Perinatal and infant early atherosclerotic coronary lesions

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OBJECTIVE: Because the fetal origin of coronary artery lesions is controversial, early atherosclerotic coronary artery lesions in late fetal still-borns and infants, as well as the possible atherogenic role of maternal cigarette smoking, were studied.

METHODS: Twenty-two fetal death and 36 sudden infant death syndrome victims were examined by autopsy. In 28 of 58 cases, the mothers were smokers. Serially cut sections of coronary arteries were stained for light microscopy and immunotyped for CD68, CD34, alpha-smooth muscle actin, proliferating cell nuclear antigen, *c-fos* and apoptosis.

RESULTS: Multifocal coronary lesions were detected in 10 of 12 fetuses and in 15 of 16 infants whose mothers smoked. Arterial lesions in infants with nonsmoking mothers were observed in only five cases (two of 10 fetuses and three of 20 infants) ($P < 0.001$). Alterations ranged from focal areas with mild myointimal thickening in prenatal life to early soft plaques in infants. Smooth muscle cells infiltrated into the subendothelium. These early lesions demonstrated *c-fos* gene activation in the smooth muscle cells of the media, and in some of these, positivity for apoptosis was observed, suggesting that *c-fos* overexpression may promote proliferation, as evidenced by proliferating cell nuclear antigen-positive cells.

CONCLUSIONS: Early intimal alterations of the coronary arteries are detectable in the prenatal and infancy period, and may be significantly associated with maternal smoking.

Key Words: Atherosclerosis; Coronary artery disease; SIDS; SIUD; Smoking

Lésions coronariennes athéroscléreuses précoces périnatales et chez le nourrisson

OBJECTIF : Étant donné que l'origine fœtale des lésions artérielles coronariennes est controversée, les auteurs ont entrepris d'étudier des lésions artérielles coronariennes athéroscléreuses précoces chez des fœtus morts-nés en fin de grossesse et chez des nourrissons, de même que le rôle athérogène possible du tabagisme maternel.

MÉTHODE : Vingt-deux bébés morts-nés et 36 victimes du syndrome de la mort subite du nourrisson ont été autopsiés. Dans 28 cas sur 58, les mères étaient fumeuses. Des sections en coupes des artères coronaires ont été colorées pour analyse microscopique et on a déterminé les immunotypes des CD68, CD34, l'actine alpha du muscle lisse, l'antigène nucléaire cellulaire proliférant, le *c-fos* et l'apoptose.

RÉSULTATS : Des lésions coronariennes multifocales ont été décelées chez dix fœtus sur douze et chez 15 nourrissons sur 16 dont les mères fumaient. Chez les nourrissons dont les mères ne fumaient pas, les lésions artérielles n'ont été observées que dans cinq cas (2 des 10 fœtus et 3 des 20 nourrissons) ($P < 0,001$). Les anomalies allaient de zones d'épaississement myointimal léger en foyers chez les fœtus, à des plaques molles précoces chez les nourrissons. Des cellules musculaires lisses avaient infiltré le sous-endothélium. Ces lésions précoces se sont révélées actives envers le gène *c-fos* dans les cellules musculaires lisses de la média et, parmi ces lésions, on a observé des signes d'apoptose, ce qui donne à penser qu'une surexpression du *c-fos* pourrait promouvoir la prolifération, comme en fait foi la présence de cellules positives à l'égard de l'antigène nucléaire cellulaire proliférant.

CONCLUSIONS : Des anomalies précoces de l'intima des coronaires sont détectables chez le fœtus et le nourrisson et pourraient significativement être associées au tabagisme maternel.

Zeek (1) had already concluded in 1930 that arteriosclerosis can occur at any age. Atherosclerosis begins very early, progressing slowly in a silent manner until approximately the fifth or sixth decade, when complications with clinical and pathological damage start. No agreement exists regarding the earliest demonstrable lesion, with which the search for the etiology should start (2). In the literature regarding the fetal origin of coronary artery lesions, reported observations are rare and controversial. However, there is a relative consensus that lipid levels in children determine, to a large extent, the rate of coronary artery disease in the adult population (3).

