Aging Influences Development and Progression of Early Aortic Atherosclerotic Lesions in Cholesterol-Fed Rabbits

Augusto Orlandi, Marcella Marcellini, Luigi Giusto Spagnoli

Abstract—The arterial wall in aged animals shows an increased susceptibility to develop atherosclerotic lesions, although the mechanisms by which aging acts are still unclear. We investigated early aortic lesions in aged rabbits (5 to 6 years old, AH group) and young rabbits (2 months old, YH group) after 2 months of 0.2% cholesterol feeding. Fatty streaks or spots mainly in the proximal segments occupied a relative surface area that was greater in AH than in YH rabbits, although plasma cholesterol and lipoprotein levels did not differ. YH lesions showed an irregular endothelial profile mainly from accumulations of large, rounded, RAM 11–positive macrophagic foam cells. There was a higher percentage of myocytic, CD-5–positive, proliferating, and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)-positive cells and larger accumulation of glycosaminoglycans in AH fatty streaks than in YH lesions. Ligation-mediated polymerase chain reaction confirmed differences in apoptosis. Early fibromuscular coats and subendothelial plasma-like insudate were also observed in AH lesions. Aged-matched normocholesterolemic rabbits showed a diffuse aortic intimal thickening composed of myocytic cells with a synthetic phenotype and extracellular matrix rich in glycosaminoglycans. In addition, in aged rabbits, we observed a spontaneous increase of monocytes adhering to the endothelial surface and a reduced expression of endothelial nitric oxide synthase in areas distant from the branches. These plasma cholesterol–independent spontaneous changes in the aortic wall of aged rabbits seem to act as a multiple atherogenic risk factor. Moreover, age-related differences in the distribution, composition, and proliferative and apoptotic rates represent crucial events during the progression of early fatty streaks to advanced plaques. (Arterioscler Thromb Vasc Biol. 2000;20:1123-1136.)

Key Words: atherosclerosis ■ aging ■ smooth muscle cells ■ glycosaminoglycans ■ endothelial nitric oxide synthase

The onset of severe or advanced fibroatheromatous plaques is a late event and represents the final result of a slow and complex phenomenon.1,2 Human and animal studies suggest that atherosclerotic plaques begin as early lesions or fatty streaks,2,3 although the mechanisms responsible for this progression are still unclear. In fact, advanced plaques are generally encountered after the fourth or fifth decade of life,2,4 and their development is at first related to the chronic exposure of the arterial wall to exogenous risk factors,5 in particular to hypercholesterolemia.6 In rabbits receiving a hyperlipemic diet, the pattern of atherosclerotic lesions depends on cholesterol content6 and duration of the diet.7 In particular, a low-dose hypercholesterolemic diet, even when extended for a long period of time, induces the development of fatty streaks.6 In addition to dietary habits, endogenous or intrinsic factors have been also considered in the pathogenesis of atherosclerosis. On the basis of the high incidence of cardiovascular disease in elderly people, McGill et al8 in 1963 first hypothesized a relation between aging and the increased onset of atherosclerotic lesions.9 The increase of the intensity of some exogenous atherogenic risk factors with aging10 and the similarity of endothelial alterations observed with hypercholesterolemic hypertension and aging11 might suggest that the latter represents the interval of time necessary for atherogenic noxae to act. However, even in the absence of recognized risk factors, aging induces intrinsic modifications in the arterial wall, which include changes of the endothelial barrier,12 the increase of intimal smooth muscle cells (SMCs),13 and the progressive vessel rigidity related to the inversion of the elastin/collagen ratio.14 We15 reported that similar to nonhuman primates,16 aged rabbits receiving a long-term low-dose hypercholesterolemic diet develop fibroatheromatous plaques, whereas in young animals, only fatty streaks are encountered in spite of similar plasma cholesterol levels. Recently, Barnes and Weinberg17 reported an increase and a different distribution of spontaneous aortic lipid deposits in old normocholesterolemic rabbits compared with very young rabbits. Nevertheless, the causes of this different susceptibility of the arterial wall and of the mechanisms supporting the progression of early lesions to fibroatheromatous plaques in cholesterol-fed aged animals remain uncertain. Similarly, comparison of the differences...
of atherosclerotic lesions between young and aged rabbits could somehow be confusing, because young animals during a long-term hyperlipemic diet grow becoming adult. 15 It is well documented that the increase of the intimal cell population by the migration and proliferation of myocytes and monocytes/macrophages 18, 19 and the accumulation of debris, 20 derived in part from apoptotic cells, 21 are mechanisms contributing to the development and progression of atherosclerotic lesions. Some experiments indicate that aging influences the proliferative rate and the effects of heparin and transforming growth factor-β1 on aortic SMCs in vitro 22, 23 or after endothelial injury in vivo. 24 Nevertheless, these age-related changes are not documented in animal models of atherosclerotic lesions by diet-induced hypercholesterolemia. The aim of the present work was to investigate how aging affects those cellular and molecular events supporting the development and the progression of lesions. Differences in the distribution, morphology, cell composition, and proliferative and apoptotic rates of aortic lesions observed in young and aged rabbits after 2 months of a moderately hypercholesterolemic diet are reported in the present study and discussed, with particular reference to their relations with normative aortic changes associated with aging.

