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Coronary Intimal Thickening Begins in Fetuses and Progresses in Pediatric Population and Adolescents to Atherosclerosis

Guerri-Guttenberg Roberto, MD, PhD[1], Castilla Rocío, ScD, PhD[1], Cao Gabriel, MD, PhD[1], Azzato Francisco, MD, PhD[1], Ambrosio Giuseppe, MD, PhD[2], and Milei José, MD, PhD[1]

Abstract

The prevalence of coronary intimal thickening (IT) was assessed in fetuses and pediatric population. We studied the coronary arteries of 63 hearts obtained from fetuses, infants, children, and adolescents, deceased from noncardiac disease or trauma. Histomorphometric analysis, planimetry, and immunohistochemical studies were conducted. Intimal thickening consisted of proliferation of smooth muscle cells and scarce monocytes embedded in amorphous deposits within the internal elastic membrane (IEM). Intermingled lesions of intimal hyperplasia and parietal nonstenotic plaques were also observed. Intimal thickening was found in 10% of 20 fetuses, in 33.3% of 18 infants, 73.3% of 15 children, and 100% of 10 adolescents. A significant correlation ($r = 0.671$, $P < 0.001$) was found between the extent of IT and age. The IEM was duplicated or interrupted in 43% of patients, showing a positive correlation with the degree of IT ($P = 0.01$). Intimal thickening was predominantly found near bifurcation sites in the left anterior descending coronary artery (55.6%) and in zones free of bifurcation in the right coronary artery (75%). In conclusion, the prevalence and extension of IT lesions are higher at older ages within a young population. Intimal thickening may be regarded as the first event occurring in coronary preatherosclerosis, preceding lipid deposition.

Keywords

coronary artery, early atherosclerosis, smooth muscle cells, intimal hyperplasia, intimal thickening, adventitia

Introduction

In 2010, in this Journal, we published the morphological characterization of intimal thickening (IT) in coronary arteries from 67 infants, in order to obtain insights into initial coronary atherogenesis.[1] Alterations ranged from focal areas with mild myointimal thickening to diffuse moderate thickening. In those lesions, smooth muscle cells (SMCs) showed loss of polarity, infiltrating the subendothelium, mostly with rupture of the internal elastic lamina.[1] However, in spite of our findings that IT might be an early event in the process of human coronary artery atherosclerosis, no articles have been published demonstrating the progression of these lesions from fetuses to adolescents.

The earliest feature of progressive atherosclerosis as described by the American Heart Association classification is pathologic IT (PIT) characterized by extracellular lipid accumulation and rich in proteoglycans and hyaluronan.[2] However, other investigators proposed a different view, with initial lesions of coronary arteries being characterized by the proliferation of intimal SMCs, which cause IT prior to any evidence of visible lipid deposition.[3-7] Virmani et al[8] and Nakashima et al[9,10] have emphasized that IT precedes lipid/macrophage deposits. Virmani et al also observed that while coronary lesions in patients older than 50 years were richer in lipids and foam cells, occurrence of IT tended to precede lipid/macrophage deposition in patients younger than 50 years.[8] Using coronary arteries obtained from patients with a mean age of 47.6 years, the same group demonstrated that in the natural progression of atherosclerosis from PIT to fibroatheroma, these lesions present early lipid accumulation, followed by

[1] Instituto de Investigaciones Cardiológicas (ININCA), Universidad de Buenos Aires-CONICET, Buenos Aires, Argentina
[2] Division of Cardiology, School of Medicine, University of Perugia, Perugia, Italy

Corresponding Author:
Castilla Rocío, Instituto de Investigaciones Cardiológicas (ININCA), Universidad de Buenos Aires-CONICET, Marcelo T. de Alvear 2270 (C1122AAJ), Buenos Aires, Argentina.
Email: rcastillalozano@fmed.uba.ar

macrophage infiltration with defective clearance of apoptotic bodies, and a decrease in hyaluronan and proteoglycan in lipid pools. In addition, in a younger cohort, Nakashima et al[9] found the earliest lesion in the coronary artery wall to be IT, consisting of SMCs and extracellular matrix, with little or no accumulated lipids and a few macrophages in the superficial layer.

