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ORIGINAL ARTICLE

Intimal preatherosclerotic thickening of the coronary arteries in human fetuses of smoker mothers

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Summary. *Background*: Many studies have described the development of preatherosclerotic coronary artery lesions in infancy. The observations reported in the literature regarding the fetal origin of coronary artery lesions are rare and controversial. *Objectives*: To identify the features of preatherosclerotic coronary artery lesions in late fetal stillborns and the possible atherogenic role of maternal cigarette smoking. *Methods*: We examined 22 stillborns (13 males and nine females), all of whom had died *sine causa* after the 32nd week of gestation. All underwent autopsy. Twelve of the mothers smoked over five cigarettes per day before and during the pregnancy. The four major epicardial coronary arteries were isolated along their whole length, embedded in paraffin and serially cut for histologic examination and immunohistochemical studies, particularly searching for the proliferating cell nuclear antigen and c-Fos expression. Alterations of chromosome 7 were also investigated by the fluorescence *in situ* hybridization technique. *Results*: In over 50% of the fetuses, almost all from smoker mothers, multifocal structural alterations of coronary walls were evident. The smooth muscle cells (SMCs) presented loss of polarity, forming columns perpendicular to the axis of the media and infiltrating the subendothelial connective tissue. Increased amounts of mucoid ground substance were also observed in the subendothelial connective tissue. In all the cases with coronary alterations, study of the biological markers showed intense c-Fos positivity of the SMCs. *Conclusions*: Preatherosclerotic intimal alterations of the coronary arteries are already detectable in the prenatal period and are significantly associated with maternal cigarette smoking.

Keywords: cigarette smoke, coronary arteries, human fetus, preatherosclerotic lesions.

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Introduction

The consequences of atherosclerosis arise due to acute mechanical complications (thrombosis or hemorrhage of the plaque) and, in a small measure, to hemodynamic complications (vasoconstriction) favored by the instability of the atherosclerotic plaque.

It has long been known that initial atherosclerotic damage of the coronaries is already recognizable in infancy [1–10]. Recently, in a systematic investigation of sudden infant death syndrome victims we observed evident preatherosclerotic lesions, which were particularly common in formula-fed infants of smoker mothers. In this study, the previous findings of musculoproliferative intimal thickening, sometimes of a severe degree even in the first days of life, prompted us to seek more precocious modifications in the coronary walls of stillborns, and to consider the role of maternal cigarette smoking before and during pregnancy in these cases.

In addition, we studied the possible presence of some biological markers, previously described by us to be present in reactivation of the atherosclerotic process in its classic adult aspects, namely high expression of the proliferating cell nuclear antigen (PCNA), activation of the proto-oncogene c-*fos*, and trisomy of chromosome 7 [11–13].

Materials and methods

We studied the coronary arteries of 22 fetuses, 13 males and nine females, ranging in age from 32 to 40 weeks of gestation, suddenly and unexpectedly deceased.

Pregnancy had run a normal course in all cases. None of the mothers had any significant pathology, nor had they used drugs or alcohol. Twelve mothers were cigarette smokers before and during the pregnancy, smoking more than five cigarettes a day.

A complete autopsy examination was carried out, including gross and microscopic evaluation of the body, the placental disk, the umbilical cord and membranes. All organs were fixed in 10% phosphate-buffered formalin, processed and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin, and Heidenhain's trichrome (Azan).

Coronary artery histologic examination

Following the protocol of Roberts *et al*. [14], the hearts were fixed in formalin for at least 1 day. The four major epicardial coronary arteries (left main, left anterior descending, left circumflex, right) were excised transversely to their longitudinal axis into segments approximately 2–3 mm long. Each segment was labeled sequentially from either its aortic ostium or from its origin from the left main coronary artery. They were dehydrated, embedded in paraffin, and serially cut. The sections of each block were stained with hematoxylin and eosin, trichromic Heidenhain (Azan), and Alcian blue for histologic examination, Weigert for elastic fibers labeling, and submitted to specific immunohistochemical methods for identification of the smooth muscle cells (SMCs) and PCNA, and for c-Fos expression. Fluorescence *in situ* hybridization (FISH) was applied to determine alterations of chromosome 7.

