related to an LDL receptor deficiency (Watanabe, 1980, Watanabe et al., 1985). This animal model is therefore suitable for investigating familial hypercholesterolaemia. Other genetic hypercholesterolaemic animals models may give further experimental support to the understanding of pathogenesis of atherosclerosis.

Until now the rat has not been a favoured model for investigating the pathogenesis of atherosclerosis due to its resistance to atherosclerotic lesions (Armstrong and Heistad, 1990; Joles et al., 1991). Only certain strains of mice show arterial lesions in response to a very high cholesterol diet (Morrisett et al., 1982). A model of spontaneous hyperlipidaemia was reported by Fantappie et al. (1992), where the dyslipidaemia was caused by a generalized rise of all lipoprotein classes. The Pittsburgh Yoshida (YOS) rat is an inbred hyperlipidaemic rat which has only recently become available. This animal model has been developed by inoculation of Yoshida sarcoma cells to Donryu rats (Omura et al., 1995). The characteristic peculiarity of Donryu rats appears as specific changes of vascular functions particularly after feeding on a high cholesterol diet (Ralevic et al., 1996). Plasma lipoprotein profiles of YOS rats have been reported recently by Fantappie et al. (1992).

Young YOS rats (about 2-months old) are characterized by the presence of sporadic adhering of leukocytes to ECs in the aortic arch and thoracic ostia (Chinellato et al., 1994a). The presence of fibrous intimal combs are seen with increasing age. At 24 months YOS rats show ovoid tuberous lesions found mainly around the entry of intercostal arteries (Chinellato et al., 1994a). Characteristic changes in the aortic wall includes the formation of rhytmic structures, described by Vikhert and Zdanov (1989) in human atherosclerotic lesions. Recently we have shown that these new endogenous hyperlipidaemic animals (YOS rats) have high serum lipid levels but no atheromatous plaques on the aortic wall (Chinellato et al., 1994a,b) and with impaired vascular function increasing with age and mainly located at the endothelial level (Chinellato et al., 1994a,b). The YOS rat, therefore, represents a new and suitable model of endogenous hyperlipidaemia, different from the WHHL rabbit in which hyperlipidaemia is clearly associated with atherogenesis (Buja et al., 1983). Moreover, young YOS rats fed with a special CCT diet (a diet enriched with 4% cholesterol, 1% cholic acid and 0,5% thiouracil) (Joris et al., 1983) showed the absence of typical atherosclerotic lesions in the aortic wall (Chinellato et al., 1994b). Previously it has been shown that normolipidaemic rats when fed on this same diet show an acceleration of adhesion of leukocytes followed by their migration through the endothelial monolayer and lipid-containing foam cells accumulated in their cytoplasm (Joris et al., 1983). Proliferation and migration of SMCs from media to intima and the formation of atherosclerotic lesions was very similar to that in WHHL rabbits. However, in the rat it was observed that a CCT diet produced atherosclerotic plaques very similar to those found in humans (Joris et al., 1983).

Recent studies have demonstrated that, YOS rats fed on a CCT diet showed a significant increase in total serum cholesterol and HDL, and no change in triglyceride levels (Pandolfo et al., 1993, 1994; Chinellato et al., 1994b). Moreover, the cholesterol content in thoracic aortic tissue was significantly increased in cholesterol-fed YOS rats (Pandolfo et al., 1993, 1994;

Chinellato et al., 1994b). Infiltration of high levels of lipids into aortic tissue is considered a main risk factor for atherogenesis as scavenger macrophages in the subendothelial level accumulate lipid material to become foam cells (Steinberg, et al., 1988; Brown and Goldstein, 1990). Interestingly, despite cholesterol infiltration in the aortic tissue of the CCT-fed YOS rats, no typical atherosclerotic plaques were found (Chinellato et al., 1994b). It may be speculated that, in this animal model, LDL is not atherogenic or that vascular tissue may be resistant to the damage induced by lipid infiltration. Pandolfo et al. (1993, 1994) suggested that significant increases of HDL in blood serum of YOS rats leads to factors which may protect ECs under high cholesterol conditions. It is noteworthy that in the YOS rats, after 2 months of CCT diet that, despite the further increases in serum cholesterol level caused by the high cholesterol diet, no atheromatous plaques on aortic wall develop although the number of adhered leukocytes increase significantly (Chinellato et al., 1994b). This investigation suggests, that CCT treatment of YOS rats may represent a new model of mixed endogenous and exogenous hyperlipidaemia, different from the well known WHHL rabbit, in which hyperlipidaemia is clearly associated with atherogenesis.