Consistent with the hypothesis that SMC proliferation can be an initial event in vascular atherosclerosis and remodeling, we and others have observed IT and its transition to atherosclerotic lesions, already present in newborn babies and younger than 1 year infants.[1,11,12] Of note, we demonstrated that IT is detectable in the prenatal and infancy period and is significantly associated with maternal smoking, suggesting that the oxidants present in the gas phase of cigarette smoke may induce the immediate and intense stimulation of the *c-fos* gene in the SMC of the media.[13]

Therefore, according to accumulating evidence,[1,8-10,14] IT may be the first sign of coronary pre-atherosclerosis, although a characterization of its progression, incidence, degree, and immunohistochemistry is still pending. Even more, it has been demonstrated that, through a phenotypic conversion, fibroblasts from the adventitia become activated and transform into myofibroblasts, developing a crucial relationship with the SMC of the tunica media and inducing proliferation, migration, and apoptosis, all of which result in IT.[15]

This background makes it essential to systematically investigate autopsy material of fetuses, infants, children, and adolescents with no evidence of structural heart disease, to obtain better insights into IT as the first pre-atherogenic event and its progression to atherosclerosis.

Methods

Population

A total of 63 hearts were examined: 20 from 12- to 32 gestational-week fetuses, 18 from infants up to 1-year old (mean age 3 months), 15 from 1- to 11-year-old children (mean age 6.4 years), and 10 from 11- to 18-year-old adolescents (mean age 14.6 years). None of them were previously studied. Sixty-seven percent were male. In all cases, autopsy had excluded structural heart disease. Deaths were due to organic diseases not related to coronary alterations and neither bearing on the study outcome; 74.4% (n = 32) were due to sudden infant death syndrome, anoxic encephalopathy, meningitis, or pneumonitis, and trauma in 25.6% of cases (n = 11). Individuals whose mother had any known diseases or history of drug or alcohol abuse during pregnancy were excluded. Written informed consent was obtained from parents by the Coroner's office (Buenos Aires, Argentina) before the autopsy.

Sample Handling

Autopsies were performed within 6 to 18 hours from death. Hearts were fixed in toto by 48 hours immersion in 10% buffered formalin (pH 7.0). The major epicardial coronary arteries were identified along their entire extension, and an average of 4 samples of 5-mm length was taken from the left main coronary artery (LM), left anterior descending coronary artery (LAD), right coronary artery (RCA), circumflex (CX), and posterior interventricular artery (PIV). Proximal and distal coronary artery segments and their location in relation to bifurcations were specifically identified. To obtain a well-defined internal elastic lamina patterns, samples from bifurcations branch vessels and curvatures were analyzed but excluded from quantifications.

Segments were dehydrated, embedded in paraffin blocks, and serially cut at 3-μm thickness in both transversal and longitudinal orientations when necessary. Sections were stained with hematoxylin & eosin (H&E), Victoria blue, Masson trichrome, Alcian Blue pH 2.5, and Sirius red and processed for immunohistochemistry.

Morphometric Study

Coronary artery histomorphometric and planimetric analyses were performed using a Nikon Eclipse E400 microscope and Image J software (National Institute of Health, Bethesda, Maryland). Sections were magnified and digitalized, and maximal intima thickness, media thickness, and intima to media ratio were measured. This ratio was used to assess the degree of IT defined as musculoelastic thickening characterized by (1) proliferation of SMCs; (2) scarce monocytes and lymphocytes embedded by amorphous deposits within the IEM; and (3) morphologically intact endothelium above the lesion, devoid of thrombi and with smooth surface. In order avoid any processing artifacts that can influence the interpretation of vascular changes, differential diagnosis of intimal ridges responsible for vessel bifurcation was carried out with the aid of serial axial and longitudinal sections.

Thickenings were classified as complete diffuse (uniform thickening that encompasses the entire circumference of the artery), incomplete diffuse (encompassing <90% of the circumference of the artery), focal, and mixed variant. Based upon appearance of IEM, IT could further be classified as intact (type I), interrupted or duplicated (type III), or intermediate status (fragmented, disrupted: type II).

