**Abstract**—Intimal masses develop in the human coronary arteries of all humans, becoming atherosclerotic in later life either because of focal accumulation of lipid or the resulting response to injury. We evaluated the time course of formation of the intimal mass in the proximal left anterior descending coronary artery in autopsy specimens from 91 patients between 17 weeks’ gestation and 23 months of postnatal age. Intima was rarely found before 30 weeks’ gestation; however, the frequency with which at least some intimal cells were observed increased to 35% between 36 weeks’ gestation and birth. By 3 months after birth, all patients had an intimal mass at this coronary location. The mean intima/media ratio was 0.1 just after birth and increased continuously to the second postnatal year. Replication of medial smooth muscle cells, indicated by proliferating cell nuclear antigen staining, was high before birth and decreased between birth and 2 years of age. However, the replication index of the intima remained at 2% to 5%. Thus, coronary intimal cells appearing in the perinatal period may arise by migration after replication of medial smooth muscle, as is seen in models of carotid artery balloon injury. In conclusion, formation of the coronary artery intima is a rapid process, beginning in the peripartum or postpartum period. Given the clonality of the adult lesion and the lack of proliferation in later stages of lesion formation, it is intriguing to speculate that this event may form the basis for atherosclerosis in later life. (Arterioscler Thromb Vasc Biol. 1999;19:2036-2040.)

**Key Words:** intima ■ proliferating cell nuclear antigen ■ monoclonality ■ infants ■ left anterior descending coronary artery

Despite studies of the mechanisms involved in the formation of intima in injured rat carotid arteries, there is no evidence that this model is valid either for the spontaneous formation of intima or for the accelerated intimal process characteristic of atherogenesis in our species. In contrast, it is difficult to explain the clonality of intimal lesions seen in adults if these lesions begin as different proliferative responses.1,2 The intima, especially in portions of the left anterior descending coronary artery, appears in childhood at sites where atherosclerosis becomes evident in later life. Stary,3-6 among others, has suggested that lipid accumulation deep in these masses may be the earliest step in the vessel wall injury of atherosclerotic lesions. Although several histological studies have focused on coronary development, only a limited number of studies have addressed the processes leading to formation of the neonatal intima.3-7,12

As reported herein, intimal formation is a rapid process beginning just before birth and appears to be associated with a period when cell proliferation is abundant. We speculate that this process may be the first event in a clonal expansion accounting for the monoclonality of advanced lesions.1,2 Moreover, the rapid time course of intimal expansion may make the process amenable to experimental manipulations in animal models designed to determine the factors responsible for spontaneous formation of the intima.

**Methods**

**Patients and Coronary Artery Samples**

Materials from 91 cases <2 years old (62% male) who underwent autopsy at the University of Nebraska Medical Center, Omaha, were included. Forty-five cases (49%) were premature (17-week fetus to 39 weeks), 22 cases (24%) were between 0 and 7 days old, and 24 cases (26%) were between 7 days and 23 months old. The major cause of death (40%) was congenital heart disease. All human tissues used in this study were procured under approval of the Institutional Review Board of the University of Nebraska Medical Center.

We chose the proximal left anterior descending coronary artery segments, 5 mm distal to the left main bifurcation, that were sectioned transversely and fixed in 10% neutral buffered formalin. We excluded bifurcated lesions because it is known that intimal cushions start at bifurcations.10

**Measurement of Intima/Media Ratio**

We determined the intima as the tissue inside the internal elastic lamina and the media as the tissue between the internal and external elastic laminae on Verhoeff–van Gieson–stained slides. We excluded sections near branches or oblique sections. We measured the intima/media ratio with a computer-assisted system. We did not measure luminal area because pressure fixation was done in only a few samples.

**Antibody**

We purchased monoclonal anti–proliferating cell nuclear antigen (PCNA) antibody (Ab-1) from Calbiochem.
Figure 1. Size of left anterior descending coronary arteries. A, Proximal left anterior descending coronary arteries at 21 weeks' gestation (×400, Verhoeff–van Gieson’s stain). B, At 8 days old (×400, Movat’s stain). C, At 15 months old (×400, hematoxylin and eosin stain). D, Same section as in A at ×40. E, Same section as in B at ×40. F, Same section as in C at ×40.

