Localization and Smooth Muscle Cell Composition of Atherosclerotic Lesions in Watanabe Heritable Hyperlipidemic Rabbits

Luca Giuriato, Marta Scatena, Angela Chiavegato, Anna Maria Cecilia Zanellato, Diego Guidolin, Paolo Pauletto, and Saverio Sartore

Morphological techniques (histology and electron microscopy), as well as immunofluorescence assays, were applied to the study of the localization and smooth muscle cell (SMC) composition of atherosclerotic lesions in Watanabe heritable hyperlipidemic (WHHL) rabbits during a 4.5-month period. Vascular segments from different arteries (carotid, coronary, and iliac arteries) or from the same vessel at different levels (aorta) of animals at days 7, 15, 30, 40, 60, 90, and 135 showed that the atherosclerotic lesion first became visible at the level of the aortic arch in 60-day-old WHHL animals. Histological examination of serial cryosections from this vascular region indicated that the vascular lesion arose from a cavity in the media layer, located anatomically at the level of the juncture of the ligamentum arteriosum with the aortic arch. This aortic arch cavity is formed during the postnatal closure of the ductus arteriosus and is characterized by the presence of a thickened intima, which was absent in the other vascular regions examined. Immunofluorescence comparison of normal and atherosclerotic tissues from the aortic arch cavity wall with the use of monoclonal antibodies specific for smooth muscle and nonmuscle myosin isoforms revealed the existence of distinct SMC populations. SMCs in the thickened intima showed a myosin isoform pattern peculiar to cells with a degree of maturation intermediate between the fully differentiated and the developing (fetal) aortic SMCs. By contrast, SMCs present in atherosclerotic lesions displayed a predominant fetal-type pattern of myosin isoform expression. The achievement of this myosin isoform content seems to be correlated with the accumulation of lipids in the intima. In the media subjacent to the intimal thickening or atherosclerotic lesion, SMCs primarily displayed an intermediate degree of maturation. In older WHHL animals and at this aortic level, the SMC composition of the atherosclerotic lesion did not change, whereas in the subjacent media, the cells of intermediate type almost disappeared. In the vascular regions in which the atherosclerotic lesion appeared at later stages, such as near the aortic bifurcation, the distribution of fetal and intermediate cell types in the atherosclerotic wall was similar to that taken at the aortic arch level. These results indicate that there is 1) a preferential anatomic site from which atherogenesis initiates in WHHL rabbits; 2) a time correlation between the accumulation of lipids in the wall and the phenotypic change of SMCs toward a poorly differentiated cell type; and 3) the tendency for SMCs to follow the same differentiation pattern in early atherosclerotic lesions, irrespective of the site and time at which they develop. (Arteriosclerosis and Thrombosis 1993;13:347-359)

KEY WORDS • smooth muscle cells • lesions • rabbits

It is well established that genetic factors can have a direct influence on the development of experimental and spontaneous atherogenesis.1 The Watanabe heritable hyperlipidemic (WHHL) rabbit2,3 represents a useful model in which the role of endogenous hypercho-lesterolemia in the development of atherosclerotic lesions can be conveniently evaluated. In homozygous WHHL rabbits,4 hypercholesterolemia is due to the absence of cell surface receptors for low density lipoprotein (LDL),5,6 a characteristic in common with the genetically based familial hypercholesterolemia in humans.7 Two primary cellular events take place in the structurally or functionally altered arterial wall: 1) attachment, migration, and subendothelial localization of monocytes and 2) migration/proliferation of medial smooth muscle cells (SMCs) into the intima. As a result of lipid deposition, cell accumulations, and cell–cell interactions, the arterial wall undergoes progressive changes from fatty streaks to atheromas.8–12 Initial lesions in WHHL rabbits are particularly evident in the proximal thoracic aorta adjacent to the aortic arch and at the level of the ostia of the branch vessels.8,9 In these vascular sites, raised intimal lesions are characterized by
the presence of numerous lipid-laden foam cells of macrophage origin, along with a variable proportion of lipid-containing intimal SMCs that show a marked morphological heterogeneity.8,9

Cytokontractile and cytoskeletal proteins13-17 can be used as reliable markers of vascular SMC differentiation because of their different patterns of expression during development15,18-20 and in the intimal thickening that follows mechanical injury,13,15 as well as in spontaneous14,21 and experimental12-22 atherogenesis. So far, studies8-11 that deal with the identification of SMC components in the atherosclerotic lesions of WHHL rabbits have relied on the use of the HHF-35 monoclonal anti-actin antibody, which is specific for an epitope shared by SM-specific α- and γ-actin.23 In this way, the SMCs of atherosclerotic lesions, which are in the "dedifferentiated,"15 "synthetic,"17,24,25 or "immature"26-27 phenotypic condition and that presumably contain β-nonmuscle (NM) isoactin, cannot be identified.