Immunohistochemical Studies

Immunophenotyping of cells present in the vessel wall was performed with monoclonal antibodies against macrophages (CD68, Dako Cytomation, Denmark), SMCs (α-SMActin; anti-mouse α-actin monoclonal antibody concentrated MU128-UC clon1A4, Biogenex), transforming growth factor β1 (TGF-β1), apolipoprotein B (Abcam, Cambridge, UK), and endothelial cells (CD31, Biogenex; Ylem-Milano Milan, Italy). Streptavidin–biotin–peroxidase (Biogenex, San Ramón, California) was used as a detection system. Regarding stem cells (CD34/CD117), fibroblasts (vimentin), endothelial cells (CD34/vimentin), endothelial cells and monocytes/macrophages (vascular endothelial growth factor [VEGF]), and cellular proliferation (Ki67), an automated immunohistochemical

system was used (Ventana BenchMark GX equipment; Roche Tissue Diagnostics, Basel, Switzerland).

The intensity and distribution of immunostaining were analyzed using Image J software (National Institutes of Health, Washington, District of Columbia). The score was expressed in terms of intimal area positive for the antibody, except for Ki-67, for which the percentage of positive nuclei within the intimal layer was expressed.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. Histological results were compared by 2-tailed unpaired *t* test. Statistics was performed using Pearson χ^2 test, Mann-Whitney *U* test or Kruskal-Wallis test samples, and Student-Newman-Keuls for multiple comparisons. Pearson coefficient was used to determine correlation. Analyses were performed using GraphPad Prism v5.03 or SPSS Statistics 19 (IBM).

Results

Fetuses

Hearts of fetuses studied were from 17- to 34-year-old mothers. Causes of pregnancy interruption were miscarriage, abortion in progress, infected abortion, oligohydramnios, eclampsia, or HELLP (**h**emolysis, **e**levated **l**iver enzymes, **l**ow **p**latelet) syndrome. Structural heart disease was excluded in all cases, and hearts had morphology consistent with their gestational age.

Only 2 cases of IT were observed in this group: 1 male fetus (estimated at 12-16 weeks of gestational age) presented focal IT in the RCA (Figure 1A and B). The fetus was from a miscarriage in a mother without disease or toxic antecedents. The second case was found on the PIV of a 15-week-old female fetus (Figure 1C and D). The 34-year-old mother, with a history of 4 births and 2 abortions, smoked 10 cigarettes/d. In this case, it is interesting to establish a difference between intimal ridges responsible of bifurcating arterial lumen and the IT found on the opposite side of the vessel bifurcation (see arrows).

Infants, Children, and Adolescents

When infants, children, and adolescents were analyzed, no differences were observed in the incidence regarding gender or cause of death. However, significant differences were noticed with respect to age. Table 1 shows the frequency of patients with coronary artery alterations and the number of arteries affected in infants, children, and adolescents, showing a progressive increase in prevalence after birth. Therefore, the number of affected vessels was related to the group ($P < 0.002$, χ^2 test). Accordingly, infants were different from the 2 other groups ($P < 0.006$ vs children, $P < 0.001$ vs adolescents), while the number of compromised vessels did not appear to go through major changes from childhood to adolescence.

The most pronounced cases are shown in Figure 1. Panels E and F show a hypercellular lesion on the RCA of a 3-month-old infant who died from anoxia. Among children arteries, the most outstanding case was observed in the RCA of a 10-year-old