The CCT-treated YOS rat presents a mild hypercholesterolaemia that may resemble many human dyslipidaemic diseases and therefore may become a useful tool in the study of early stages of atherogenesis and possible pharmacological intervention.

Functional changes in the vessel wall in WHHL rabbits

A role of endothelial dysfunction in the pathophysiology of atherosclerosis continues to emerge. The first principal insight into the importance of the endothelium as a modulator of vascular tone was by Furchgott and Zawadski (1980). These investigators demonstrated the obligatory role of endothelium in the relaxation to acetylcholine of rings of rabbit aorta contracted with noradrenaline.

Several recent studies have shown that endothelium dependent vascular relaxation is impaired in human and animal models of atherosclerosis including in the WHHL rabbit (Ragazzi et al., 1989, 1993, 1995; Stewart-Lee et al., 1991, 1992; Stewart-Lee and Burnstock, 1991; Brizzolara et al., 1992; Cirillo et al., 1992; Caparrotta et al., 1993; for review see Chinellato and Ragazzi, 1995).

Responses to acetylcholine, thrombin and other agents are markedly abnormal in atherosclerotic monkeys and rabbits (Freiman et al., 1986; Verbeuren et al., 1986, 1994; Chappel et al., 1987; Jayakody et al., 1987, 1988; De Meyer et al., 1991, 1992). The weakening of endothelial-mediated relaxation has been demonstrated in many animals during diet-induced hypercholesterolaemia both with and without balloon damage of the endothelium (Freiman et al., 1986; Yamamoto et al., 1987a,b; Verbeuren et al., 1986), as well as increased constrictor response to the various neurohumoral agents *in vitro* (Yokoyama et al., 1983, 1987) and *in vivo* (Kawachi et al., 1984; Tagawa et al., 1991). Similar observations were seen with vessels obtained from WHHL rabbits (Stewart-Lee et al., 1991, 1992; Stewart-Lee and Burnstock, 1991; Brizzolara et al., 1992; Cirillo et al., 1992; for review see: Chinellato and Ragazzi, 1995). It was shown that relaxation of the aorta in response to acetylcholine and some other vasoactive substances (including ATP) is reduced with age; this may be connected with injury of the endothelial monolayer (see: Chinellato and Ragazzi, 1995). The contractile response of arteries to phenylephrine (an activator of α-adrenergic receptors), histamine and serotonin remains unchanged when the endothelium is mechanically removed (Kolodgie et al., 1990). An increased contractile response was shown when the endothelium was removed from vessels of rats, dogs and pigs (Cocks and Angus, 1983; Van de Voorde and Leusen, 1983, 1986; Martin et al., 1986). However, earlier observations (Furchgott, 1983; Verbeuren et al., 1986) showed that removing ECs from the aorta of the rabbit does not lead to any increase in the contractile response. It is most probable therefore that increased contraction of the vascular wall is a response to the influence of histamine and serotonin in WHHL rabbits connected with the changing function of SMCs, rather than with the loss of ECs (Kolodgie et al., 1990).

The dynamic role of the endothelium in the regulation of vascular tone was further established when it was observed that relaxation of isolated blood vessels by vasoactive substances, such as acetylcholine and the calcium ionophore A23187, was dependent on an intact endothelium and a diffusible factor (endothelium-derived relaxing factor, EDRF) that stimulated cyclic guanosine monophosphate (cGMP)-dependent relaxation of vascular smooth muscle cells (Chappel et al., 1987). Endothelium-dependent relaxation in response to acetylcholine, ATP and calcium ionophore A23187 progressively decreases with the progress of atherosclerotic damage (Kolodgie et al., 1990; Ragazzi et al., 1993) and is connected with the loss of ECs in the region of the plaques, rather than a change of function of ECs. Endothelium-independent relaxation in response to nitroglycerin remains unchanged in all regions, although the vessels with severe atherosclerotic damage become less sensitive to the action of vasoactive stimuli (Kolodgie et al., 1990).