In a previous article,22 we analyzed the differentiation pattern of SMCs from the atherosclerotic aortas of 90-day-old New Zealand White (NZW) rabbits (maintained on a cholesterol-enriched diet) by means of monoclonal anti-myosin isoform antibodies and immunofluorescence techniques. The majority of the SMC population of the plaques showed a profile of myosin isoform expression that was identical to that of a minority of an SMC population present in the underlying media22 and normally developing vascular SM.18,22 Our more recent finding,28 that rabbit aortic SM tissue follows three developmental stages of maturation (fetal, postnatal, and adult), poses the problem of establishing the degree of differentiation of SMCs in the atherosclerotic lesions and the underlying media, as well as the potential differences between the fat-fed and genetic models of atherogenesis.

Methods

Experimental Atherogenesis

Homozgyous male and female WHHL rabbits used in this study were supplied by Dr. Marco Prosdocimi and were obtained from a colony established at Fidia Research Laboratories, Abano, Italy. Animals were used and maintained in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, 1985) and the guidelines of the Animal Care Advisory Committee of the Italian Ministry of Public Health. Their ages were 7 days (three rabbits), 15 days (four rabbits), 30 days (three rabbits), 40 days (three rabbits), 60 days (four rabbits), 90 days (five rabbits), and 4.5 months (three rabbits). A set of three NZW control rabbits was killed at 7, 15, 30, 40, 60, and 90 days and 4.5 months after birth. Plasma cholesterol levels in the WHHL animals examined ranged from 900 in 7-day-old rabbits to 700 mg/dL in 60-day-old rabbits to 600 mg/dL in 4.5-month-old rabbits. The animals were killed under sodium pentobarbital anesthesia, and the aortic arch, the thoracic and abdominal aortas, the proximal coronary arterial system, and the carotid arteries, as well as the iliac arteries close to the bifurcation, were immediately excised. The vessels were rinsed in phosphate-buffered saline (PBS), pH 7.4, dried on paper towels, and subsequently injected with Tissue-Tek OCT (Miles Inc., Diagnostic Division, Elkhart, Ind.), while maintaining a constant infusion pressure of 100 mm Hg for 5 minutes. The various vascular specimens were then frozen in liquid nitrogen, and small rings from each specimen were stored at −80°C until use.

Histology

Frozen specimens of the aortic arch (about 5 mm long) from 60-day-old WHHL or aged-matched control NZW rabbits28 were serially sectioned, in a transverse plane, at 20-μm intervals. Cryosections were fixed for 10 minutes in 10% formalin buffered in PBS and then processed for hematoxylin-eosin staining. A few cryosections were kept for lipid histochemistry and were stained with Sudan black or oil red O–hematoxylin or were used for the histochemical demonstration of elastic membranes (Weigert–van Gieson staining). Some
FIGURE 2. Light photomicrographs of hematoxylin-eosin-stained aortic arch region near the ligamentum arteriosum from a 60-day-old New Zealand White rabbit. The cavity (aac) in the wall is shown in panel A; a magnification of the vascular region that surrounds the cavity (small square in panel A) is shown in panel B. Note the marked thickening of the intima (it) and the different orientation of smooth muscle cells in the intima and underlying media (m). I, Lumen; e, endothelium; iel, internal elastic lamina. Bars: A=140 μm, B=35 μm.

Transmission Electron Microscopy
For transmission electron microscopy, specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C for 24 hours. Glutaraldehyde-fixed tissue samples (about 1 mm³) from the aortic arch region of 60-day-old WHHL and NZW rabbits were postfixed in 1% OsO₄, dehydrated through graded ethanols, and embedded in Epon 812 (Fluka, Buchs, Switzerland). Thin sections were cut on a diamond knife, collected onto copper grids, and doubly stained with uranyl acetate and lead citrate before examination in a Phillips EM 301 electron microscope. Images were taken using Kodak electron microscope film 4489 and developed in HRP Kodak developer (Eastman Kodak Co., Rochester, N.Y.).