Traditionally, fatty streaks have been considered to be the earliest manifestations of atherosclerosis, because they represent the earliest macroscopic lipid-containing lesion. They consist of extracellular lipids with scattered macrophages that differ from those found in early xanthelasmata of familial hypercholesterolemia and cholesterol-overfed rabbits (2).

Fatty streaks develop into fibrofatty plaques that merge, producing intimal thickening. The present investigators and others (4-6) recently described the scenario of complex atherosclerotic lesions. The site of

plaque rupture is associated with an extensive infiltrate of macrophages and T lymphocytes. These plaques, as well as those with large amounts of lipid and thin fibrous caps, are 'plaques at risk'. Intraplaque hemorrhage without plaque rupture is characterized by neovascularization of the plaque, and is caused by the breakdown of neofomed vessels in the core and periphery of the plaque (5). In complicated plaques and at the cap regions, trisomy and tetrasomy of chromosome 7, monosomy of chromosome 11, amplifications of fibroblast growth factor 3 and apoptosis, as well as overexpression of p53 protein, proliferating cell nuclear antigen (PCNA) and *c-fos* positivity, were observed in smooth muscle cells (SMCs) (4,6-8) – main factors in atherosclerosis (5,6).

On the other hand, it is known that initial atherosclerotic lesions of coronary arteries are recognizable in infancy (9-14). Some authors have stated that intimal SMC proliferation precedes lipid deposition (2), and that the initial atherosclerotic lesions are then characterized by intimal thickness due to the infiltration of SMCs, monocytes and rare leukocytes, as well as interstitial glycosaminoglycan deposits (15).

Some papers from the 1990s reported that exposure to environmental tobacco smoke increases the risk of atherosclerosis (16).

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Moreover, multicentre studies have emphasized the need for intervention to prevent atherosclerosis and its complications by the age of 15 years and possibly earlier (17).

The aim of the present study was to perform immunophenotyping characterization of early atherosclerotic lesions in autopsy specimens belonging to victims of fetal death or sudden infant death syndrome (SIDS) for insight into the mechanisms involved in early atherogenesis and into the relevance of maternal smoking in early atherosclerosis.

METHODS

Twenty-two sudden intrauterine unexplained deaths (fetal deaths) and 36 SIDS victims, all between the 32nd week of gestation and one year of age, were examined. In 28 of 58 cases, the mothers were smokers before and during pregnancy, and during the postnatal period. In all cases, the pregnancies had been proceeding normally. Clinical data collected for each case included any parental cigarette smoking habit (mother's cigarette smoking before and/or during pregnancy, and in all cases, the number of cigarettes smoked daily). Written, informed consent was obtained from parents at the coroner's office before autopsy.

All fetal death and SIDS victims underwent autopsy. The four major epicardial coronary arteries were isolated along their whole length (left main coronary artery, left anterior descending branch, circumflex artery and right artery) and excised transversely to their longitudinal axis in segments of approximately 3 mm to 4 mm. Each segment was labelled sequentially from either its aortic ostium or from its origin from the left main coronary artery. The arteries were dehydrated, embedded in paraffin blocks and serially cut. The sections of each block were stained with hematoxylin-eosin and Heidenhain trichrome (Azan) for histological examination, Alcian blue (at pH 0.5 and 2.5) for acid mucopolysaccharide analysis and acetic orcein for elastic fibre identification. The sections were then subjected to specific immunohistochemical methods for analysis of CD68, CD34, α -SM actin, PCNA, *c-fos* and apoptosis.

From the histological point of view, arterial lesions were classified as 'musculoelastic intimal thickenings' and 'soft plaques' (see the 'Results' section for the definitions of these terms).

Immunocytochemical staining

Immunophenotyping of cells in early lesions was performed with monoclonal antibodies to identify lymphocyte subsets (T cell, CD45RO [BioGenex Inc, USA]; B cell, CD20 [Dako, Denmark]; cytotoxic/suppressor, CD8 [Dako]; helper/inducer, CD4 [BioGenex Inc]); macrophages (CD68 [Dako]), endothelial cells (CD31, CD34 [BioGenex Inc]; and factor VIII [Ylem, Italy]) and SMC hyperplasia (α -SM-actin, anti-mouse α -actin monoclonal antibody concentrated MU128-UC clone 1A4 [BioGenex Inc]).