It has been demonstrated that 12-14 month-old WHHL rabbits have a significant loss of ECs in the central region of atherosclerotic plaques (36%). Before 12 months of age the rupture of the endothelial monolayer is rare. The contractile response to histamine increases with age (Kolodgie et al., 1990). At 8-12 months (Yokoyama et al., 1983), and between 10-22 months, the contractile response to serotonin (Yokoyama et al., 1987) increases in the aorta with atherosclerotic changes. It is supposed that the destruction of ECs may

be a dominant mechanism in altering vascular vasodilatation although the importance of dysfunction of endotheliocytes is not excluded. At the same time, ECs in WHHL rabbits may adapted to the high level of circulating cholesterol, since they exist in these surroundings from the early period of embryogenesis.

The percentage of the surface of the arch which is occupied by atherosclerotic damage increases with age in WHHL rabbits; from 11% (3-5 months), to 28% (6-9 months), and to 54% and more (12-14 months). Only isolated plaques were observed in the thoracic aorta in all age groups. The contractile response to phenylephrine decreases with increased damage. The contractile response to histamine is increased in all age groups, while only certain responses to serotonin are increased (Kolodgie et al., 1990). Aged WHHL rabbits with pronounced atherosclerotic damage show severe disturbances of haemodynamic indices (Watanabe et al., 1985; Paro et al., 1991, 1992).

The damage or loss of EC can predispose the atherosclerotic vessels to vasospasm (Kolodgie et al., 1990). As soon as the function of ECs is damaged, the absence of a vasodilator response can make the vessel unprotected against vasoconstrictor stimuli (Shepherd and Vanhoutte, 1986). Thus, the functional features of myocardium in WHHL rabbits are also disturbed (Clozel et al., 1988; Paro et al., 1991, 1992). The coronary reserve in Watanabe rabbits of 300 days of age is reduced at the expense of stenosis of the coronary arteries (Clozel et al., 1988). It is well known that coronary spasm is of great pathogenic importance in the pathophysiology of ischaemic heart disease. In patients with angina pectoris, coronary spasm is seen angiographically after the administration of pharmacological agents (Schroeder et al., 1977; Kaski et al., 1986; Yasue et al., 1986).

Impairment of muscarinic endothelium-dependent relaxation induced by acetylcholine has also been found *in vitro* in WHHL rabbit thoracic aorta (Ragazzi et al., 1989; Kolodgie et al., 1990). Despite endothelium damage, muscarinic-mediated relaxation and the relaxant response to ATP in the WHHL rabbit aorta was not found to be impaired when compared with that in age-matched normolipidaemic NZW rabbits (Ragazzi et al., 1989; for detail see Chinellato and Ragazzi, 1995). Only a part of the relaxant effect of ATP is mediated by ECs (Verbeuren et al., 1986; De Meyer et al., 1991, 1992). It has been demonstrated, that the endothelium-dependent relaxation produced by ATP in atherosclerotic WHHL rabbit aorta is not impaired (Chinellato et al., 1993). In diet-induced atherosclerotic rabbit aorta (Bossaller et al., 1987a,b) and in rabbit carotid artery after mechanical damage (De Meyer et al., 1991), muscarinic endothelium-dependent relaxation is impaired while endothelium-dependent relaxation to the calcium ionophore A23187 is completely preserved, demonstrating a selective alteration of receptors. ATP induced endothelial relaxation in the WHHL rabbit aorta is not mediated by the P2Y receptor because the potent-agonist 2-Me-S-ATP was without effect (Ragazzi et al., 1993). However, the exact receptor site of action of ATP at the endothelial level is not yet clear.

Wines et al. (1989) demonstrated that aortic vascular responses to serotonin were increased in 1- and 6-month-old WHHL rabbits, with the increase being greater in aortas from 6-months-old animals. Those authors suggested that the augmented vasoconstrictor response to serotonin in WHHL rabbits precedes the development of gross, but not microscopic, atherosclerotic changes of the vessels, and a specific and selective increase in the vasoconstrictor response of the isolated aorta to serotonin. These findings suggest that enhanced vasoconstriction to serotonin may occur during the earliest stages of atherosclerotic plaque morphogenesis.