**Methods**

**Study Design**

Nine aged (5 to 6 years old, AH group) and 9 young (2 months old, YH group) New Zealand White rabbits received a standard chow enriched with 0.2% (in weight) crystalline cholesterol (Merck) ad libitum. As a control, 8 aged (AC group) and 8 young (YC group) rabbits received only standard chow. The hypercholesterolemic diet was prepared by adding cholesterol dissolved in heated olive oil to the ingredients of standard chow before preparation. To increase tolerance to this diet, it was prepared fresh every 7 days. On chemical analysis, standard chow contained (by weight) 11.3% water, 17% fibers, 16.8% proteins, 1.6% lipids, 7.8% ash, and 45.4% nitrogen-free extract (mainly carbohydrate). Cholesterol and triglyceride contents of the standard diet were 0.06% and 0.83% (by weight). The hypercholesterolemic diet contained 0.21% cholesterol (by weight); the latter induces in rabbits a progressive moderate hypercholesterolemia without relevant signs of lipid storage-type disease. 15 Food consumption, body weight, and systolic arterial pressure were checked, as previously reported. 15 To overcome the variable response to the hyperlipemic diet, rabbits whose cholesterol after 1 month was significantly lower than the overall mean value were considered to be hyporesponders 25 and were cut off from the study. 26 For cryopreservation of aortic tissue, 4 hypercholesterolemic rabbits randomly selected were killed by intravenous injection of sodium thiopental (100 mg/kg body wt, Abbot SpA). Tissue rings from the arch and thoracic aortas were rolled up and embedded in paraffin, as previously reported. 15 For morphological, morphometric, and some immunohistochemical studies (see below). The remaining organs were macroscopically and microscopically examined to exclude the presence of any disease.

**Plasma Analysis**

Blood samples were drawn from the marginal vein of fasted rabbits (12 hours), with the addition of EDTA (Merck). Plasma was separated by centrifugation at 3000 rpm for 20 minutes, and an aliquot was used for lipoprotein separation, as we previously reported. 17 Briefly, VLDL (density [d] 1.006 g/mL), LDL (d 1.006 to 1.019 g/mL), LDL (d 1.019 to 1.065 g/mL), and HDL (d 1.065 to 1.21 g/mL) were separated by ultracentrifugation in a discontinuous salt gradient by an IEC-60 ultracentrifuge (International Equipment Co) at 41 000 rpm for 24 hours at 14°C. Fractions were collected by using a tube slicer (Beckman Inc) and stored at −20°C. Plasma lipemic pattern and lipoprotein composition were determined by enzymatic methods 27, 28 with the use of an Auto-analyser Cobas Mira S (Roche).

**Morphometric Studies**

To evaluate the size and the extent of lesions, 15 the intimal volume and surface area relative to the arterial wall were calculated on Movat’s pentachrome-stained sections of entire rolled aortas of perfused rabbits by using a Quantimet 920 image analyser (Cambridge Instruments) connected to a Polyvar microscope (Reichert Jung) by a Hamamatsu HC3077 camera. To evaluate the possible differences in the distribution, 17 a macroscopic mapping of lesions of the thoracic aorta was manually performed on each double-sized photograph by 2 different researchers using a 30×30-cm grid with 2×2-mm squares and a line spacing of 0.11 mm. Their intervariability was 1±5%. Lesions were arbitrarily classified as adjacent or distant (situated at ≥2 mm from the ostia) from branches. The prevalent spatial relation with the ostia (ie, upstream, lateral, and downstream) was also recorded.

Differences in phenotype, proliferation, and apoptosis were quantified by calculating the percentage of positive cells or their number per unit of area. The number of fields required to obtain a significant difference was calculated according to a stereological formula. 29 Intimal cellularity was obtained by calculating the number of cells per unit area at ×200 magnification. A minimum of 20 random fields and 5 serial sections were evaluated by 2 different researchers, with intervariability <5%.

A semiquantitative evaluation of glycosaminoglycans (GAGs) of the extracellular matrix was performed by using Alcian blue (8GX, Sigma; pH 2.7)—stained paraffin sections of rolled perfused aortas according to the method of Wight et al, 30 with modifications. 31 Specificity of Alcian blue staining was controlled on serial sections by digestion with 0.1% testicular hyaluronidase (Sigma) in sodium acetate buffer, pH 5.4, and 0.5 U/mL chondroitinase ABC (Sigma) in Tris buffer before staining. Alcianophilia was estimated at ×200 magnification by 2 different researchers who used a grading system in arbitrary units as follows: 0, absent staining; 0.5, equivocal staining; 1, faint staining; 2, moderate staining; and 3, intense staining. The interobserver reproducibility was >95%. For each animal, the ratio of the resulting score with the total number of fields analyzed was calculated.

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Ultrastructural Study
For transmission electron microscopy, small aortic samples selected from the arch and the thoracic tract of perfused aortas were postfixed in 1% OsO4 for 2 hours and dehydrated through an alcohol series and propylene oxide before they were embedded in Epon 812. Thin sections were stained with toluidine blue to verify the absence or presence of lesions. Ultrathin sections were cut by an 8800 Ultra-microtome III (LKB), counterstained with uranyl acetate and lead citrate, and studied with a Philips 301 electron microscope. To verify the presence of GAGs, small samples after fixation were transferred to wash buffer (0.15 mol/L sodium cacodylate) containing ruthenium red (0.75 mg/mL) for 16 hours. After postfixation in 1% OsO4 plus ruthenium red (0.75 mg/mL) for 2 hours and en bloc staining in 10% aqueous uranyl acetate for 1 hour, dehydration and embedding were performed as reported above.

For scanning electron microscopy (SEM), arterial fragments were dehydrated in ethanol and acetone series and dried in an E3100 drier (Polaron Equipment Limited) with CO2 transition fluid. Specimens were mounted on aluminum stubs with silver print (Polaron). Photographs were made by using a SCAN100 scanning electron microscope (Cambridge Instruments) at 10 kV. The number of monocytes attached per 1 mm2 of endothelial surface was counted on photographs at 3,400 magnification of at least 12 randomly selected areas for each rabbit, excluding sites at the edge of the arch and the thoracic tract of perfused aortas were postfixed in 1% OsO4 plus ruthenium red (0.75 mg/mL) for 16 hours. After postfixation in 1% OsO4 plus ruthenium red (0.75 mg/mL) for 2 hours and en bloc staining in 10% aqueous uranyl acetate for 1 hour, dehydration and embedding were performed as reported above.