Detection systems used include streptavidin biotin peroxidase or/and alkaline phosphatase (BioGenex Inc), and enhanced polymer one-step staining kit (EPOS; Dakopatts, USA). When double immunostaining was required, a single step with peroxidase-conjugated primary antibodies was performed with the EPOS system, followed by a second detection with streptavidin-biotin alkaline phosphatase. Development of peroxidase reactions was carried out with 3,3'-diaminobenzidine, and Fast Red was used to visualize alkaline phosphatase reactions. Thus, the first antigen was labelled in brown and the second in bright red. Negative controls were run simultaneously with irrelevant antibodies of the same isotype.

DNA nick end-labelling

Immunohistochemical visualization of apoptotic cells was obtained by the terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-conjugated deoxyuridine nick end-labelling (TUNEL) method, which identifies early DNA fragmentation in the nucleus on the basis of the specific binding of TdT to 3'-OH ends of DNA. Deparaffinized sections were incubated with 20 μ g/mL proteinase K (Sigma, USA).

After the endogenous peroxidase treatment, the enzyme TdT (0.3 U/mL) was used to incorporate digoxigenin-conjugated

deoxyuridine (0.01 mM/mL) into the ends of DNA fragments. The signal of TUNEL was detected by an antidigoxigenin antibody conjugated with peroxidase. Cell counting was performed under a light microscope. Cells stained with TUNEL exhibited dark brown nuclei. Nonapoptotic cells were counterstained with methyl green, and their nuclei were stained blue-green.

PCNA immunohistochemistry

Sections were air-dried overnight at room temperature and immunostained with the monoclonal antibody PC 10 at a dilution of 1:200 using an immunoperoxidase method (avidin-biotin-peroxidase complex) with light hematoxylin counterstaining. All immunostained sections were examined at a magnification of $\times 100$.

c-fos immunohistochemistry

Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase was blocked by incubating sections with 3% hydrogen peroxide for 5 min. After being washed in phosphate-buffered saline, the sections were incubated with 10% normal goat serum and then with a 1:100 diluted polyclonal anti-*c-fos* antibody (SC-52P; Santa Cruz Biotechnology, USA) at room temperature for 1 h. After being washed in phosphate-buffered saline for 5 min, sections were incubated for 30 min with the biotinylated goat anti-rabbit immunoglobulin G antibody supplied in the kit, incubated with streptavidin peroxidase for 30 min, stained with 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma) (30 mg, along with 0.5 mL of 0.3% hydrogen peroxide in 100 mL of 0.05 M Tris Hydrochloride, pH 7.6) and counterstained with Mayer's hematoxylin. Only cells with very intense brown staining were considered to be positive.

Statistical analysis

The significance of differences between group parameters was evaluated by Fisher's exact test. The selected level of significance was $P < 0.05$ (two-tailed).

RESULTS

None of the mothers had dyslipidemia, nor had they used drugs or alcohol. They were 20 to 39 years of age. In 28 of 58 cases (48%), the mother smoked between five and 20 cigarettes per day. The smoking habit had started before pregnancy and had been maintained or even increased after the child's birth.

In 10 of 12 fetuses and in 15 of 16 infants of smoking mothers, multifocal coronary lesions of varying nature were detected (see below). Arterial lesions in infants with a nonsmoking mother were observed in only five cases (two of 10 fetuses and three of 20 infants) ($P < 0.001$). The alterations ranged from focal areas with mild myointimal thickening in prenatal life to parietal plaques in infants, which partially reduced the arterial lumen. The anterior descending coronary artery was always involved to varying degrees. These lesions were classified into two categories:

- Musculoelastic intimal thickening: these were marked by slight to moderate focal myointimal thickening (Figures 1A, 1B and 1C). In some cases, the thickening was localized as a cushion (Figures 1D and 1E). The subendothelial connective tissue was infiltrated to varying extent by SMCs, monocytes, rare lymphocytes and amorphous deposits. Some SMCs presented loss of polarity, forming columns perpendicular to the axis of the media and infiltrating the subendothelial connective tissue, mostly with split and/or interrupted internal elastic lamina (Figures 1D and 1E, and Figure 2). An increased amount of mucoid ground substance was also observed at this level. Macrophages were detected in the intimal border of the lesions, penetrating the endothelium (Figure 1F). No neoangiogenesis was observed. Monocytes and/or foam cells were present in low numbers; B lymphocytes were rare. The endothelium was morphologically intact.