Changes in vasoconstrictor and vasodilator responses during progression of atherosclerotic lesions differ in different vessels (Stewart-Lee et al., 1991, 1992; Brizzolara et al., 1992; Cirillo et al., 1992). In the basilar artery, the action of vasoconstrictor factors (KCl) at basal tone showed no difference at 4, 6, and 12 months of age in either WHHL or NZW rabbits. Contractile responses to both histamine and neuropeptide Y were significantly greater in 12-month-old WHHL rabbit preparations when compared with responses measured at 4 and 6 months (Stewart-Lee and Burnstock, 1991).

Endothelium-dependent relaxations to acetylcholine in raised-tone preparations from WHHL rabbits were significantly greater at 6 months than at either 4 or 12 months of age. In contrast, endothelium-independent relaxations to calcitonin gene-related peptide and vasoactive intestinal polypeptides showed no change over the age range studied (Stewart-Lee and Burnstock, 1991). The significance of these changes in the rabbit basilar artery in atherosclerosis has been discussed in relation to the 'protection' of intracranial arteries from atherosclerosis and their subsequent susceptibility to cerebral ischaemia and stroke (Stewart-Lee and Burnstock, 1991).

Brizzolara et al. (1992) have suggested that with the progression of atherosclerosis in the hepatic artery of WHHL rabbits, a 'compensatory vasodilatation' is established, initially through changes of the endothelium and subsequently, as the disease develops, extending to changes in SMC.

Cirillo et al. (1992) showed that blood flow was significantly reduced and hindlimb vascular resistance increased in aged WHHL rabbits after local intra-arterial bolus injection of increasing doses of acetylcholine, bradykinin, serotonin, sodium nitroprusside and phenylephrine. The only marked alteration found in WHHL rabbits was a clear impairment to bradykinin stimulation. Therefore, peripheral circulation in WHHL rabbits shows some unusual features, such as increased basal vascular resistance and a selective impairment of bradykinin responses (Cirillo et al., 1992). Together with these abnormalities, it seems that responses to various vasoactive agents are normal suggesting that, in the

WHHL model of atherosclerosis, the alterations are more specific than in other models (Cirillo et al., 1992).

A reduction of sympathetic neurotransmission in WHHL rabbits at 12 months of age was shown to be due to a reduction in the release of the cotransmitters noradrenaline and ATP. The reduced contractile response to nerve stimulation in 12-months-old WHHL rabbits is suggested as a 'protection' of the mesenteric artery from potential vasospasm and atherogenesis (Stewart-Lee et al., 1991).

The role of the endothelium in the regulation of vascular tone has attracted considerable interest since the discovery that many substances may exert effects on vessel tone via receptors located on ECs (Furchgott and Zawadski, 1980). The importance of endothelial integrity is emphasized by the fact that in many vessels the same vasoactive substance may produce opposite effects on vascular tone, depending on the presence or absence of intact endothelium and the corresponding exposure of different receptor subtypes on the underlying SMC. Activation of endothelial receptors initiates a chain of events, a key part of which is the release of EDRF, endothelium-derived constricting factor, or prostaglandins, which subsequently affect vessel tone via the smooth muscle (for review see, Moncada et al., 1991; Ralevic and Burnstock, 1993).

In 1988, Furchgott and Ignarro independently suggested that EDRF was nitric oxide (NO) (Ignarro et al., 1988; Furchgott, 1990). Additional studies have shown that NO is synthesized from the conversion of L-arginine to citrulline by a least two enzymes: a) constitutive NO synthase (cNOS; predominantly membrane bound and calcium- and calmodulin-dependent), and b) inducible NO synthase (iNOS; predominantly cytosolic and calcium- and calmodulin-independent), but both utilizing at least 5 co-factors, including nicotinamide adenine dinucleotide, flavin mononucleotide, and heme (Ignarro et al., 1988; Palmer et al., 1988; Forstermann et al., 1993, 1994; for reviews see Moncada et al., 1991; Morris and Billiar, 1994; Dusting, 1995). NO accounts for the biological activity of EDRF (Palmer et al., 1988; Schmidt et al., 1991; Moncada et al., 1991; Marletta, 1994). NO causes relaxation of vascular smooth muscle by interacting with soluble guanylate cyclase which leads to an increase in cGMP levels (Forstermann et al., 1993, 1994). Inhibition of endogenous NO synthesis in rat aortic rings and in the anaesthetized rat induces specific supersensitivity to the vasodilator effect of nitrovasodilators. The concept that vascular tone is partly modulated by the level of basal NO synthesis has important implications for the understanding of the physiological and pathological regulation of vascular tone (Moncada et al., 1991; Schmidt et al., 1991). Reduced synthesis and/or inactivation of EDRF has been suggested to be involved in impaired endothelium-dependent relaxation in WHHL rabbits (Tagawa et al., 1991). Recently it has been demonstrated that the vascular content of L-arginine is significantly lower in WHHL rabbits compared with NZW rabbits (Chinellato et al., 1992). Reduced endothelium-dependent NO-mediated relaxation also occurs in human atherosclerotic coronary arteries *in vitro* and *in vivo* (Forstermann et al., 1988; Lefer and Sedar, 1991).