Immunohistochemistry and Terminal Deoxynucleotidyl Transferase–Mediated dUTP-Biotin Nick End-Labeling
Serial sections of rolled perfused aortas were deparaffinized, rehydrated, treated in sequence with 3% H2O2 and normal rabbit serum, and incubated with RAM 11, a monoclonal antibody to rabbit macrophages,32 or anti-α-smooth muscle actin (α-actin).33 Lymphocytes were quantified on ethanol-fixed cryostatic sections from aortic rings (Table 1) by an overnight reaction with a mouse anti-rabbit CD5 (clone KENB-5, Serotec), which recognizes rabbit T lymphocytes.34 Previous studies on serial sections that reacted with α-actin and RAM 11 demonstrated that the percentage of positive cells did not significantly differ from paraffin section values (data not shown). The expression of endothelial nitric oxide (NO) synthase (eNOS) was evaluated on methanol-fixed cryostatic sections by using a monoclonal antibody (Zymed Laboratories Inc). Preliminary studies demonstrated the specific positive eNOS reaction of rabbit endothelial cells and the negativity of SMCs and fibroblasts in various organs. Intimal proliferating cells were evaluated by incubating aortic and control gut sections with an anti-BrdU monoclonal antibody (Ylem), as previously reported.26 All immunostainings were performed at room temperature. Biotinylated rat-adsorbed anti-mouse IgG (Vector Laboratories Inc), StreptABC-POD-complex (Ylem), and diaminobenzidine (Sigma) were used as secondary antibody, revelation complex, and final chromogen, respectively.

To indicate apoptosis, rehydrated sections were stripped from proteins by incubation with 300 U/mL proteinase K (Sigma) for 15 minutes at 37°C and endogenous peroxidase blocked with 0.1% H2O2 in methanol (20 minutes) at room temperature. Apoptotic nuclei were revealed by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling (TUNEL) according to the method of Gavrieli et al,35 with positive controls.

DNA Isolation and Electrophoresis
For genomic DNA isolation, tissue was finely minced in liquid nitrogen, suspended in 2 mL of digestion buffer (70 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 25 mmol/L EDTA, and 1% SDS) with 0.2 mg/mL protease K (Sigma) for 16 hours at 50°C. After inactivation of protease K by boiling, samples were incubated with DNase-free RNase from bovine pancreas (100 μg/mL, Sigma) for 1 hour at 37°C and extracted twice with phenol/chloroform. DNA was ethanol-precipitated with 0.3 mol/L sodium acetate (pH 5.0), pelleted by centrifugation, washed in 70% ethanol, air-dried, resuspended in Tris-HCl plus EDTA buffer (pH 8.0), and quantified at 260 nm by

| TABLE 1. Plasma Cholesterol and Lipoprotein Levels of Young and Aged Rabbits After 2 mo of Standard Chow Supplemented with 0.2% Cholesterol (YH and AH) or Standard Chow Alone (YC and AC) |
|-----------------|-----------------|-----------------|-----------------|
|                  | YH              | AH              | YC              | AC              |
| Total cholesterol| 178.3±32.9†     | 148.2±26.7†     | 27.9±3.3*       | 34.4±3.1†       |
| VLDL cholesterol | 57.1±6.5*       | 48.4±7.2*       | 1.9±0.4*        | 2.2±0.3†        |
| IDL cholesterol  | 35.7±4.5*       | 28.8±4.2*       | 1.6±0.4*        | 2.7±0.3†        |
| LDL cholesterol  | 12.5±2.2*       | 11.5±1.7†       | 4.1±0.5*        | 3.9±0.6†        |
| HDL cholesterol  | 37.4±4.5*       | 29.4±5.1‡       | 21.1±3.1*       | 19.8±2.8‡       |
| VLDL triglycerides| 15.2±4.2*       | 13.8±5.0‡       | 6.1±1.2*        | 4.8±1.0†        |
| IDL triglycerides| 8.8±1.5*        | 8.5±1.8*        | 3.1±0.5*        | 2.9±0.4†        |
| LDL triglycerides| 7.5±1.3*        | 7.0±1.5*        | 4.4±0.6*        | 3.9±0.3†        |
| HDL triglycerides| 25.2±5.1        | 23.7±4.8        | 21.1±3.0        | 22.3±2.4        |
| VLDL apoproteins | 12.1±1.5*       | 11.1±2.1†       | 4.2±0.8*        | 2.6±0.6†        |
| IDL apoproteins  | 19.0±2.4*       | 17.5±3.3*       | 24.1±6.2*       | 4.8±0.9†        |
| LDL apoproteins  | 14.6±1.6*       | 13.6±1.8*       | 20.1±8.2*       | 4.1±0.8†        |
| HDL apoproteins  | 126.0±14.2       | 118.3±12.9      | 138.8±21.2      | 129.5±18.2      |

Values with the same symbol for each parameter are significantly different: *P<0.01, †P<0.01, ‡P<0.05.
Values are mean±SEM.
using an Ultraspec 3000 spectrophotometer (Pharmacia Biotechnol-
ogy). DNA extraction and quantification were checked by electrophoresis on 1% agarose gel containing 1 μg/mL ethidium bromide under UV light.