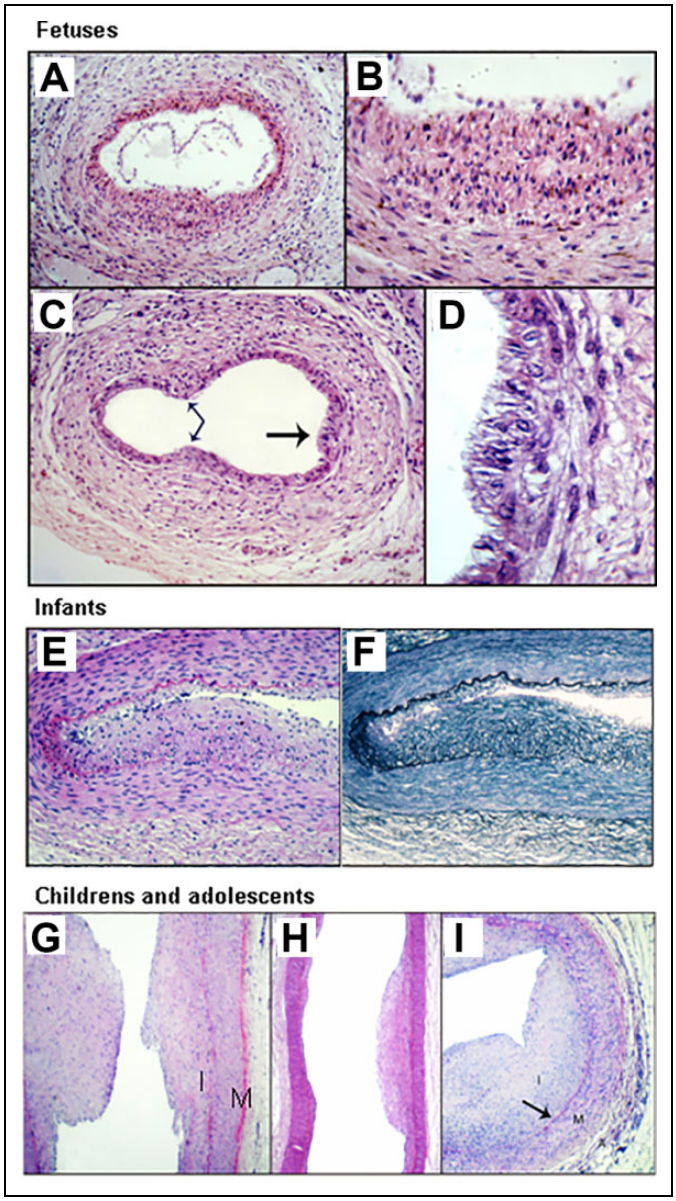

Figure 1. Intimal thickening in fetuses, infants, children, and adolescents. (A and B) Right coronary artery (RCA) of a 12-16-weeks-old male fetus. A marked intimal thickening is shown consisting of smooth muscle cells (SMCs) perpendicularly oriented to the lumen. Hematoxylin & eosin (H&E) ×40 and ×100. (C) Posterior interventricular artery of a 15-weeks-old female fetus. The difference between intimal ridges belonging to the bifurcation of the vessel (arrows) is clearly distinguished, H&E ×40, and in (D) intimal thickening pointed out on the opposite side of the vessel (arrow). H&E ×200. (E) Left anterior descending coronary artery of a 3-month-old baby sectioned in the center of the vessel. A severe, large hypercellular lesion occluding the artery lumen is shown. H&E ×40. (F) Internal elastic membrane (IEM; in black) is severely interrupted and fragmented. Victoria blue ×40. (G) Longitudinal section of RCA of a 10-year-old patient. A total of 76.7% of the arterial lumen was obstructed by a large intimal thickening constituted by SMCs and amorphous intercellular substance. Note the thickening, fragmentation, and rectification of the IEM. (H) Longitudinal section of the RCA of a 12-year-old male patient. On the right, a loose fibrous plaque partially occluding the artery lumen with large amounts of ground substance can be observed. (I) RCA of a

Table 1. Frequency of Intimal Thickenings in Coronary Arteries.

	Infants	Children	Adolescents
Patients with coronary artery alterations	6/18 (33.3%)	11/15 (73.3%)	10/10 (100%)
Patients with 2 or $\geq$3 arteries affected	3/18 (16.6%)	8/15 (53.3%)	9/10 (90%)

female patient who had died from a central nervous system infection. A longitudinal section shows that the intima–media ratio was of 3.4 on the left side of the arterial wall and 2.12 on the right one. A total of 76.7% of arterial lumen (assessed in a transverse section) was obstructed by bilateral heterogeneous IT constituted by SMCs and amorphous intercellular substance. Thickening, fragmentation, and rectification of the IEM are worth mentioning (Figure 1G). Evaluation of full-length IT in longitudinal sections showed small lesions that focally obstructed the arterial lumen on one side and diffuse IT of a greater extension on the other. In Figure 1H, the RCA of a 12-year-old male patient is shown. On the right, a loose fibrous plaque partially occluding the artery lumen with large amounts of ground substance can be observed. However, the greatest alteration was found in the RCA of a 16-year-old patient who had died from traumatic brain injury (Figure 1I). At its maximum thickness, the intima was 4.32 times the size of the media. It contained significant amounts of SMCs irregularly arranged with type II IEM in some areas and type III in others.