PCNA immunohistochemistry

Sections were air-dried overnight at room temperature and immunostained with the monoclonal antibody PC10 at a dilution of 1 : 200, using an immunoperoxidase method (avidin–biotin complex) with light hematoxylin counterstaining. All immunostained sections were examined using a × 50 lens. Only nuclei with intense immunostaining were considered to be PCNA positive.

c-Fos immunohistochemistry

Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide for 5 min. After washing in phosphate-buffered saline (PBS), the sections were incubated with 10% normal goat serum and then with 1 : 100 diluted polyclonal anti-c-Fos antibody (SC-52P; Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h. After washing in PBS for 5 min, the sections were incubated for 30 min with the biotinylated goat antirabbit Ig G antibody supplied in the kit, then incubated with streptavidin-peroxidase for 30 min, stained with 3–3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, MO, USA) solution (30 mg of DAB and 0.5 mL of 0.3% hydrogen peroxide in 100 mL of 0.05 mol L^{-1} Tris-HCl pH 7.6) and counterstained with Mayer's hematoxylin. All immunostained sections were examined using a × 50 lens. The cells with intense brown immunostaining were considered to be c-Fos positive.

α-actin immunohistochemistry

To identify neointimal cells as SMCs, additional immunohistochemical staining (the avidin–biotin method) was performed with a monoclonal antibody against α-actin (Renner, Dannstadt, Germany).

Fluorescence in situ *hybridization*

We used an α-satellite DNA probe specific for the centromeric region of chromosome 7, labeled with biotin (Oncor). The centromeric probe was prepared by mixing 1.5 μL of the probe with 30 μL of Hybrisol VI (Oncor). The probe was applied to the prepared air-dried slides (15 μL) and coverslipped. Both probes and target DNAs underwent denaturation on the slides on a 67 ± 2 °C hot plate for 5 min and then incubation overnight in a prewarmed humidified chamber at 37 °C. The hybridized signals were detected using a commercial kit (FITC avidin detection kit, Oncor). Propidium iodide 2.5 μg mL^{-1} in antifade was used for counterstaining. For scoring, a Leitz Orthoplan with a Ploemopak incident-light fluorescence microscope was used, equipped with ultraviolet excitation filter sets. Only interphase cell nuclei with intact morphology were scored. The number of hybridization spots in each cell was considered.

The statistical value of the results was determined using the analysis of the variance test (*F*-test). The selected level of significance was $P < 0.05$.

Results

At autopsy, all 22 stillborns were described as well developed, with body length and weight corresponding to gestational age.

No intimal proliferations emerged from the histologic examination of the coronary walls in 10 cases. In 12/22 fetuses (55%), all deceased after the 35th week, multifocal structural alterations of all the coronaries were revealed, more severe along the anterior descending branch of the left coronary. More specifically, in seven of these cases (32%), foci of gross subversion of the media with thinning and fiber fragmentation were observed, also in fields far from the bifurcations. The smooth muscle cells (SMCs) presented loss of polarity, forming columns of myocytes located perpendicularly to the axis of the media itself and infiltrating the subendothelial connective tissue (Fig. 1). In five additional cases (23%), besides this intense reaction of the SMCs of the media, increased amounts of mucoid ground substance were observed in the subendothelial connective tissue, with formation of intimal preatherosclerotic lesions of proliferative aspect (Fig. 2). Such processes also seem to determine fragmentation and detachment of the internal elastic membrane. Sometimes SMCs appeared in the gaps of this lamina.

The clinical data collected disclosed that in 10 of the 12 cases with intimal thickenings the mothers were smokers before the beginning of pregnancy.