**Ligation-Mediated PCR**

To better identify and quantify DNA fragmentation associated with apoptosis, we used a ligation-mediated polymerase chain reaction (PCR) of blunt DNA ends, according to Staley et al., with modifications. Genomic DNA (1 μg) was ligated to 0.1 μmol each of 24-bp (5′-AGCACC1TGAGCCCTTCACCCGCA-3′) and 12-bp (5′-TGGGGTGAAGG-3′) unphosphorylated oligonucleotides. Optimal concentration of oligonucleotide linker has been previously checked by serial dilution and by using genomic DNA from AC aortas that demonstrated the highest percentage of apoptotic nuclei by TUNEL. Oligonucleotides were annealed by heating to 55°C for 10 minutes. T4 DNA ligase (3 U, Boehringer-Mannheim) was added, and ligation was performed at 16°C for 16 hours. Reactions were diluted with Tris-HCl plus EDTA buffer to a final concentration of 5 ng/μL. Ligated DNA (150 ng) was used in the PCR assay (100 μL volume) containing 124 pmol of the 24-bp linker primer, 67 mmol/L Tris-HCl, pH 8.8, 3 mmol/L MgCl₂, 16 mmol/L (NH₄)₂SO₄, 10 mmol/L β-mercaptoethanol, 100 μg/mL BSA, and 320 μmol/L dNTPs (GIBCO). After heating to 72°C for 3 minutes, Taq poly-
merase (5 U, GIBCO) was added and incubated at 72°C for an additional 5 minutes. Samples were amplified by 25 PCR cycles (Perkin Elmer CETUS) of 1 minute at 94°C and 3 minutes at 72°C. PCR products (15 μL) were analyzed by 1.2% electrophoretic agarose gels, equilibrated in 45 mmol/L Tris borate and 1 mmol/L EDTA buffer (pH 8.0), and stained by ethidium bromide (1 μg/μL).

**Statistical Analysis**

Statistical analysis was performed by use of the SPSS program (Statistical Package for the Social Sciences, 4th ed, MJ Norusis/SPSS Inc.). For each parameter, the mean, standard error of the mean, and range were calculated. Differences were evaluated by t tests and nonparametric Mann-Whitney tests; values of P<0.05 were consid-
ered statistically significant.

**Results**

Macroscopic and microscopic examination of organs did not show any relevant diseases. The mean daily intake of chow of YH rabbits (111.0±3.4 g) was similar to that of AH rabbits (108.9±4.0 g) and less than that of AC and YC rabbits (122.5±2.21 and 123.2±1.8 g, respectively; P<0.01). Systolic arterial pressure value in YH rabbits (121.0±4.5 mm Hg) did not significantly differ from that of AH rabbits (132.8±5.2 mm Hg); these values were similar to those of respective normolipemic control rabbits and did not vary at the end of the experiment (data not shown).

As reported in Table 1, plasma cholesterol and lipoprotein levels in the AH and YH groups were similar and greater than respective normolipemic control levels. No significant differences were observed when plasma cholesterol and lipoprotein pattern of the AC and YC groups were compared.

**Morphological and Morphometric Findings**

Macroscopically, aortic lesions in AH (Figure 1A) and YH (Figure 1C) rabbits appeared as small faint blue or whitish streaks and spots, mainly in the arch and thoracic tracts. No relevant differences were observed when AH and YH lesions were compared by Evans blue staining. AH lesions appeared frequently (58.8±7.2%) distant from arterial ostia (Figure 1A); when adjacent, they did not demonstrate any predilec-
tion for upstream, lateral, or downstream margins of the branches. Instead, 62.3±5.2% of YH lesions were near arterial branches, generally downstream from the ostia (Figure 1C). The difference in the distribution of AH and YH lesions was statistically significant (P<0.05). Morphometric analysis demonstrated that the relative surface area of AH lesions was greater than that of YH lesions (P<0.02, Figure 2B), whereas the intimal relative volume (Figure 2A) and the cellularity per millimeter squared (Figure 2C) did not differ. No lesions were macroscopically detected either in AC (Figure 1E) or YC aortas.

Microscopically, in YH lesions, an endothelial layer with an irregular profile covered variable accumulations of large rounded foam cells (FCs) interspindled with rare elongated cells and scarce extracellular matrix (Figure 3A). Early lesions by a few FCs bulging from the endothelial surface could also be detected. In AH lesions, the endothelial profile appeared more regular than that in YH lesions. Groups or single rounded FCs were mixed with abundant, sometimes foamy, small elongated cells and a discrete amount of extracellular matrix, in the absence of extracellular lipidd necrotic accumulations (Figure 3B). These AH lesions resembled mature fatty streaks or human type II atherosclerotic lesions. Moreover, Movat’s pentachrome stain demonstrated the presence of brilliant red subendothelial accumulations of plasma-like substance in 80% of AH rabbits and in 46.9% of the observed lesions of AH rabbits (Figure 3C). Very small areas of insudate were detected in only 3.1% of lesions in 1 YH rabbit.

In the AC group, a diffuse intimal thickening (Figure 3G) was observed in the proximal aortic segments of 62.5% of rabbits, without any predilection for areas adjacent to arterial ostia. The intimal thickening appeared to be composed of rounded or elongated cells associated with extracellular matrix, including small elastic lamellae; no brilliant red areas were observed by Movat’s pentachrome stain. In YC rabbits, the tunica intima resulted from endothelial cells separated from the inner elastic lamina by a scarce subendothelial space. Two YC rabbits showed rare accumulations of 1 or 2 subendothelial cells.

As reported in Table 2, morphometric analysis demon-
strated that the amount of alcianophilic GAGs of intimal lesions, such as those underlying the tunica media, was greater in AH rabbits than in YH rabbits (P<0.01, Figure 1B and 1D). In AC rabbits, intimal alcianophilia was marked and more prevalent than in the underlying tunica media (P<0.02, Figure 1F). In YC intima, alcianophilia was practically absent; in the media, it was scarce and less prevalent than in AH intima (P<0.02, Figure 1I).