Figure 1) Musculoelastic intimal thickening. **A** Epicardial coronary artery (left anterior descending branch). Marked intima-medial thickness and increase in the ground substance and mononuclear cells. Smooth muscle cells are not parallel, and some seem to migrate into the intima (hematoxylin and eosin stain, original magnification $\times 40$). **B** Epicardial coronary artery (left anterior descending branch). Intima-medial thickness with increase in ground substance and mononuclear cells can be observed at the bottom. The internal elastic membrane is fairly preserved, except in a small area, where migrating mononuclear cells can be noted (bottom) (hematoxylin and eosin stain, original magnification $\times 40$). **C** Intima-medial thickness with subendothelial proliferation of smooth muscle cells in an epicardial coronary artery of a sudden infant death syndrome patient (azan trichrome stain, original magnification $\times 40$). **D** Left anterior descending coronary artery of a baby 2.5 months of age who died from sudden infant death syndrome. Marked intima-medial thickness with disappearance of the internal elastic membrane. Subintimal proliferation of smooth muscle cells, with medial atrophy (azan trichrome stain, original magnification $\times 100$). **E** Same section as in D. Intima-medial thickness showing positivity for alpha-actin. Immunohistochemistry (anti-smooth muscle actin stain, original magnification $\times 100$). **F** Coronary artery. Multiple intimal macrophages are clearly marked in dark brown (black in this photograph). The internal elastic membrane can easily be identified (anti-CD68 stain, original magnification $\times 400$)

Figure 2) Intimal thickening in the left anterior coronary artery, with evident splitting and fragmentation of the internal elastic membrane (acetic orcein stain, original magnification $\times 200$)

- Soft plaques (Figures 3A, 3B, 3C and 3D): these are rich, cellular plaques with extensive infiltration by SMCs, associated with slight infiltration by monocytes/foam cells and rare lymphocytes. An increased amount of mucoid ground substance was also observed. There was evident rupture of the media elastic fibre system, and the tunica media was focally thinner, especially in the areas of greater proliferation. SMCs lost their polarity, with their axis perpendicular to the media, and

Figure 3) **A** Epicardial coronary artery showing a soft plaque with loose subendothelial connective tissue infiltrated with proliferating smooth muscle cells (anti-smooth muscle actin stain, original magnification $\times 10$). **B** Enlargement of 2A (anti-smooth muscle actin stain, original magnification $\times 80$). **C** Coronary artery soft plaque partially occluding the artery lumen (46%). Large amounts of mucoid ground substance can be observed in the subendothelial connective tissue (azan trichrome, original magnification $\times 10$). **D** Same section as in C. Left anterior descending coronary artery of an infant who died of sudden infant death syndrome. Severe damage, with diminution of muscular fibres and fibrosis at the media (right). Perpendicularly oriented smooth muscle cells crossing the internal elastic membrane, which is fragmented (centre), can be observed. On the left, marked subintimal fibrosis can be also observed (azan trichrome stain, original magnification $\times 400$)

Figure 4) **A** High apoptotic index in a coronary artery with myointimal thickness. Apoptotic nuclei are stained dark brown (black in this photograph); negative cells can be seen in green (gray in this photograph) (original magnification $\times 200$). **B** High proliferating cell nuclear antigen index in myointimal thickness in coronary artery (original magnification $\times 400$). **C** High positivity for c-fos in smooth muscle cells of a coronary artery with myointimal thickness. Perpendicularly oriented smooth muscle cells can be observed (original magnification $\times 200$)

migrated into the subendothelial tissue (Figure 3D). The endothelium was morphologically preserved. Reduction of the lumen diameter varied from 15% to 20%, and in one case, it reached 46%.

Intermingled lesions with components of both categories were frequently observed.