In a recent study of endothelium-dependent vascular relaxation of aortic isolated preparations, it has been shown that preincubation for 45 min with L-arginine (1 mM) did not modify the effect of acetylcholine. This lack of effect of L-arginine indicates that amino acid deficiency is not the main cause of impairment of endothelial function (Caparrotta et al., 1993).

There are three possible explanations for abnormal endothelium-dependent modulation of vascular SMC tone in atherosclerosis. Firstly, production or release of EDRF may be decreased in atherosclerotic vessels, including WHHL rabbits (Caparrotta et al., 1993). This abnormality may be secondary to alterations of membrane receptors or dysfunction of the pathways leading to EDRF synthesis and release. Secondly, atherosclerotic vessels may be less sensitive to EDRF. This may be due to thickened intima or increased lipids in the vessel wall of atherosclerotic vessels acting as a barrier to diffusion of EDRF from the endothelium to the underlying vascular smooth muscle (for review see Rubanyi, 1993). Alternatively, EDRF may be inactivated by oxygen radicals released by inflammatory cells, including foam cells which have a great amount of lipids in the cytoplasmic matrix of the atherosclerotic vessel wall (Rubanyi, 1988; Aliev et al., 1995a,b). Thirdly, the endothelium of atherosclerotic vessels may store and release a constrictor factor, namely endothelin-1 (ET-1) (Lerman et al., 1991; Lerman and Burnett, 1992; Aliev et al., 1995a-c; Jones et al., 1996) which may negate or diminish the effect of concomitantly released EDRF (Lerman and Burnett, 1992).

ET-1 is the most potent vasoconstrictor known and produces contraction of isolated arteries and veins (Yanagisawa et al., 1988; Miller et al., 1989; Lerman and Burnett, 1992). In addition, endothelin possesses mitogenic properties *in vitro*, stimulating DNA synthesis in cultured vascular SMCs in a dose-dependent manner (Hirata et al., 1989). DNA synthesis is markedly inhibited by antibodies to endothelin (Takagi et al., 1990), suggesting a role for endothelin as a growth factor. A recent study (Lerman and Burnett, 1992) showed that plasma and tissue levels of endothelin in humans with symptomatic atherosclerotic vascular disease requiring arterial revascularization were correlated with the number of disease sites involved, and plasma endothelin concentrations were elevated in advanced atherosclerosis. Specific immunohistochemistry staining for ET-1 in human atherosclerotic aorta demonstrated the presence of ET-1 in the cytoplasm of both vascular smooth muscle and ECs (Lerman and Burnett, 1992; Aliev et al., 1995b; Jones et al., 1996). This suggests a role for endothelin as a marker for arterial vascular injury and as a participant in atherogenesis (Lerman et al., 1991; Lerman and Burnett, 1992; Aliev et al., 1995b; Jones et al., 1996). Similar

findings were found in the thoracic aorta of WHHL rabbits, particularly in aged groups, where ECs, subendothelial and medial lipid-laden macrophages and SMCs show a high immunopositive reaction for antibody against ET-1 (Aliev and Burnstock, 1995). Therefore, our studies and other recent observations (for references see above) suggest that endothelium damage which occurs in atherosclerosis disturbs the balance between EDRF and endothelin, leading to unopposed vasoconstriction and SMC proliferation.