**Ultrastructural Study**

Investigation by transmission electron microscopy confirmed in AH and YH lesions the variable presence of elongated myocytes and rounded macrophages, respectively. In addition, rare cells showed a scanty cytoplasm, a few organelles, and a round nucleus with clumped chromatin, resembling mature lymphocytes. In large AH lesions, semithin and thin sections sometimes revealed elongated or foamy SMCs surrounded by fibrous extracellular matrix–covered clusters of
rounded macrophagic FCs, resembling an early fibrous cap (Figure 3D). No deep accumulations of extracellular lipidd necrotic material were observed. At higher magnification, subendothelial rounded macrophagic FCs were observed “floating” into AH insudate (Figure 4A). In deep portions of AH fatty streaks underlying the insudate, SMCs were prevalent and sometimes appeared foamy (Figure 4C). However, YH lesions showed a very irregular profile and a marked thinning of endothelial cytoplasm over the bulging FCs surrounded by scarce extracellular matrix and rare SMCs (Figure 4B). We observed abundant irregularly shaped granules, sometimes showing filamentous projections, inside the extracellular matrix of AH lesions. Ruthenium red staining confirmed granules corresponding to large (20- to 50-nm diameter) and small (10- to 20-nm diameter) GAGs. GAGs were also present inside the insudate, decorating blocks of amorphous material (Figure 4D).

In AC aortas, intimal thickening resulted from the accumulation of rounded SMCs, with large amounts of rough endoplasmic reticulum and cytoplasmic organelles. Extracellular matrix was composed of small elastic fibers or blocks associated with collagen microfibrils and ground substance.

Figure 1. Photographs of rabbit aortic intimal surfaces (A, C, and E). One hour after intravenous injection of 3 mL/kg of a 0.5% solution of Evans blue in physiological saline, rabbits were anesthetized and perfused through the femoral artery. Aortas were dissected, opened longitudinally, and pinned on a Teflon board. After 2 months of 0.2% cholesterol feeding, rabbits show small faint blue or whitish streaks and spots, mainly in arch and thoracic segments, occupying a greater surface area in aged (A) than in young (C) cholesterol-fed rabbits. In aged cholesterol-fed rabbit (A), thoracic lesions appear generally far from ostia and, when adjacent to ostia, without any predilection for upstream, lateral, or downstream margins of branches. Thoracic aortic lesions in young cholesterol-fed rabbit (B) are mainly near arterial branches, generally downstream from ostia. No aortic lesions are detectable in aged normocholesterolemic rabbit (E). Alcian blue (pH 2.7) staining on paraffin sections (B, D, F, and I) reveals marked GAG accumulation in aortic intimal lesion as well as in underlying tunica media of aged rabbit after 2 months of 0.2% cholesterol feeding (B). Scarcie GAG accumulation is present in lesion from young cholesterol-fed rabbit (D). In aged normocholesterolemic rabbit (F), intimal thickening shows intense Alcian blue staining, as does the underlying tunica media, whereas staining in young normocholesterolemic rabbit aortic wall (I) is weak. Immunohistochemical evaluation of eNOS expression on cryostatic sections (G and H) reveals the weak positivity of endothelial cells in an area far from branches of aged normocholesterolemic rabbit (G), in contrast with the marked positive reaction in the same area in young normocholesterolemic rabbit aorta (H). Bar = 25 μm.
Ruthenium red staining confirmed that GAGs were scarce in the subendothelial space of YC aortas (Figure 5A) and abundant in the extracellular matrix of AC intimal thickening (Figure 5B).

SEM showed the irregular endothelial surface of AH and YH lesions, with enlarged endothelial intercellular junctions compared with the adjacent normal tunica intima and single raised cells that were somehow more evident in YH than AH lesions (Figure 6). In YC rabbits, endothelial cells with a regular, smooth, and intact profile covered the luminal surface. In AC aortas, the endothelial cell profile appeared somehow irregular and prominent. Quantification on SEM photographs (Table 3) demonstrated an increased number of monocytes with microvilli attached to the aortic endothelial surface in hyperlipemic animals compared with respective normolipemic control animals. As reported in Table 3, although the overall number of adherent monocytes on lesions did not differ, those adhering on nonlesioned areas were more numerous in the AH than in the YH group (P<0.05, Figure 6). Monocytes adhering on AH lesions were sometimes grouped, whereas in YH lesions, they appeared almost single. In AC rabbits, the total number of adherent monocytes was greater than that in YC rabbits (P<0.01). This difference was more evident in endothelial areas far from the branches.

Immunohistochemistry

Immunohistochemical study on paraffin sections demonstrated a higher percentage of α-actin–positive cells in AH than in YH lesions (P<0.02, Figure 2D). In the latter, RAM 11–positive cells were prevalent (P<0.02, Figure 2E). In the YH group, α-actin–positive cells tended to be more frequent in large than in small lesions, whereas the opposite was true in AH lesions (Figure 3E and 3F). Immunohistochemical study on cryostatic sections revealed the presence of limited CD-5–positive T-lymphocyte infiltrates in AH and YH lesions. The percentage of CD-5–positive cells was higher in AH than in YH lesions (P<0.02, Figure 2F). This difference was more marked considering the number of CD-5–positive cells per millimeter squared (19.6±4.9 versus 3.4±1.2, P<0.01). In AC rabbits, intimal cells appeared to be α-actin positive.

Immunohistochemical evaluation of eNOS demonstrated a faint positivity of endothelial cells covering AH and YH lesions, such as in those of YC areas adjacent to branches, and diffuse positivity in AC endothelium. In contrast to AC cells (Figure 1G), YC endothelial cells far from the aortic ostia showed marked positivity (Figure 1H). Control α-actin immunoreactivity of underlying medial SMCs on serial sections was similar (data not shown).
Figure 3. Movat’s pentachrome staining of an aortic lesion in a young rabbit after 2 months of 0.2% cholesterol feeding (A), showing an irregular endothelial profile covering variable accumulations of large rounded FCs and scarce extracellular matrix. In an aged cholesterol-fed rabbit (B), the endothelial profile appears regular and covers groups or single rounded FCs mixed with abundant, sometimes elongated, cells and a discrete amount of extracellular matrix. In aged cholesterol-fed rabbit lesions, brilliant red subendothelial serofibrinous insudate (C) is also observed. Semithin section of a fatty streak in an aged cholesterol-fed rabbit (D) is characterized by the presence of an early fibromuscular cap covering groups of rounded FCs without fibrin-necrotic accumulations. Immunostaining for α-actin reveals rare positive cells in early lesion of a young hypercholesterolemic rabbit (E) and numerous, sometimes foamy, positive cells in fatty streak of an aged hypercholesterolemic rabbit (F). Movat’s pentachrome staining of an aged normocholesterolemic rabbit aorta (G) shows a diffuse myointimal thickening. Apoptosis as revealed by TUNEL demonstrates positive nuclei in the inner portion of fatty streak of aged cholesterol-fed rabbit (H). Bar=25 μm.
Proliferation and Apoptosis