These early atherosclerotic lesions presented *c-fos* gene activation in the SMCs of the tunica media, and in some of these, positivity of the SMCs for apoptosis was also observed, suggesting that *c-fos* gene overexpression can promote a proliferative process, as supported by the PCNA positivity in many of the lesions observed in the SIDS victims (Figures 4A, 4B and 4C) (Table 1).

TABLE 1
Demographic data and biological markers

	Fetal death (n=22)	Sudden infant death syndrome (n=36)	P
Age (mean $\pm$ SD)	37.09 $\pm$ 2.6 weeks	4.03 $\pm$ 2.4 months	–
Sex	13 male/9 female	23 male/13 female	–
Maternal smoking, n (%)	12 (54.5)	18 (50)	NS
Preatherosclerotic lesion	12	13	NS
Soft plaque	0	11	**
<i>c-fos</i>	10	7	*
Proliferating cell nuclear antigen	0	10	**
TUNEL method	5	5	NS

Significance determined by Fisher's exact test. * $P < 0.05$, ** $P < 0.01$. NS Not significant; TUNEL Terminal deoxynucleotidyl transferase-mediated digoxigenin-conjugated deoxyuridine nick end-labelling

DISCUSSION

In the present paper, we have shown that early atherosclerotic lesions of the coronary arteries are already detectable in the prenatal and infancy periods, and that they may be significantly associated with maternal cigarette smoking.

Observations regarding the fetal origin of coronary artery lesions are controversial. Stary (18) reported that approximately one-half of infants within the first six months of life presented small collections of foam cells in susceptible segments of the coronary arteries. In subsequent years, fewer children were observed to have such accumulations larger in size than those found in infants. Later, these lesions occurred in 69% of adolescents 12 to 15 years old (14).

Despite this, it has also been emphasized that the coronary arterial intima undergoes a sequence of changes following injury before the appearance of foam cell accumulation. Studies of coronary arterial pathology in the transplanted heart demonstrated that the first stage is that of intimal hyperplasia and disruption of the internal elastic lamina. The second stage involves migration of medial SMCs into the thickened intima, and in the third stage, the deposits of lipids result in atheroma (19).

The same sequence can be sought and found in coronary artery disease-prone societies, the first being commonly identifiable in infancy and childhood. These changes, reported over many years, have been assumed to be benign accompaniments of growth and development. However, they are likely to be the precursors of atherosclerosis and, later, the seat of lipid deposition, without which that deposition would not occur (14). These lesions, which may represent the missing link between normality and foam cell accumulation, and atheromas, have been identified in a subgroup of highly susceptible locations (ie, main branch bifurcations) (18).

Fetal and infant arteries usually adjust to normal asymmetries in hemodynamic forces to maintain an optimal flow equally at all points along the artery. Therefore, increases in thickness occur through the activation of a subgroup of local intimal SMCs. 'Thick segments' develop in fetal life (20), and although variable in degree, they are found in everyone at birth (21). Accordingly, a 'thick segment' can be seen at and near bifurcations of arteries and at the ostiums of even the smallest vessels, where it is focal and eccentric. A thick intima is also found at some sites that are not obviously related to a branch vessel, where it is more diffuse. These 'thick segments' have been called "adaptive intimal thickening", "intimal pad", "mucoid fibromuscular plaque", "focal intimal hyperplasia" and "musculoelastic intimal thickening", among others (21).

Conversely, other authors consider 'thick segments' to be preatherosclerotic lesions, because they are located where atheromas are

usually found, they are circumscribed and eccentric, and they project into the lumen of vessels when these are collapsed after death (18). The findings reported herein seem to support this suggestion.

Nevertheless, when atherogenic lipoprotein concentrations are extremely high, foam cells and lipids accumulate, and plaques develop without adaptive thickening.

Thus, clinically overt atherosclerotic lesions may develop through a sequence of morphological changes, beginning as minimal lesions. However, these lesions may stabilize at that morphology without progressing further. When risk factors change favourably, these lesions can regress (18) or, more conceivably, decrease or be functionally minimized by arterial remodelling (22).

Intimal proliferation has been observed in 95.3% of coronary arteries in infants between one and five years of age who died from causes unrelated to the cardiovascular system. It should be noted that the site in which plaques were predominantly found was the proximal left anterior descending coronary artery (23), a very frequent location of stenotic plaques in adults.