Antibodies
Monoclonal anti-desmin and anti-vimentin antibodies were purchased from Boehringer Mannheim, Mannheim, FRG. NM-G2 and NM-F6 anti-human platelet myosin heavy-chain (MHC) and SM-E7 anti-bovine aortic SM-MHC antibodies were produced in our laboratory and have been described elsewhere.¹⁹,²⁰,²²,²₈,³₀

FIGURE 3. Light photomicrographs showing hematoxylin-eosin staining of the aortic arch region close to the ligamentum arteriosum at three different levels in 60-day-old Watanabe heritable hyperlipidemic rabbits. At the caudal level with respect to the juncture of the aorta with the ligamentum arteriosum (panel A), the atherosclerotic lesion (al) appears as a formation of tissue originating in the intima and protruding into the lumen; at more cranial levels the lesion is localized within the media (panels B and C). Cells can be grouped together when embedded deeply in the media (panel C, upper level) or dispersed when the lesion approaches the luminal surface (panel B, intermediate level). The large arrowhead in panels A and B indicates the position of the atherosclerotic lesion; small arrowheads in all three panels indicate the position of the internal elastic membrane. Bar=350 μm for all panels.

The anti-α-actin SM-type antibody was from Sigma Chemical Co., St. Louis, Mo. Fluorescent secondary antibodies (immunoglobulin G [IgG] anti-mouse conju-
gated with fluorescein isothiocyanate [FITC] or rhodamine isothiocyanate [RITC]) were from Dako, Dakopatts a/s, Glostrup, Denmark.

Immunofluorescence

Unfixed cryosections (4 μm thick) from the various vascular specimens of 60-, 90-, and 135-day-old WHHL and age-matched control NZW rabbits were processed for indirect and double immunofluorescence as follows. In the indirect procedure, cryosections were first incubated with about 50 μL of the first antibody (anti-vimentin, anti-desmin, anti-α-actin, SM-E7, NM-G2, or NM-F6) appropriately diluted in PBS–1% bovine serum albumin (BSA) for 30 minutes at 37°C in a humidified chamber. After two washes with PBS, the cryosections were incubated with a 1:70 dilution of rabbit IgG anti-mouse IgG coupled with RITC or FITC in PBS/BSA under the conditions reported above. After an additional two washes, the cryosections were fixed for 10 minutes in 1.5% p-formaldehyde in PBS, pH 7.4, and mounted in Elvanol. Controls were nonimmune IgG instead of the first antibody or the second antibody alone.

Double immunofluorescence with anti-myosin antibodies was performed, in sequence, with SM-E7 directly labeled to FITC followed by NM-G2 indirectly revealed by rabbit IgG anti-mouse IgG coupled with RITC (Dako). In detail, after incubation of the cryosections with NM-G2 antibody followed by the application of anti-mouse IgG coupled with RITC (see above), they were treated with nonimmune mouse IgG (100 μg/mL) for 30 minutes at 37°C to saturate the potential free binding sites on the second antibody. Subsequently, the cryosections were reacted with the appropriate dilution of SM-E7 coupled with FITC. Conjugation of the anti-myosin IgG with the fluorochrome was performed as described in Reference 31. Controls were SM-E7 coupled with RITC and NM-G2 revealed by anti-mouse IgG conjugated with FITC or reactivity of nonimmune IgG instead of anti-myosin antibodies in the first step of the double immunofluorescence procedure. In the immunofluorescence experiments carried out with NM-G2 and NM-F6 antibodies, 2-μm-thick serial cryosections were cut and compared with the antibodies in indirect assay by using the rhodaminated secondary antibody.

Cryosections were examined with a Zeiss Axioplan microscope (Zeiss, Oberkocken, FRG) equipped with a 100-W mercury lamp, and specific barrier filters were used for observation of double-stained cryosections. Photomicrographs were taken using Kodak technical pan film.