Anti-BrdU immunostaining revealed that the percentage of proliferating cells was reduced in YH lesions compared with AH lesions ($P<0.02$, Figure 2G). Rare BrdU-positive nuclei were present in the intima of AC lesions. Apoptotic cells as detected by TUNEL were rare. They were more frequent in AH than in YH lesions ($P<0.02$, Figure 2H). In AH lesions, rounded and elongated positive nuclei could be detected, suggesting the presence of both macrophagic and myocytic apoptotic cells (Figure 3H). Very rare apoptotic cells were observed in the tunica media of the AH ($0.17\pm0.03\%$) and YH ($0.15\pm0.04\%$) groups. These values did not differ from those of respective normolipemic controls. No apoptotic intimal cells were detected in examined AC sections. To confirm the presence of apoptosis, we used a modified ligation-mediated PCR. The use of blunt-end linkers in this procedure allows us to amplify and quantify the DNA ladder that is not distinguishable when standard genomic DNA gel electrophoresis is used. $^{37}$ Quantification of ligation-mediated PCR products on agarose gel (Figure 7) showed that the integrated optical density value of AH sections was greater than that of YH sections ($P<0.01$). No significant differences were observed when integrated optical density values were compared in AC and YC aortic tissue.

### TABLE 2. Alcianophilia of Tunica Intima and Tunica Media in Young and Aged Rabbit Aortas After 2 mo of Standard Diet Supplemented With 0.2% Cholesterol (YH and AH) or Standard Diet Alone (YC and AC)

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<tr>
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<th>YH</th>
<th>AH</th>
<th>YC</th>
<th>AC</th>
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<tbody>
<tr>
<td>Tunica intima</td>
<td>1.1±0.16*‡</td>
<td>2.6±0.16†‡</td>
<td>0.2±0.15*</td>
<td>2.0±0.14†</td>
</tr>
<tr>
<td>Tunica media</td>
<td>0.7±0.08*</td>
<td>1.5±0.17*</td>
<td>0.65±0.07$</td>
<td>1.4±0.16§</td>
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Values are mean±SEM. Amount of GAGs was morphometrically evaluated with a grading system of alcianophilia in arbitrary units as follows: 0, absent; 0.5, equivocal; 1, faint; 2, moderate; and 3, intense staining.

Values with same symbols for each tunica are significantly different: *$P<0.01$, †$P<0.01$, ‡$P<0.01$, and §$P<0.02$.

**Proliferation and Apoptosis**

Anti-BrdU immunostaining revealed that the percentage of proliferating cells was reduced in YH lesions compared with AH lesions ($P<0.02$, Figure 2G). Rare BrdU-positive nuclei were present in the intima of AC lesions. Apoptotic cells as detected by TUNEL were rare. They were more frequent in AH than in YH lesions ($P<0.02$, Figure 2H). In AH lesions, rounded and elongated positive nuclei could be detected, suggesting the presence of both macrophagic and myocytic apoptotic cells (Figure 3H). Very rare apoptotic cells were observed in the tunica media of the AH ($0.17\pm0.03\%$) and YH ($0.15\pm0.04\%$) groups. These values did not differ from those of respective normolipemic controls. No apoptotic intimal cells were detected in examined AC sections. To confirm the presence of apoptosis, we used a modified ligation-mediated PCR. The use of blunt-end linkers in this procedure allows us to amplify and quantify the DNA ladder that is not distinguishable when standard genomic DNA gel electrophoresis is used. $^{37}$ Quantification of ligation-mediated PCR products on agarose gel (Figure 7) showed that the integrated optical density value of AH sections was greater than that of YH sections ($P<0.01$). No significant differences were observed when integrated optical density values were compared in AC and YC aortic tissue.

Figure 4. Transmission electron micrographs of a fatty streak in aged cholesterol-fed rabbit showing subendothelial rounded macrophagic FCs floating into subendothelial insudate (A) and a smooth muscle cell (deeply underlying insudate) with abundant rough endoplasmic vesicles and cytoplasmic organelles and a foamy appearance in the absence of adjacent macrophagic FCs. Early lesion in young cholesterol-fed rabbit (B) is seen with an irregular bulging endothelial profile indicating presence of underlying large rounded macrophagic FCs and, deeply, a smooth muscle cell (SM) apparently emerging through the inner elastic lamina. Ruthenium red staining of insudate in fatty streak from aged cholesterol-fed rabbit (D) demonstrates presence of small and large GAG granules decorating blocks of amorphous fibrinous material. Bar=1 μm.
Discussion