Immunohistochemistry

After hydration, antigen retrieval was performed by microwaving the slides for 10 minutes in an antigen unmasking solution (Vector Laboratories). Endogenous peroxidase was blocked with 3% H$_2$O$_2$ for 5 minutes at room temperature. Blocking was performed with 10% normal horse serum in PBS containing 1% BSA. Slides were incubated with the primary antibody for 1 hour at room temperature; the titer was 1:200 for PCNA. A biotinylated horse anti-mouse secondary antibody was then applied for 30 minutes, followed by an avidin-biotin-peroxidase conjugate (ABC Elite, Vector Laboratories) for 30 minutes at room temperature. Then 3,3′-diaminobenzidine with NiCl$_2$ was added to yield a black reaction product at 37°C for 10 minutes, and methyl green was used as a nuclear counterstain.

Counting of PCNA-Positive Nuclei

In performing immunohistochemistry, we took care to avoid over-staining with PCNA. We counted clearly positive nuclei as positive. Borderline staining was designated negative. Positivity was supported by the independent observations of 2 pathologists.

Results

Histology of Proximal Left Anterior Descending Coronary Artery

Coronary arteries grow rapidly with age through the perinatal and postnatal periods. Figure 1 shows the difference in size for arteries of different age. Figure 1A, 1B, and 1C shows the same high-power magnification of vessels at 21 weeks of gestation, at 8 days old, and at 15 months of age. Figure 1D, 1E, and 1F shows a low-power magnification of the same series of vessels. Near the time of birth, coronary arteries begin to develop an intima that is initially eccentric (Figure 1B and 1E). By 15 months of age, intima was found surrounding the entire lumen (Figure 1C and 1F).

At 21 weeks of gestation (Figure 2A), the endothelium rests on an already formed internal elastic lamina. The vessel has an adventitia and a thin media, but no intima. The media consists of 2 to 3 cell layers. In vessel cross sections, smooth muscle cell nuclei are elongate, suggesting a circumferential orientation in the media (Figure 2A). Between 17 and 30 weeks of gestation, the earliest time frames that we studied, we saw intimal cells in only 1 of 17 cases (Figure 2B). Such vessels showed splitting of the internal elastic lamina in areas of intima formation at 28 weeks of gestation (Figure 2B).

A frequent confounding issue in the determination of the site of intimal growth is the presence in many vessels of a second layer of media in these young coronary arteries (Figure 2C and 2D). The second layer of media has been reported previously and has been called the “musculoelastic layer.”11,12 This layer is formed before intima formation in many cases (Figure 2C). The musculoelastic layer can be distinguished from the outer media by the orientation and density of smooth muscle cells (Figure 2C). Moreover, this layer can be distinguished from true intima because the former lies beneath the internal elastic lamina (Figure 2C and 2D). Because we determined the intima as a layer above the internal elastic lamina, the musculoelastic layer belongs to the media.

Presence of Intima (Figure 3)

Other than the inner media, the first detectable intima was found at 3 months before birth (28 weeks’ gestation). Of 13 specimens between 1 and 2.5 months before birth (30 to 36 weeks’ gestation), only 2 showed intima (15%). In contrast, 2 of 6 specimens (33%) at 1 month before birth (36 to 40 weeks’ gestation) showed intima. Intima was found in 8 of 21 specimens (38%) just after birth (0 to 7 days old). At 3 months of age, intima was detected in all specimens. Thus, intimal formation begins spontaneously near the time of birth.

Intima/Media Ratio

The intima/media ratio increased with age (Figure 4). Just after birth, the intima/media ratio was nearly 0.1. We consid-
The prenatal intima/media ratio of coronary arteries from fetuses with congenital heart disease versus other causes of death was 0.0214 ± 0.0567 versus 0.0247 ± 0.0670, respectively (NS). The postnatal intima/media ratio of congenital heart disease patients versus those with other causes of death was 0.249 ± 0.264 versus 0.103 ± 0.193, respectively (NS). Thus, statistically significant differences were not observed in association with cause of death.

Intimal Cell Numbers
Figure 5 shows the scatterplot of intimal cell numbers as counted in cross sections. The newly formed intima is rich in cells, and intimal cell number increases with age.

Cell Replication in Infant Coronary Arteries
Figure 6 shows PCNA staining of coronary arteries. A similar staining pattern was confirmed by another proliferation marker, MIB-1. Figure 7 shows ratios of PCNA-positive cells to total cells in each vessel wall layer. Medial replication was quite high before birth. Intimal cells were rare, and it was not possible to estimate a replication frequency before birth, as shown in Figure 5. After birth, replication in the outer media gradually declined. However, the inner media and intima retained their cell replication rates between 2% and 5% until 2 years of age. The endothelium had a high rate of PCNA positivity prenatally, which gradually diminished in a pattern similar to that in the media. From staining the slides with cell markers, it was found that almost all cells in the intima and media were smooth muscle cells.