Intermingled lesions consisting of IT and parietal nonstenotic plaques were also observed at sites of underlying sub-IT and provided evidence of a progression toward atherosclerotic lesions (Figure 2A and B).

Regarding the degree of IT, no significant differences were found between sexes and causes of death. Of note, mean IT increased with patient age (infants vs children $P < 0.05$, infants vs adolescents $P < 0.001$, and children vs adolescents $P < 0.05$; Figure 3A) and, accordingly, a high correlation (Pearson correlation coefficient $r = 0.671$, $P < 0.001$) was found between the extent of IT and the age of patients (Figure 3B).

Distribution of IT in Coronary Arteries

Intimal thickening was more predominantly found near bifurcation sites in LAD and in zones free of bifurcation in RCA, as it was present in 75% of RCA, 55.6% of LM, 65.5% of LAD, and 37.5% of CX. In most cases, we found complete diffuse IT (59%) encompassing the entire circumference of the artery. Fifteen percent were incomplete diffuse, 21% were mixed (focal components with diffuse IT), and the remaining 5% were isolated focal intimal lesions.

Figure 1. (Continued). 16-years-old patient. It contains significant amounts of SMCs irregularly arranged embedded in abundant loose connective tissue with type II and III involvement and even destruction of IEM (arrow). H&E ×35. A indicates adventitia; I, intima; M, media.

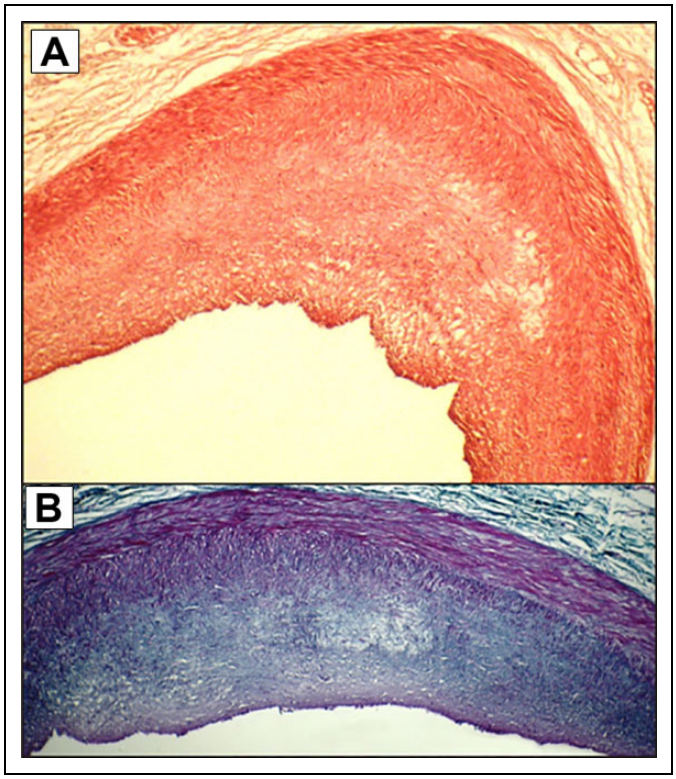

Figure 2. Intermingled lesions consisting of intimal thickenings and parietal nonstenotic plaques. Left coronary artery from a 16-year-old adolescent accidentally fell from a vehicle suffering severe head trauma causing his death. Intermingled lesions consisting of diffuse intimal thickening and a nonstenotic plaque rich in a lipid core are observed in semiserial sections. (A) Sirius red staining ×50; (B) Masson's trichrome ×50.

Internal Elastic Membrane

There was a progression in structural alterations in the IEM, from an undulated and continuous membrane (type 1, found also in normal coronary arteries, 43%), then a rectification and fragmentation of the IEM (type 2, 14%) toward the most disrupted type where the IEM is interrupted or duplicated (type 3, 43%; see Figure 1). Of note, we found that the thicker the intima (higher degree of IT), the more disrupted the IEM. Intimal thickening evaluated as the mean of intima–media ratio were 0.08, 0.089, and 1.63 for arteries with IEM type I, type II, and type III, respectively. Differences were found to be statistically significant according to the Kruskal-Wallis test for nonparametric independent samples ($P = 0.01$).