We do not agree with Blackburn (24), who undervalued the importance of these preatherosclerotic lesions in infancy and explained their occurrence based mainly on hypercholesterolemia due to lifestyle patterns adopted during youth, while Strong (25) claimed that the lesions have their origin in childhood.

Napoli et al (26) demonstrated that fatty streaks were prevalent in the aortas of premature infants (fetal age 6.2 $\pm$ 1.3 months), particularly in fetuses whose mothers were hypercholesterolemic during pregnancy. These authors found preatherosclerotic, macrophage-rich lesions in a distribution that reflected that of more advanced atherosclerosis in adults (26). Conversely, we did not find macrophage-rich lesions, and macrophages were detected only in the intimal border of the lesions, penetrating the endothelium. Of note, mothers were normocholesterolemic in our study. It is conceivable that in the cases studied by Napoli et al (26), the increased amounts of oxidized low-density lipoprotein enhanced the recruitment, activation and proliferation of monocytes.

It has been reported that a mother who is a heavy smoker (more than 20 to 30 cigarettes per day) should avoid breastfeeding to stop the passage of tobacco products through the milk (27). Furthermore, because most infants in the present study had been born in large cities, we can hypothesize that atmospheric pollution might have contributed to the development of early atherosclerotic lesions. Air pollution, particularly in the Lombardy region of northern Italy, where the subjects studied came from and which features high rates of carbon monoxide, nitrogen dioxide, ozone, sulphur dioxide and particulate matter (especially particulate matter with a median diameter smaller than 10 μ m) (28), might have had an important influence on the rate of SIDS.

The current knowledge of cigarette smoking as a risk factor for arterial disease is based on the results of epidemiological studies (29). Only a few morphological observations of the atherogenic effects of cigarette smoking have been reported (30). Tobacco smoking has been implicated in the infiltration and the progression of atherosclerotic lesions, but the precise mechanism by which cigarette smoke induces atherosclerosis has not been fully established.

From our immunohistochemical findings, it seems that the migration, proliferation and differentiation mainly of SMCs, but also of macrophages, are important pathological responses to tobacco injury that contribute to the development and progression of early atherosclerotic lesions (4,15).

In some of these primary lesions, immunopositivity of the SMCs for apoptosis was also observed. This finding may be interpreted as physiological attempts to prevent the evolution of the atherogenic process and to preserve the wall structure under normal conditions (31).

In the coronary wall of young infants, we found a moderate to high number of PCNA-positive SMCs and rare cells, with both activation of the *c-fos* gene and apoptosis. It can therefore be suggested that *c-fos*

gene overexpression can promote a proliferative process (32). It is possible that the oxidants present in the gas phase of cigarette smoke may have determined the sequence of biological events in the arterial walls observed in the present study. First, the oxidants may pass through the endothelium, without causing morphologically identifiable alterations, and induce the immediate and intense stimulation of the *c-fos* gene in the SMC of the media. Thus, it can be hypothesized that through several signal transduction pathways, different molecules (PDGF platelet-derived growth factor, EGF epidermal growth factor, interleukin 1 beta, tumour necrosis factor-alpha, etc) activate transcription factors, such as the nuclear factor kappa B, or proto-oncogenes such as *c-fos* and *c-myc*, which regulate the expression of genes involved in the inflammatory and proliferative response of the preatherosclerotic lesions (33).

In connection with this, it can be suggested that the activation of the *c-fos* gene may promote a process of SMC change, leading to the loss of the differentiated state and acquisition of ameboid movements, with migration toward the intima (34). Unpublished observations in

our laboratory have shown the presence of this type of activated 'myofibroblast' in preatherosclerotic lesions with a typical switch in actin expression from alpha to beta form, as well as increased synthetic and proliferative activity.

Limitations

Because specimens were collected from forensic autopsies, it was not possible to perfuse and fix the arteries near physiological pressure to preserve arteries from collapse and contraction after death. For the same reason, it was not possible to obtain a complete clinical record of parental diseases or the genetic background of all cases to make sure that the observed association with smoking was not confounded by other factors, such as maternal feeding versus artificial feeding.

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