Results

Localization of Vascular Wall Changes in WHHL Rabbits

Gross examination of the various vascular segments under investigation from rabbits aged 7–60 days revealed no significant evidence for the presence of vascular lesions in the wall of the thoracic and abdominal aortas or the coronary, carotid, or iliac arteries; an exception was the aortic arch. In fact, in this vascular region of 60-day-old WHHL rabbits, a grossly visible, raised lesion was consistently found (Figure 1). Light microscopy analysis of hematoxylin-eosin–stained cryosections from this vascular region confirmed that the lesion was localized at the level of the juncture of the ligamentum arteriosum with the aortic wall (not shown). In NZW rabbits this small cavity, which is located within the medial layer of the aortic arch (Figure 2A), is visible throughout postnatal development as a remnant of the original ductus arteriosum–aortic juncture. In fact, in concomitance with ductus arteriosus closure, which is completed a few days after birth, the original aperture of the ductus in the aortic wall is transformed into a pocket-like cavity (Figure 1). The innermost layer of the aortic arch cavity (AAC) wall that faces the lumen of the cavity is characterized by the presence of a marked intimal thickening (Figure 2B), which is not visible in other regions of the aorta. The thickened intima is not reactive with Sudan staining (not shown).

Examination of hematoxylin-eosin–stained cryosections from different levels of the aortic arch region close
FIGURE 5. Photomicrographs showing indirect immunofluorescence staining (with various monoclonal antibodies) of cryosections of the aortic arch (panels A, C, and E) taken at the level shown in Figure 2B and of the aorta near the bifurcation (panels B, D, and F) from a 60-day-old New Zealand White rabbit. Cryosections were reacted with SM-E7 anti-smooth muscle myosin (panels A and B), NM-G2 anti-nonmuscle (NM) myosin (panels C and D), and NM-F6 anti-NM myosin (panels E and F) antibodies. SM-E7 antibody stains the medial (m) and intimal (it) smooth muscle cells homogeneously and brightly. Conversely, NM-G2 antibody gives a heterogeneous pattern of staining, and NM-F6 is completely negative with both vascular tissues. Note that both anti-NM myosin antibodies react with endothelium (e) and the absence of intimal thickening in the aortic region near the bifurcation. iel, Internal elastic lamina. Bar=45 μm for all panels.

to the juncture with the ligamentum arteriosum in 60-day-old WHHL rabbits (Figure 3) indicates that the AAC is filled with hematoxylin-reactive tissue (Figure 3C). At a more caudal level, amorphous material containing a varying proportion of cells is present either within the media (Figure 3B) or as a true, thickened intima (Figure 3A).

The material that occupies the AAC and gives rise to the thickened intima inferiorly was also studied by electron microscopy. Figure 4 shows the ultrastructural features of the AAC wall from 60-day-old WHHL rabbits. Cells present in the atherosclerotic lesions contain numerous lipid-laden foam cells as well as SMCs that show rare, small lipid droplets. The abundant accumulation of lipid in the foam cells hampers their identification; i.e., whether they were derived from macrophages or SMCs. In the underlying media, the first rows of SMCs display very small lipid deposits, the organization and orientation of medial SMCs being essentially intact (Figure 4B), similar to that seen in the thoracic aorta (not shown).

By 90 days after birth, lesions become visible in all of the other arterial regions examined, particularly near the bifurcations and ostia of branching vessels.

Immunofluorescence Analysis of Vascular Lesions in WHHL Rabbits

The cytoskeletal and cytocontractile protein content of the vascular SMCs in normal and WHHL rabbits was examined at two distinct sites (at the AAC level and near the aortic bifurcation) and at two different ages (60 and 135 days after birth). A panel of monoclonal antibodies specific for vimentin, desmin, and SM α-actin, as well as SM and NM myosins, was applied to freshly prepared, unfixed cryosections as described in "Methods." In particular, the use of anti-myosin antibodies should allow us to evaluate the degree of SMC differentiation in the various sets of specimens; i.e. to establish whether fetal, intermediate, or adult-type SMCs are involved in this model of atherogenesis. The combined reactivity of NM-G2 and NM-F6 antibodies permits the identification of SMCs of an intermediate degree of differentiation, whereas the reactivity of SMCs with NM-F6 alone can identify SMCs of the fetal type.