Immunohistochemistry

Macrophages (CD68+) were at times detected on the intimal border of the lesions, penetrating the endothelium; however, no macrophages were seen within the lesions. Neoangiogenesis evaluated by the use of endothelial cell markers (CD31/CD34/VEGF) was not detected (data not shown).

α-SMC actin allowed us to establish that ITs were mainly due to the presence of SMCs. With this antibody, it could be

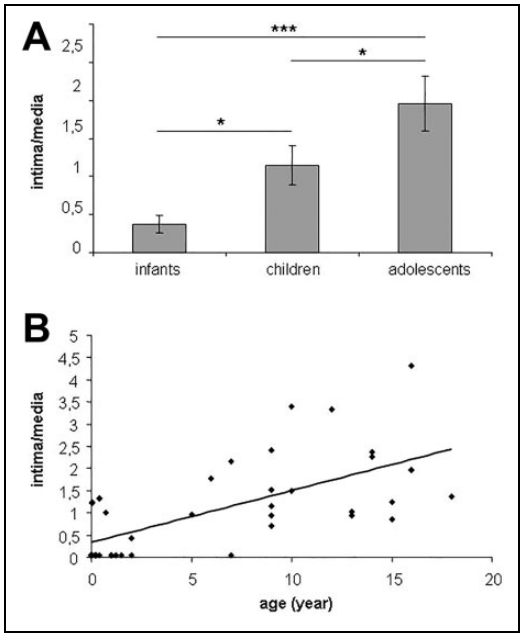

Figure 3. Intimal thickening extent in the pediatric population. The intima to media ratio was measured in arteries presenting intimal thickening from infants, children, and adolescents. (A) Data represent mean ± SEM. Student-Newman-Keuls multiple comparisons test: *** $P < 0.001$, * $P < 0.05$. (B) Correlation analysis between degree of intimal thickening and age of patient. Scatter plot is shown. Pearson correlation coefficient (r) = 0.671, coefficient of determination (r^2) = 0.450. SEM indicates standard error of the mean.

established that, in IT, SMCs lose polarity even in fetuses and orient in a perpendicular manner toward the IEM in some cases or in a disarranged form ("higgledy piggledy") in others (Figure 4A and B).

The antibody Ki67showed SMC proliferation within the thickened intima with a mean index of 6% (range: 0%-15%). It was interesting to note the presence of Ki67-positive endothelial cells in bifurcation zones, while less active lesions were found away from these regions (Figure 4B). The TGF-β1 at a dilution of 1:200 was almost negative in all cases (data not shown).

Apolipoprotein B cross-reactive for B-48 and B-100 was found to be positive either in cases with greater expression over the thickened intima or in the area around the intima–media transition (Figure 4C).

Immunohistochemical expression was observed for vimentin in the normal vascular endothelium and fibroblasts located in areas of IT, smooth muscle layer, and adventitia (Figure 5), while CD34/CD117 stem cells did not show immunohistochemical expression in the walls of the coronary vessels studied (data not shown).

Discussion

In the present study, ITs of coronary arteries were already detectable in fetuses and infants; however, their prevalence rose significantly with age in children and adolescents. The SMCs showed loss of polarity, infiltrating the subendothelium, mostly with rupture of the IEM[16] and devoid of neoangiogenesis. Coronary alterations ranged from focal areas with mild myointimal thickening to diffuse moderate thickening and even intermingled or nonstenotic plaques. In general, diffuse ITs were localized in the nonbranching long segments of arteries, while eccentric ITs were found around branches. These 2 types were contiguous and could not always be clearly distinguished, suggesting that they essentially represent the same condition. Intimal thickening was predominant near bifurcation sites and in zones free of bifurcation in the LAD. This finding had already been described by others,[13] and it had been interpreted as a reaction of these cells against shear stress due to alterations in blood flow. Accordingly, Ki67-positive endothelial cells were mostly found in bifurcation zones of LAD in our studies.