Aged hypercholesterolemic rabbits develop, after a long-term moderately hypercholesterolemic diet, diffuse and confluent aortic fibroatheromatous plaques\textsuperscript{15} resembling human type V lesions.\textsuperscript{38} Conversely, young animals, even in the presence of similar plasma cholesterol levels acting for the same length of time, show only fatty streaks occupying an intimal relative volume 3 times smaller than that of aged animals (Spagnoli et al\textsuperscript{15}). The experiment of Spagnoli et al demonstrated the role of aging in atherogenic processes but did not clarify the mechanisms through which aging influences the progression of lesions and, consequently, plaque complexity. We report that after 2 months of cholesterol feeding, fatty streaks in aged rabbits show a regular endothelial profile, with a prevalent myocytic cell population and abundant GAGs, resembling human type II fatty streaks.\textsuperscript{37} YH lesions were mainly composed of variable accumulations of large rounded monocyctic/macrophagic FCs with an irregular bulging endothelial profile and scarce extracellular GAG accumulation. YH lesions were similar to those previously reported for rabbits of comparable age and diet.\textsuperscript{39} Even though the intimal relative volume did not differ, AH fatty streaks occupied a greater aortic surface area than did YH lesions, because the latter occupied arterial areas adjacent and downstream from the branches, whereas AH lesions were also frequently present far from the ostia. These differences in the distribution and frequency of early lesions resembled those previously reported for spontaneous aortic lipid deposition by comparing weanling and old rabbits.\textsuperscript{17} We did not observe any difference in plasma cholesterol and lipoprotein levels between AH and YH rabbits. A significant decrease in plasma HDL cholesterol levels contributing to the increase in the severity of lesions has been reported in aged hypercholesterolemic rabbits but only after a very prolonged period of cholesterol feeding.\textsuperscript{15} These findings support the hypothesis that age-related differences in the distribution and morphology of lesions during early phases of atherogenesis are derived, at least in part, from parietal plasma-independent preexisting factors. Among these, we observed the presence of a diffuse myointimal thickening\textsuperscript{31} in aged normocholester-
oleric rabbit aortas, as found in human aortas. The distribution of AH fatty streaks and AC myointimal thickening was similar and resembled that of advanced plaques obtained through a longer period of hypercholesterolemic diet. This confirms the role of myointimal thickening in the development of fatty streaks in sites distant and not susceptible to the development of lesions in young rabbits. In the latter, a hypercholesterolemic stimulus is followed by the adhesion of circulating monocytes to the endothelial surface and their subendothelial accumulation downstream from the ostia; SMCs appear later. The presence of a preexisting diffuse myointimal thickening in aged animals also explains the higher percentage of SMCs than RAM 11-positive cells in AH fatty streaks. RAM 11-positive cells are not detected in rabbit aortic intima, at least during the first 2 weeks of cholesterol feeding. It is likely that in the presence of a mild hypercholesterolemia, the number of intimal monocytes increases slowly and progressively. In fact, fibroatheromatous plaques of aged rabbits after a long-term hypercholesterolemic diet show a percentage of macrophagic cells double that of SMCs.

It is evident that the diffuse aortic intimal thickening, as a simple favorable site for the development of fatty streaks, is not enough to explain the increased severity of lesions observed in aged rabbits after a long-term hypercholesterolemic diet. It is likely that other age-related intimal changes play an additional atherogenic role. We observed increased GAGs in AH lesions compared with YH lesions and AC intima and the scarcity of GAGs in YC subendothelial space, suggesting a preexisting plasma cholesterol-independent and age-related accumulation of GAGs. Because relative volume and cellularity did not differ, the increased amount of GAGs in AH lesions seems balanced by the hypertrophy of macrophagic FCs that constitute the majority of cells in YH lesions. GAGs are of great importance in influencing arterial properties, including permeability. Ultrastructural studies revealed that GAGs decorate blocks of amorphous and fibrin-like ma-
terial inside AH insudate, suggesting a local role of GAGs in the pathogenesis of insudate. Circulatory plasmatic molecules and lipids accumulate in regions of blood vessels with high concentrations of GAGs. The intense brilliant red staining of insudate by Movat's pentachrome indicates the presence of fibrin and plasma-derived material accumulations. The association of plasmatic molecules and GAGs may promote the development of lipoprotein-GAG complexes and the clearance of GAG-associated molecules in the AC group does not appear as the result of a generalized aging process of the arterial wall, because it was more marked than that of the underlying tunica media. Intimal SMCs displayed a "synthetic" phenotype. Similar to experimental models of intimal thickening, they may derive migration and proliferation of specific subsets of medial SMCs into the intima with aging. The presence of GAGs associated with SMC exocytotic cytoplasmic vesicle of AH lesions suggests that SMCs further synthesize GAGs when subjected to an atherogenic stimulus. The relevant role of SMCs is strengthened by the finding of early fibromuscular caps in AH fatty streaks. The latter may contribute to accelerate the progression of lesions by limiting the survival of FCs entrapped below. Early and large accumulation of extracellular lipidic-necrotic material favors the development of an atheromatous core and the progression of some fatty streaks to atheromatous plaques. It is evident that age-related accumulations of GAGs could not explain by themselves the plasmatic insudate in AH lesions. Serofibrinous insudates have been reported in Watanabe or hypercholesterolemic New Zealand rabbits as well as in humans. Rosenfeld and Ross have suggested that insudates are the consequence of endothelial retractions after the hypertrophy of bulging subendothelial FCs after a marked hypercholesterolemic stimulus. The insudate that we observed is not the result of the same phenomenon because in the presence of a similar mild hypercholesterolemia, they were absent in YH lesions in spite of a more irregular lesion surface. AH insudate may derive from an exaggerated arterial transmural flux of plasmatic macro-molecules in response to hypercholesterolemia. An age-related progressive thickening and fibrosis of the arterial wall and/or alterations of endothelial function may contribute to the development of insudate. SEM demonstrated an increase of monocytes adhering to endothelial surface areas far from the ostia similar to that found in aged rats, with an absence of variations of plasma lipemic parameters compared with conditions found in young rats. This suggests an intrinsic age-related dysfunction of endothelial cells. To confirm this hypothesis, we observed in the same sites a reduced eNOS expression, similar to that in aged rats. An impaired eNOS activity is considered a marker of endothelial dysfunction and one of the possible mechanisms leading to the reduction of endothelial levels of NO with aging. NO plays a relevant role in the

| TABLE 3. Morphometric Evaluation of Density of Monocytes Adhering to Endothelial Surface in Young and Aged Rabbit Aortas After 2 mo of Standard Chow Supplemented with 0.2% Cholesterol (YH and AH) or Standard Chow Alone (YC and AC) |
| Density, Cells/mm² |
| YH       | AH       | YC       | AC       |
| Lesioned areas | 19.5±4.4* | 21.7±4.3† | ...      | ...      |
| Nonlesioned areas | 4.0±0.5‡  | 7.3±1.3†‡ | ...      | ...      |
| Areas adjacent to ostia | ...      | ...      | 2.7±0.9  | 4.6±1.1§ |
| Areas distant from ostia | ...      | ...      | 1.1±0.4|| | 3.2±0.3§|| |
| Overall mean value | 12.3±3.3¶  | 15.5±3.2#  | 1.7±0.3¶| 4.1±0.6#** |

Values are mean±SEM. Density of monocytes with microvilli adhering to aortic endothelial surface was evaluated by manual counting on scanning electron micrographs of aortic endothelial surface at ×400 magnification.