We first tested the immunoreactivity of SMCs at the two aortic levels in 60-day-old NZW animals (Figure 5). At the level of the AAC, the thickened intima and subjacent media appear homogeneous with respect to SM myosin distribution (Figure 5A). NM-G2 anti-NM myosin antibody gave a heterogeneous pattern of immunostaining with both the medial and intimal SM tissues (Figure 5C). By contrast, NM-F6 anti-NM myosin antibody was able to react only with the endothelium (Figure 5E). At the level of the bifurcation, there was no evidence for an intimal thickening, and SM-E7 antibodies brightly and homogeneously stained the media (Figure 5B). NM-G2 recognizes a number of medial SMCs, which are mainly localized close to the internal elastic
FIGURE 6. Photomicrographs showing indirect immunofluorescence staining of cryosections of aortic arch from a 60-day-old Watanabe heritable hyperlipidemic rabbit, taken at the level shown in Figure 3B, with anti-vimentin (panel A), anti-desmin (panel B), anti-smooth muscle \(\alpha\)-actin (panel C), SM-E7 (panel D), NM-G2 (panel E), and NM-F6 (panel F) antibodies. Almost all intimal cells are stained with anti-vimentin, SM-E7, NM-G2, and NM-F6 antibodies. Fewer intimal cells are recognized by anti-actin, and only a very few cells are desmin-positive (panel B, small arrowheads). In the subjacent media (m), all smooth muscle cells are labeled with anti-vimentin, SM-E7, or anti-\(\alpha\)-actin antibodies. With NM-G2, all medial smooth muscle cells are stained, although a gradient of immunoreactivity appears in the media. With NM-F6, a few cells can be observed close to the internal elastic lamina (iel; asterisks), e, Endothelium; al, atherosclerotic lesion. Bar=45 \(\mu\)m for all panels.

lamina (Figure 5D), whereas NM-F6 is able to stain the endothelium exclusively (Figure 5F). Anti-vimentin and anti-\(\alpha\)-actin of the SM type homogeneously label the vascular wall, whereas anti-desmin does not stain the intima and is heterogeneous with respect to medial SM tissue (not shown).

The immunocytochemical reactivity, with the various monoclonal antibodies, of AAC wall tissue from 60-day-old WHHL rabbits at a level similar to that shown in Figure 3B is presented in Figure 6. Anti-vimentin antibody homogeneously and brightly stained the atherosclerotic tissue and the surrounding media (Figure 6A), whereas anti-desmin antibody was reactive with only a few intimal cells (Figure 6B). Some desmin immunostaining could occasionally be observed in the medial SMC region near the internal elastic lamina (Figure 6B). Although at different intensity, anti-\(\alpha\)-actin (Figure 6C) and SM-E7 (Figure 6D) were homogeneously reactive with all medial SMCs but heterogeneously reactive with the thickened intima. NM-G2 antibody (Figure 6E) was positively and uniformly reactive with all intimal cells (see also Figure 7) and with the medial SMCs, although in this last compartment, SMCs with the higher level of intensity were localized close to the internal elastic lamina. In the intimal areas in which NM-F6 antibody was reactive (Figure 6F) cells appear very bright. In the underlying media only very few SMCs were immunostained by this antibody. These immunofluorescence experiments indicate that in WHHL rabbits 1) a considerable degree of cellular heterogeneity is expressed at both sides of the internal elastic lamina, whereas 2) NM-G2-positive cells are present both in the atherosclerotic tissue and the subjacent media and
NM-F6–positive cells are almost exclusively localized to the thickened intima, and 3) the thickened intimas from endogenously derived hypercholesterolemic rabbits and those from normocholesterolemic, control rabbits display a different reactivity with NM-F6 antibody.

In light of the particular functional significance of SMCs with a double SM and NM myosin isoform content in atherosclerotic lesions of cholesterol-fed rabbits and of the different abilities of SM-E7, NM-G2, and NM-F6 antibodies to recognize SMCs in distinct stages of differentiation (adult, intermediate, and fetal), we tested cryosections obtained from AAC wall tissues of 60-day-old WHHL rabbits with all three anti-myosin antibodies in double-immunofluorescence or serial-cryosection experiments. To test whether NM-G2–positive and SM-E7–positive SMCs colocalized in the atherosclerotic tissue and in the underlying media, we performed double-immunofluorescence experiments (Figure 7). Results indicated that two cell populations were present in the thickened intima of the AAC wall, namely SMCs that were doubly reactive with SM-E7 and NM-G2 and cells that were strongly labeled by NM-G2 and weakly or negatively with SM-E7. Cells with a double myosin content can display different levels of fluorescence intensity with SM-E7 (Figure 7A), and cells positive with NM-G2 were particularly numerous in the intimal region facing the internal elastic lamina (Figure 7B). A portion of the cells stained by NM-G2 in the intima were also reactive with OKM*1 antibody, which is specific for monocytes/macrophages (data not shown). In the “surrounding” media, the majority of SMCs were doubly stained by the two anti-myosin antibodies, and a minority were reactive with SM-E7 alone (Figures 7C and 7D).