It is accepted that SMCs can display at least 2 different phenotypes in the pathogenesis of atherosclerotic lesions[17]: (1) highly differentiated contractile cells of the media and (2) synthetically active and proliferating cells invading the intima. Consistent with this view, we have observed that, in the media, SMCs present positive reaction for Ki-67, which indicates that they are actually proliferating. This is in agreement with previous observations in which SMCs in coronary arteries of infants present *c-fos* gene activation in the media.[18] In fact, SMCs respond to environmental stimuli by dedifferentiating, downregulating SMC markers and contractile proteins (eg, α-smooth muscle actin, transgelin, smooth muscle–myosin heavy chain), migrating into the neointima, proliferating, and secreting matrix and remodeling factors.[19]

On the other hand, adventitial fibroblasts may transdifferentiate to myofibroblasts, triggered by factors such as hemodynamics, low oxygenation, and metabolic disturbances and then migrate through the middle muscle layer toward the intima as shown by the expression of α-smooth muscle actin and vimentin. All 3 layers of the vessels, the intima, media, and adventitia, contain resident progenitor cells, including endothelial progenitors and stromal cells.[15] In this sense, it is of utmost importance to determine the possible signal pathways operating cell differentiation toward SMCs and endothelial cells.

From the immunohistochemical point of view, TGF-β1 was negative in all cases, indicating that IT does not respond to an increment in this factor known to modulate proliferation of vascular cells and regulate their interaction with the extracellular matrix.[20,21] However, we cannot rule out the activation of TGF-β1 signaling cascade, as this result may only represent nondetectable activity under the conditions and antibody dilutions used in this study. Indeed, we have previously reported an increment in TGF-β1 related to IT in children with congenital heart disease.[22] Likewise, we were unable to detect CD34/CD117 progenitor cells in the coronary walls of the cases studied, suggesting pathophysiological events different from the formation of the atherosclerotic plaque in adults.[23,24]

According to our results, as intimal width increases, the IEM first rectifies, then discontinues, and finally disappears completely or appears duplicated in the areas of greater thickening.[16] Consistent with this is the finding of greater disruption of

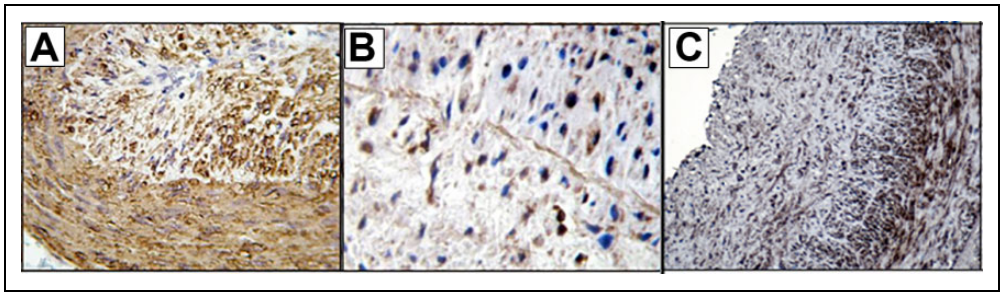

Figure 4. Immunostaining of actin, Ki67, and apolipoprotein B (apoB). (A) Immunohistochemistry for α-actin shows smooth muscle cells (SMCs) expressing α-muscle actin with loss of polarity, some cells are oriented in a perpendicular manner toward the internal elastic membrane, while others are oriented in a disarranged form. ×100. (B) The antibody Ki67 analyzed in the right coronary artery of a 9-year-old patient who died in a traffic accident showed proliferation of SMCs within the thickened intima. ×200. (C) Immunohistochemistry for apoB 48 and apoB 100 performed in the left right coronary artery of a 14-year-old patient who died from brain trauma produced by gunshot wound. ×100.