Artificial damage of the vascular wall in WHHL rabbits

The main attention in modelling atherogenic processes concentrates on two factors; reproduction of hyperlipidaemia and induction, by different means, a region of endothelium with functional changes. Various approaches have been used in modelling diet-induced hypercholesterolaemia in different species (rabbits, pigs, monkeys, etc.) (Jokinen et al., 1985); activation of the processes of peroxide oxidation of lipids (Rosenfeld et al., 1990); simulating intake deficit of natural antioxidants (Mironov et al., 1989); endothelium denudation with or without intravascular manipulation of the vessel wall, such as by balloon catheter de-endothelialization or local exposure to liquid nitrogen or by using endotoxin treatment (Malkzak and Buck, 1977; Reidy and Schwartz, 1983; Reidy, 1985; Mironov et al., 1985, 1988); use of WHHL rabbits as described by Watanabe and colleagues (Kondo and Watanabe, 1975; Watanabe, 1980; Watanabe et al., 1981, 1985, 1987). The WHHL rabbit is the most widely used model for studying atherogenic processes in hereditary hypercholesterolaemia (Buja et al., 1983, 1990).

However, none of these methods of atherosclerosis takes into account the morphological peculiarity of human arteries in which the structure of the aortic intima is essentially different from those in experimental animals (Rekhter et al., 1991). In most of experimental animals SMCs are located in the intimal layer (Schwartz et al., 1990; Aliev et al., 1995c) whereas the aortic intima of the human has a network of SMC of various forms and origins. Morphologically cells are mostly stellate in form in the luminal area of the elasto-hyperplastic layer and are spindle-shaped (fusiform) in musculoelastic layer of aortic intima (Rekhter et al., 1991). Rekhter and Mironov (1987) showed possible restoration of the muscular layer of the vessel wall resulting from migration of SMCs from edges of wound to the zone of injury in rat abdominal aorta after liquid nitrogen induced damage. During this process myointimal thickening developed in the disturbed area and consisted of a stellate form of SMCs in a network, although the thickness of the vessel wall remained the same (Rekhter and Mironov, 1987). Recent studies (Mironov et al., 1992) have shown that cryo-destruction of abdominal aorta and carotid artery in young (6-8-months-old) WHHL rabbits had accelerated the formation of fatty streak and fibrous plaque after 2 months. Moreover, ECs in undamaged areas were characterized by the presence of a large number of adhered leukocytes and intraluminal foam cells (Mironov et al., 1992). The plaque contained many dispersed SMCs surrounded by connective tissue and thus containing the structural elements seen during early atherosclerotic change in man (Rekhter et al., 1991).

SMC migration into the intima and their proliferation are essential steps for the development of atheromatous lesions. The mechanisms of these processes have been extensively investigated in experimental animals after endothelial denudation (Clowes and Schwartz, 1985; Schwartz et al., 1990; for review see Ross, 1986, 1993). Clowes and Schwartz (1985) have shown in rat carotid artery that only small proportions of SMCs from the media are altered in their growth kinetics following balloon catheter damage. In humans, it has been suggested that the initial SMC proliferative event leading to the formation of the atheromatous plaque involves only a small proportion of the media population, possibly one cell (Benditt and Benditt, 1973).

In human aorta, in the vicinity of fatty streaks, changes are seen in cell-cell contacts and very large cells are contained in a fibrillar matrix (Rekhter et al., 1991). These changes are very similar to the changes observed in both infrarenal segments of abdominal aorta and carotid artery of WHHL rabbits after cryo-damage (Mironov et al., 1992). This simple technique in the WHHL rabbit provides a model of human atherosclerosis in which there is a high degree of morphological similarity between the artificially induced plaque and human atherosclerotic plaque, thus providing a suitable model for the study of antiatherosclerotic drugs.

Experimental therapy of atherosclerosis in WHHL rabbits

The model of familial hypercholesterolaemia became of intensive use in testing various methods of experimental therapy of atherosclerosis. At present there are a large number of papers devoted to this area and we will outline only a few below.

It has been demonstrated that introduction of LDL, isolated from a normal rabbit, to the WHHL rabbit leads to reduced cholesterol levels in blood (Kanazawa et al., 1991). Nifedipine also decreases the concentration of cholesterol in the blood serum in WHHL rabbits (Van Niekerk et al., 1984a). However, oral administration of nifedipine has no effect on the development of atherosclerotic plaques in WHHL rabbits (Van Niekerk et al., 1984a; Watanabe et al., 1987).