Since the possibility remains open that the NM-F6–positive intimal cells may not be of the SM type but instead belong to the monocyte/macrophage cell lineage, we performed a double-immunofluorescence experiment with SM-E7 and NM-F6 antibodies (Figure 8). Two cell populations of the SM type were identified in the intima, namely SMCs immunoreactive with SM-E7 exclusively and SMCs doubly immunostained with SM-E7 and NM-F6 antibodies (Figures 8A and 8B). Very rare cells, however, were also seen that were recognized by NM-F6 alone. Similarly in the underlying media, SMCs that were stained by NM-F6 were also recognized by SM-E7 (Figure 8). In light of the fact that all SM-E7–positive SMCs in the thickened intima of WHHL rabbits were also labeled by NM-G2 (Figures 7A and 7B), we can say that intimal SMCs that are immunoreactive with both SM-E7 and NM-F6 are actually triply reactive; i.e., they are of the fetal type and that the thickened intima contains another small SMC population, which is reactive with SM-E7 and NM-G2 and is of the intermediate type (see Figures 7A and 7B). Since a number of intimal cells were reactive with NM-G2 alone (see Figures 7A and 7B), it was of interest to determine whether they were unreactive with NM-F6. To test this hypothesis, we performed an immunofluorescence experiment on serial cryosections (Figures 8C and 8D). A small number of cells in the thickened intima were found to be negative with NM-F6, whereas the majority of these cells were found to be doubly reactive. In the media subjacent to the atherosclerotic tissue, the few NM-F6–positive SMCs were also reactive.
with NM-G2 antibody (Figures 8C and 8D). Since in this vascular compartment all SMCs were labeled by SM-E7, the cells doubly labeled with both anti-NM myosin antibodies actually contain a triple myosin isoform content; i.e. they are of the fetal type.

The SMC composition of atherosclerotic lesions in 60-day-old WHHL rabbits at the level of the A AC was compared with that of more mature lesions at the same site (Figure 9) and with that of lesions that developed in older animals at different sites (Figures 10 and 11). Figure 9 shows the immunostaining pattern of anti-myosin antibodies in atherosclerotic tissue from 4.5-month-old WHHL rabbits taken at the A AC level. SM-E7 intensely stained both the atherosclerotic tissue and the underlying media (not shown), whereas NM-G2 (Figure 9A) and NM-F6 (Figure 9B) reacted almost exclusively with the intimal cells. Occasionally, small clusters of medial SMCs (Figures 9C and 9D) appeared to have been labeled by both antibodies. These small vascular regions were characterized by the presence of sudanophilic deposits of lipids (not shown). Thus, more mature lesions are characterized by the disappearance or grouping(?) of medial SMCs that show an intermediate degree of differentiation and persistence of the fetal SMC phenotype in the atherosclerotic tissue.

In another set of experiments, we studied the SMC composition of an early atherosclerotic lesion localized near the aortic bifurcation, i.e., in a vascular region particularly enriched with SMCs of intermediate type (about 12% of the total number of medial SMCs; see also Figure 5D) and lacking the “natural” intimal thickening (see Figures 5B, 5D, and 5F). Here, the raised intimal lesion is characterized by splitting of the internal elastic membrane (Figure 10) and a large accumulation in the intima of vimentin- (Figure 10A) and α-actin- (Figure 10C) positive cells. Desmin distribution is heterogeneous and mainly restricted to the media, although some reactivity could also be detected between the split laminae (Figure 10B). The presence of cells of the SM type in the intima was confirmed by the reactivity of this tissue with SM-E7 antibody (Figure 11A). In this compartment a number of SM myosin-positive cells were also stained by NM-F6 (Figures 11A and 11B), and part of the cell population recognized by NM-G2 (Figure 11C) was also reactive with NM-F6 (Figure 11D). SMCs of the fetal type are restricted to the intima. Conversely, SMCs of the intermediate type...
can be found in the underlying media. Thus, the composition and distribution of SMC populations in this locus are quite similar to those found in the AAC of 60-day-old WHHL rabbits.