Discussion
We analyzed the left anterior descending coronary arteries of 91 patients from 17 weeks’ gestation to 23 months old. We histologically judged the intima as tissue above (luminal to) the internal elastic lamina. Several reports have shown that a second medial layer is observed during coronary development, which is called the “musculoelastic layer.” This layer has sometimes been misinterpreted as intima. If the musculoelastic layer is thought of as part of the intima, then the internal elastic lamina has completely disappeared with the appearance of a subendothelial elastic layer (Figure 2C). It is reasonable to conclude that the musculoelastic layer belongs to the media because the former lies beneath the internal elastic layer, a concept that is supported by much literature.

The intimas of these young coronaries consisted of smooth muscle cells. We stained all of the samples with CD68, a macrophage marker; however, we did not find any CD68-positive cells. In contrast, Stary reported that macrophages were found in the coronary arteries of young children. We cannot exclude the possibility that macrophages existed in certain regions of the intima because we studied only the proximal left anterior descending coronary arteries. However, virtually all of the proliferating cells we observed were smooth muscle cells.

The perinatal spontaneous formation of an intima in the left anterior descending coronary artery shows a startling similarity to the histological descriptions of another spontaneously formed intima, the lining of the ductus arteriosus.
coronary artery. In the rat model, anti–fibroblast growth factor antibody^{25–27} can inhibit medial proliferation and delay intimal formation after injury. The studies of Campbell et al^{8–30} have suggested that this medial proliferation is part of the change in smooth muscle phenotype that is required before medial cells can migrate and form an intima. If so, this part of the response to injury may be recapitulated during normal development.

Finally, rapid formation of the intima at this coronary site offers a possible explanation for the clonality of atherosclerotic lesions.^{1,2} It is possible that masses like that shown in Figure 2 arise either by migration of a replicative medial cell or by trapping of an intimal cell as the internal elastic lamina forms. In either case, replicative growth of the intima continuing for 2 years postnatally, even at levels of only 2% to 4% as shown here, would be adequate to produce a clone as has been observed in the adult lesion. If so, clonal expansion may be a normal early event that precedes atherosclerosis but is neither causal, as proposed by Benditt,^{31,32} Fabricant,^{33} and Casalone et al,^{34} nor a result of mutations in the plaque, as proposed by McCaffrey et al,^{35} Spandidos et al,^{36} and others.

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References


Even in patients with persistent ductus arteriosus, intima formation occurs spontaneously before birth.^{15} The major difference is that the ductus intima begins to form earlier than in the coronary artery, presumably as a prerequisite to the requirements of postpartum ductus closure. One wonders whether formation of the left anterior descending coronary artery intima represents a similar response to the same demand of early postnatal physiology. Like the left anterior descending coronary artery, the ductus also forms an inner medial layer.^{15–17} Similarly, splitting of the internal elastic lamina has been reported during intima formation in both the human coronary artery^{18} and human ductus arteriosus.^{15} In contrast, in murine models, the ductus intima mainly forms over a period of 1 to 2 days after closure, although the first intimal cells were found at 17 days of gestation.^{19} Whereas there is a general impression that the ductus intima forms as a response to injury occurring during closure, ductus intima in humans forms spontaneously before birth.^{15–17}

The similar spontaneous formation of the ductus intima and the coronary intima should be contrasted with what is known about experimental models of intima formation after angioplasty. Most important, there is no evidence of endothelial injury in the spontaneous process that involves the left anterior descending coronary artery, whereas endothelial denudation is believed to be critical for the response-to-injury model. In the best-studied injury model, the rat carotid artery, smooth muscle cell proliferation reaches a maximum at 48 hours in the media (46%) and at 96 hours in the intima (73%).^{20} Subsequently, the thymidine index declines to baseline (0.06%) by 4 weeks in the media and by 8 weeks in the intima covered by endothelium.^{20} Such massive levels of medial smooth muscle cell replication are not seen in the spontaneous formation of the intima. In human coronary arteries, cell proliferation as determined by PCNA staining was 3% to 5% in the media before birth and decreased with age. We also stained all of the samples with MIB-1, another proliferation marker. The staining pattern was similar to that with PCNA. Finally, formation of the second medial layer before intima formation and multiplication of the internal elastic lamina, typical of spontaneous formation but not seen in the responses to angioplasty in the rat,^{21} rabbit,^{22} monkey,^{23} or pig,^{24} have been frequently described as a characteristic feature of the advanced atherosclerotic lesion.

The spontaneous-development and response-to-injury models are similar in 1 way. Medial proliferation precedes intimal formation in both the rat model and the human


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