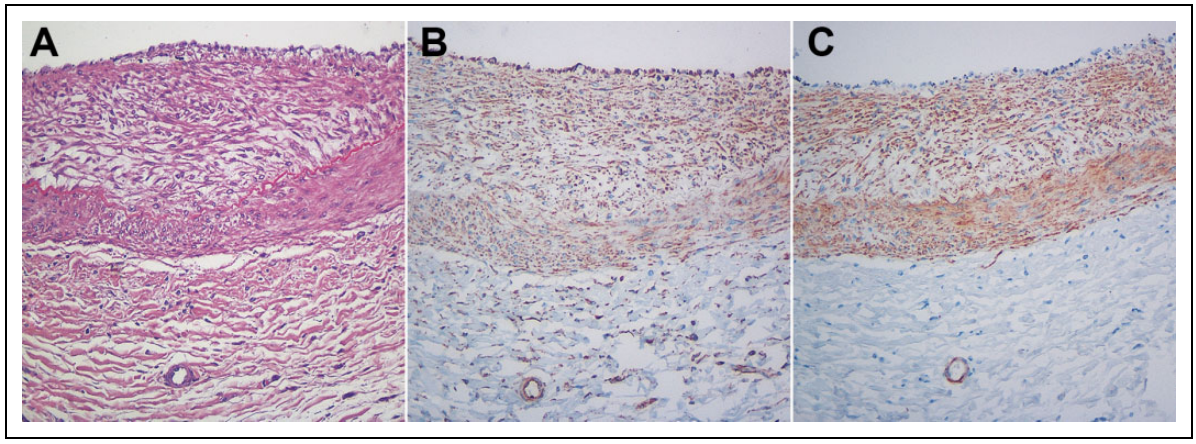

Figure 5. Immunostaining of vimentin. Analysis of serial sections of the 3 coronary layers. (A) Coronary artery with marked intimal thickening and thinning of its muscular layer, as well as partial disruption of the internal elastic lamina. Adventitia with eosinophilic collagen fibers, fusiform cell elements, and a small transversely sectioned vessel (vasa vasorum). Hematoxylin & eosin (H&E) ×50. (B) Expression of vimentin in fibroblastic cells both in the intima and in the muscular and adventitia layers suggesting cell migration from the adventitia to the intima. ×50. (C) α-smooth muscle actin expression in mature cells of the muscle layer and fusiform elements at the level of the intima (myofibroblasts that may originate from the transdifferentiation of fibroblasts of adventitial origin). ×50.

the IEM at higher degrees of IT. An intact IEM underlying endothelial cells appears to be essential for cell anchorage, mainly to act as a barrier against the entry of macromolecules and cells into the intimal layer.[25]

In addition, the fact that apolipoprotein B was positive both in cases with greater expression over the thickened intima and in the area around the intima–media transition point at the transition from IT to PIT.[2] It is noteworthy that no differences in apolipoprotein B among groups of patients were observed.

The progression of early atherosclerosis was correlated with IT rather than with the presence of T lymphocytes and macrophages. In agreement with Nakashima et al,[9] in the present study macrophages were barely seen in advanced lesions.

Overall, in agreement with previous studies,[26,27] thickening may be regarded as a prerequisite for retention and accumulation of lipids and thus for plaque formation. Nevertheless, if lipoprotein concentration is particularly high, macrophage/foam cells and lipids accumulate and plaques may develop also at sites without previous thickening[28] as happens in familial hypercholesterolemia. Our data further document, for the first time, that IT is rare in fetuses but frequent in pediatric patients, which indicates an increase in prevalence after birth and in the extension of lesions with age.

Our findings help to clarify several issues. Namely, the fact that IT is already present in early childhood but almost absent in fetuses clearly indicates that (1) IT is not a "remnant" of changes derived from embryological development, as in this case they would be much more present in fetal life, and subsequently decrease and (2) IT cannot be a consequence of alterations in fetal circulation/oxygenation. Finally, although we cannot rule out that maternal risk factors or other maternal conditions during pregnancy might predispose to the development of IT later in life, it is clear that their effects are not evident during fetal life, when they should be expected to be mostly operative.

The present results, coupled with our previous findings[1,14,29] and those of others,[8,10,30] strongly indicate that IT/hyperplasia in the context of the retention hypothesis is the first event occurring in coronary atherosclerosis and that it precedes lipid deposition. In this connection, the adventitia seems to be the initial battlefield and significant promoter of arterial phenotype change.[30,31]

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Castilla Rocío https://orcid.org/0000-0003-4951-2464

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