**Discussion**

The results presented in this study concerning the chronology of the appearance of early atherosclerotic lesions in a number of arterial vessels or vascular regions (aortic arch; thoracic and abdominal aortas; and coronary, carotid, and iliac arteries) in WHHL rabbits establish that the first lesion becomes detectable in the aortic arch around the 60th day after birth. More precisely, the lesion is localized at the level of the AAC that remains after closure of the ductus arteriosus.29 The wall of this cavity is uniquely characterized in adult NZW rabbits by the presence of a marked intimal thickening29 (Figure 2B). This finding might be consistent with the tendency for the atherosclerotic lesions in WHHL rabbits to develop from preexisting intimal thickenings. A close topographical relation between fatty streaks and intimal SMC masses has also been reported to occur in the vascular wall of cholesterol-fed swine.33

A unique composition and distribution of cellular and extracellular elements can also occur in the vascular intima at specific arterial sites1-34 as a consequence of the "response to injury."7 For example, in the rabbit aorta the atherosclerotic lesions are distributed in a diffuse manner at the level of the aortic arch and the ascending aorta, or focally in the proximal portions of the branching artery ostia in the thoracic and abdominal aortas.8,9,15 In the human aorta, fatty streak formation, which is regarded as the first macroscopically recognizable lesion, occurs in the region of the aortic valve, the ostia of intercostal vessels, and the aortic arch area in close proximity to the ductus arteriosus scar.34 The unique distribution of these lesion-prone and lesion-resistant vascular regions could be related to site-specific hemodynamic characteristics36-37; to differences in arterial cholesterol content and metabolism38-40; and possibly to local differences in endothelial cell composition,29-41-42 turnover, or integrity.43

Two hypotheses can be put forward to explain the major susceptibility of the AAC in the formation of atherosclerotic lesions compared with other vascular sites. Both hypotheses rely on the existence of specific local conditions that might favor the development of vascular disease, i.e., the presence of an intimal thickening well before the appearance of the atherosclerotic lesion. In the first hypothesis, the unique structure of the cavity is such that low shear stress in the wall of the cavity and the relatively high turbulence of hemodynamic flow at the level of the AAC aperture could induce some cell damage to endothelial cells lining the cavity.7,37 Such an event in normcholesterolemic animals gives rise to a proliferation/migration of medial SMCs into the intima27-28 and, thus, to the formation of an intimal thickening.

In the second hypothesis, which does not deny the first, endothelial cell injury and the subsequent atherosclerotic lesion would be the consequence of a local ischemic event. The three-dimensional structure of the pocket-like cavity29 is such that blood flow is likely to be scarce in the deeper part of the cavity and, thus, conditions of hypoxia could develop locally. Exposure of cultured endothelial cells to hypoxia results in a significant increase in their production of platelet-derived...
growth factor (PDGF) mRNA. It is well known that SMC migration into the intima is driven by some growth factors, such as PDGF, which can also act as mitogens. Medial ischemia induced by occlusion of the vasa vasorum has also been proposed as playing a crucial role in the pathogenesis of atherosclerosis.

The relatively high vulnerability of this vascular locus to the potential hemodynamic and ischemic insults compared with the other sites, e.g., the aortic region near the bifurcation, might also be related to the fact that the AAC is actually of ductus arteriosus origin and may have endothelial cells with unique structural and functional properties. Cultured endothelial cells from the aorta and ductus arteriosus of fetal lambs display unique properties in terms of extracellular matrix proteins and two specific soluble proteins synthesized by these cells.

The results obtained from the study of cytoskeletal and cytocontractile protein distribution in vascular SMCs of NZW and WHHL rabbits at two different susceptible loci (AAC and the aortic bifurcation) indicate the existence of a marked SMC heterogeneity in the atherosclerotic lesion and underlying media. In fact, when the early lesions from the AAC of 60-day-old WHHL rabbits and from the bifurcation of 4.5-month-old WHHL animals were compared, both appeared to predominantly contain an SMC population of the fetal type, whereas in the subjacent media the SMCs were of the intermediate or adult type. The identification of these distinct SMC populations with specific differentiation patterns has been achieved using the combination of three monoclonal anti-myosin antibodies, i.e., SM-E7, NM-G2, and NM-F6. Since NM myosin isoforms in rabbit aortic SM have been shown to be downregulated in a different manner with development, they can be conveniently applied to the study of SMC differentiation in the atherosclerotic lesion. Less information about the differentiation of these cells can be obtained using other markers. For example, only a few or no desmin-positive SMCs can be found in the atherosclerotic lesions of WHHL rabbits. Conversely, the majority of SMCs in the two vascular compartments contain SM α-actin, and almost all of the SMCs are homogeneous for vimentin (Figures 6 and 10). Previous studies performed with anti-cytoskeletal protein antibodies on the atherosclerotic lesions of WHHL rabbits relied mainly on the use of anti-actin antibodies and focused on the differences between SMC and SM-derived foam cells on the one hand and monocytes/macrophages and macrophage-derived foam cells on the other. Similar to myosin, actin isoforms are also regulated during development, but antibodies specific for all isoforms are not yet available. Therefore, the antibodies specific for myosin isoforms are actually a reliable tool to follow the phenotypic changes that occur in the arterial wall during atherogenesis.