Values with same symbols are significantly different: *P<0.01, †P<0.01, ¶P<0.01, §P<0.01, ||P<0.01, #P<0.01, **P<0.01, and ††P<0.05.
maintenance of vascular homeostasis.\textsuperscript{52,53} A chronic inhibition of NO production accelerates neointimal formation and impairs endothelial function in hypercholesterolemic rabbits.\textsuperscript{54} An age-related reduction of NO production may predispose blood vessels to atherosclerosis\textsuperscript{55} and dramatically influence the transport properties of the rabbit aortic wall.\textsuperscript{56} Age-related endothelial dysfunction amplifies the hypercholesterolemia-induced decrease of eNOS bioavailability and may increase arterial permeability, thus favoring the accumulation of plasmatic substance and the development of lesions also in those areas that are non-susceptible in young rabbits. Increased adhesion of circulatory monocytes and impaired eNOS levels may also be related phenomena, because NO gene therapy reduces adhesion molecular expression and, consequently, inflammatory cell infiltration.\textsuperscript{57}

We also observed an increased intimal cell proliferative rate in AH compared with YH lesions. Besides confirming that cell proliferation is a phenomenon present during early phases of rabbit atherogenesis,\textsuperscript{58} the age-related higher cell proliferation rate contributes to the progression of lesions by increasing the intimal cell population in vivo\textsuperscript{19,20} as in other experimental conditions.\textsuperscript{22–24} In aged rats subjected to a hypercholesterolemic stimulus, aortic intimal cells, compared with underlying medial SMCs, associated phenotypic changes with a double proliferation.\textsuperscript{59} Preliminary studies by double immunohistochemistry demonstrated that macrophagic cells mainly proliferate in early as well as in advanced lesions of cholesterol-fed young rabbits, whereas in aged rabbits, myocytic and macrophagic cells proliferate (L.G.S. et al, unpublished data, 1999). The cell proliferation rate in AH lesions was similar to that previously reported in advanced plaques of aged rabbits after long-term cholesterol feeding.\textsuperscript{26} This differs from young cholesterol-fed rabbits, in which the proliferative index has been reported to decline with time.\textsuperscript{41} All these data suggest a double effect of aging: increasing the fatty streak proliferative rate and maintaining it at high levels during the progression to advanced plaques. Apoptosis also contributes to the modulation of the size of the intimal cell population.\textsuperscript{60} Apoptosis was more frequent in AH than in YH lesions, suggesting that this is an age-related phenomenon. We could clarify whether the modulation of apoptosis is intrinsic to the arterial wall of aged animals or is mediated by other conditions. However, the absence of apoptotic cells but not of proliferating cells in AC intimal tissue confirms that mechanisms regulating programmed cell death differ, at least in part, from those inducing growth arrest.\textsuperscript{61,62} The presence of apoptotic cells in AH fatty streaks excludes the possibility that they represent an adaptive intimal thickening.\textsuperscript{63} Humoral and cellular factors (in particular, locally delivered inflammatory cytokines) contribute to the control of apoptosis.\textsuperscript{61,64} We documented the presence of T-lymphocyte infiltration in AH and YH lesions, confirming this to be an early event in rabbit atherogenesis.\textsuperscript{59} T-lymphocyte infiltration was greater in AH than in YH lesions. This difference was sharper considering the lymphocyte percentage per total mononuclear cells, with the number of nonmyocytic cells being lower in AH fatty streaks. In YH lesions, lymphocyte percentage was similar to that reported for rabbits of comparable age and on similar diets.\textsuperscript{65} Because nonmyocytic cells were less prevalent in AH than in YH lesions, we conclude that the age-related increase of lymphocytes is selective and not dependent on a possible, spontaneous, generalized increase of adhering mononuclear cells to the endothelial surface with aging.\textsuperscript{13,66} Mechanisms responsible for this increased recruitment of T lymphocytes are still to be clarified. Because T lymphocytes have many relevant biological properties, including the secretion of inflammatory cytokines,\textsuperscript{67} it is likely that greater lymphocyte infiltrates deliver locally increased amounts of cytokines, thus favoring the progression of lesions.\textsuperscript{1,67} One may hypothesize that an age-related increase of lymphocytes contributes to the modulation of the intimal cell population of fatty streaks by triggering apoptosis. Successively, apoptotic cells may facilitate the progression to advanced plaques by further stimulating the adhesion of circulating monocytes and the activation of local factors, such as thrombin.\textsuperscript{68}

In conclusion, the relation between aging and atherosclerosis appears to be a complex phenomenon. Parietal aging contributes to the development of fatty streaks in cholesterol-fed rabbits as a plasma-independent, prelesional, multifactorial risk factor. In addition, age-related influences on composition, proliferation, and apoptosis represent some of the mechanisms promoting the progression of fatty streaks to advanced plaques.

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Aging and Progression of Atherosclerosis


Aging Influences Development and Progression of Early Aortic Atherosclerotic Lesions in Cholesterol-Fed Rabbits
Augusto Orlandi, Marcella Marcellini and Luigi Giusto Spagnoli

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