The protective action of calcium antagonists and ω-3-fatty acids which is seen in animals with diet-induced hypercholesterolaemia has not been confirmed in WHHL rabbits (Clubb et al., 1989). It has been shown that when ω-3-fatty acids are added to the diet of immature WHHL rabbits, a reduction of cholesterol

levels and concentration of triglycerides is seen. In addition, there is incorporation of ω-3-fatty acids into platelets, increased luminal concentration of collagen and arachidonic acid which can induce platelet aggregation, and a reduction in atherosclerosis (Clubb et al., 1989). Reduced cholesterol levels in the plasma does not affect the progress of atherosclerosis. Hypercholesterolaemia itself is sufficient for initiating the development of atherosclerosis in rabbits or in patients with FH (Buja et al., 1990).

Treatment with the antioxidant probucol (dibutylphenol derivatives) (Carew et al., 1987; Kita et al., 1987; Steinberg et al., 1988) and with the cholesterol lowering agents HMG-CoA reductase inhibitors (Watanabe et al., 1981) has been shown to retard the development of atherosclerotic plaque formation. The inhibitory effect on atherosclerosis of the latter seems to be dose-dependent and more pronounced in young WHHL rabbits (Watanabe et al., 1981).

Probucol, which predisposes oxidation of LDL *in vitro*, reduces manifestation of atherosclerosis in WHHL rabbits without altering the level of lipids in plasma (Kita et al., 1987). The same result was found in diet-induced hypercholesterolaemia. It was shown that probucol used *in vivo* in WHHL rabbits strongly inhibits the development of atherosclerosis (Carew et al., 1987; Steinberg et al., 1988). It reduces the level of hypercholesterolaemia and inhibits the oxidative modification of LDL, thus reducing the speed of atherogenesis (Steinberg et al., 1988; Finckh et al., 1991). No reduction of lipid deposits in the aorta is seen with the control and probucol treated animals, and plasma levels of cholesterol remain largely unchanged (Stein et al., 1989). Later studies (Kleinveld et al., 1994) showed that vitamin E (an antioxidant) in lower doses was more effective than probucol for preventing the progression of atherosclerotic lesions in WHHL rabbits.

Oral administration of pravastatin (a 3-hydroxy-3-methylglutalyl coenzyme A (HMG-CoA) reductase inhibitor) to 3-month-old WHHL rabbits in doses up to 40 mg/kg per day for the period of 9 months significantly decreased plasma cholesterol by 51% when compared with untreated WHHL rabbits (Kroon et al., 1993). Similar protective effects were demonstrated when WHHL rabbits were treated with verapamil (Tilton et al., 1985).

Basal coronary flow and bradykinin-induced increase in coronary flow in Langendorff hearts of the pravastatin-treated animals were significantly greater than flow in control untreated animals. The incidence of atherosclerotic lesions in main coronary arteries and the aorta was significantly lower in pravastatin treated animals (25% and 53% respectively) than in untreated WHHL rabbits (34% and 80% respectively). The mean percentage of narrowing in the aorta was also significantly lower in the pravastatin treated group (12%) than in the controls (25%) (Kroon et al., 1993). From these findings it was suggested that long term cholesterol lowering treatment with pravastatin, starting at an early age, retards the progression of plaque formation in the aorta and coronary artery and preserves the endothelium-dependent relaxation of the coronary arteries. These results are encouraging with regard to lipid lowering treatment in familial hypercholesterolaemia in humans (Kroon et al., 1993).

Conclusions

The use a new line of rabbits with heritable hypercholesterolaemia can be considered a major step in the investigation of the pathogenesis of human familial hypercholesterolaemic atherosclerosis. The model illustrates the main points of pathogenesis of atherosclerosis which are characteristic for people with familial hypercholesterolaemia. It should be noted, however, that this model of hereditary hypercholesterolaemia has some limitations. Morphogenesis of atherosclerotic lesions in the arterial wall in WHHL rabbits does not differ from the morphogenesis of the development of atherosclerotic lesions in rabbits with diet-induced hypercholesterolaemia. Moreover, morphogenesis of the development of atherosclerotic lesions differs in the YOS rat, being characterized by atypical atherosclerotic lesions. The WHHL rabbit model does not take into account the peculiarities of the structure of the arterial wall in man. However, extensive artificial damage of the arterial wall in young WHHL rabbits, which accelerates the development of atherosclerotic lesions in that region of the vessel wall, results in lesions that are essentially similar to human atherosclerotic lesions. This model is therefore useful in the study of potent antiatherosclerotic agents.

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