Comparison of differentiation patterns displayed by SMCs in the atherosclerotic lesions from the AAC and the aortic bifurcation reveals that they are very similar. Thus, hypercholesterolemia induces the same cellular response in vascular SMCs irrespective of the presence (AAC) or absence (aortic bifurcation) of an intimal thickening. In light of this finding, migration/proliferation of medial SMCs into the intima and the myosin isoform transition toward the fetal type may not be two coordinate events. The presence of medial SMCs with different levels of maturation and the existence of a fetal SMC population pose the problem of the origin of intimal SMCs that are found in the lesion. Poorly differentiated SMCs might be obtained from preexisting resident or migrated intermediate
SMCs\textsuperscript{26,43} by a dedifferentiation process.\textsuperscript{15} These hypotheses do not deny the possibility that the fully immature SMC phenotype may be obtained directly from the adult-type SMCs, possibly by a dedifferentiation process\textsuperscript{15} or by phenotypic modulation.\textsuperscript{17,24,25} Since the presence of the fetal SMC population in the atherosclerotic lesion is paralleled by the accumulation of sudanophilic deposits of lipids in the wall, it might be that the oxidized lipoproteins\textsuperscript{50} are among the factors involved in myosin isoform switching and, thus, in SMC heterogeneity. As suggested by some authors,\textsuperscript{51,52} the achievement of an immature phenotype and ability to ingest and metabolize LDL by SMCs in culture seem to be correlated.

Comparison of the SMC differentiation patterns in the atherosclerotic lesions that developed at the AAC level at day 60 and 4.5 months after birth indicates that in older rabbits, there is a diminution of cells of the intermediate type in the media, whereas the fetal SMCs are still evident in the lesion. We have no explanation for this phenotypic change, which has also been observed in chronic renovascular hypertension (authors’ unpublished observations). The marked decrease of intermediate cells in the media underlying the lesion is also in agreement with data from the cholesterol feeding model, in which NZW rabbits were kept on a fat diet for a time ranging from 1 to 3 months.\textsuperscript{22} Moreover, in this model of atherogenesis, the differentiation pattern of SMCs in the atherosclerotic tissue and underlying media is identical to the WHHL model, i.e., fetal-type SMCs in the lesion and SMCs of intermediate or adult type in the media\textsuperscript{22} (not shown). The presence of fully immature SMCs in the atherosclerotic plaque but not in the media (R. Nagai, personal communication) has been observed in cholesterol-fed rabbits by using a polyclonal antibody prepared against a short peptide located at the carboxyl-terminal end of the “embryonic” SM myosin sequence, which actually corresponds to the so-called “brain”-type NM myosin isoform.\textsuperscript{53}

Our results are also in agreement with those of Rosenfeld and Ross,\textsuperscript{11} who found that the immunophenotypic composition of SMCs in the atherosclerotic lesion and underlying media in both WHHL and cholesterol-fed rabbit models was the same. The two models of hypercholesterolemia display some differences in the localizations of lipid deposits,\textsuperscript{8} phagocytic cells,\textsuperscript{8} level of triglyceridemia,\textsuperscript{54} and β-very low density and intermediate density lipoproteins.\textsuperscript{9,55} These differences seem to play no significant role in establishing the potentially distinct patterns of SMC response to the progressive accumulation of cholesterol in the arterial wall. Studies are under way to evaluate more precisely the pattern of myosin isoform expression in cultured SMCs from different aortic regions taken from NZW and WHHL rabbits, as well as the role of oxidized LDL as a potential factor capable of influencing the mechanism of myosin isoform switching.

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L Giuriato, M Scatena, A Chiavegato, A M Zanellato, D Guidolin, P Pauletto and S Sartore

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