In all the lesions, immunohistochemical study of the biological markers showed intense c-Fos positivity of the SMCs (Fig. 3), while PCNA-positive cells were not detected. The search for chromosome 7 alterations using the FISH technique gave negative results, showing only two normal hybridization spots per nucleus.

Table 1 shows the relationship between the results obtained from the morphological examination and the biological marker study, compared with the mothers' smoking habit before and during pregnancy. A significant correlation ($P < 0.05$) is evident between the presence of intimal preatherosclerotic lesions, c-*fos* gene activation and maternal smoking. In fact, in 10 of the 12 fetuses of smoker mothers (cases 1, 4, 5, 10, 12, 14, 17, 18, 20, 21), c-Fos-positive preatherosclerotic lesions were present. Only in two cases (3 and 13) of non-smoking mothers were coronary wall alterations demonstrated.

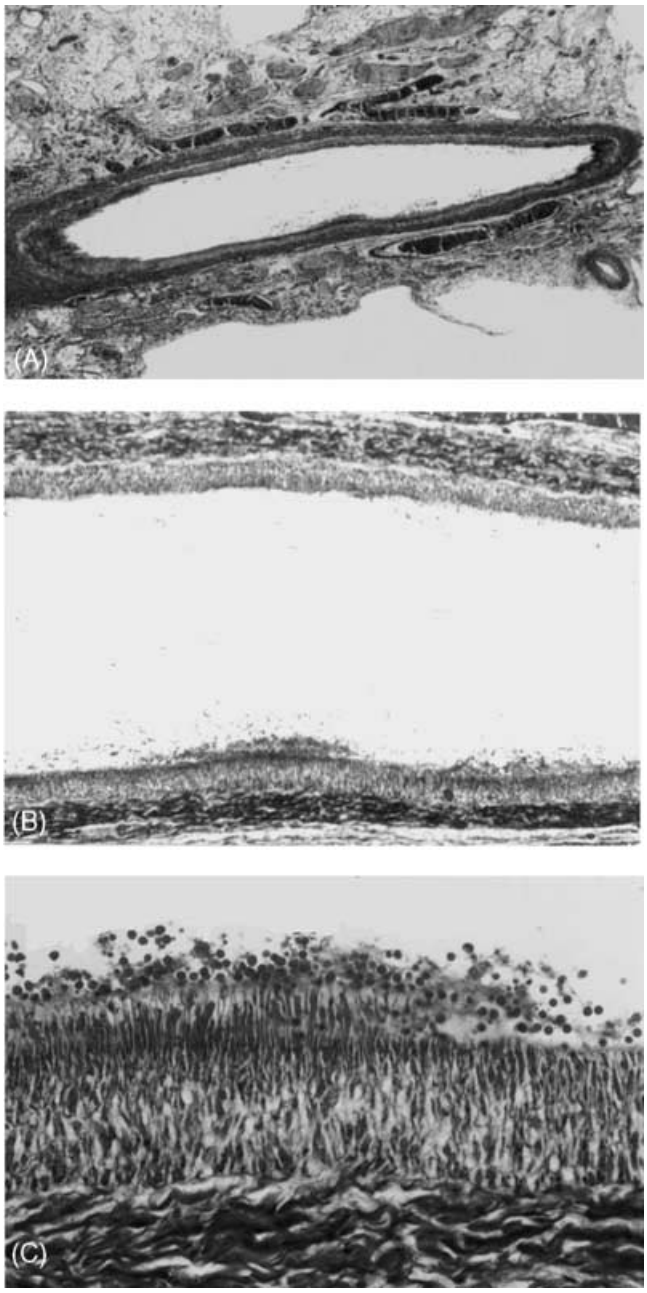

Fig. 1. Epicardial coronary artery (left anterior descending branch) of a fetus of 38 gestational weeks (case no.1). The focal thickness consists of medial SMCs, perpendicularly oriented. Azan stain. Original magnifications: A, × 20; B, × 100; C, × 400.

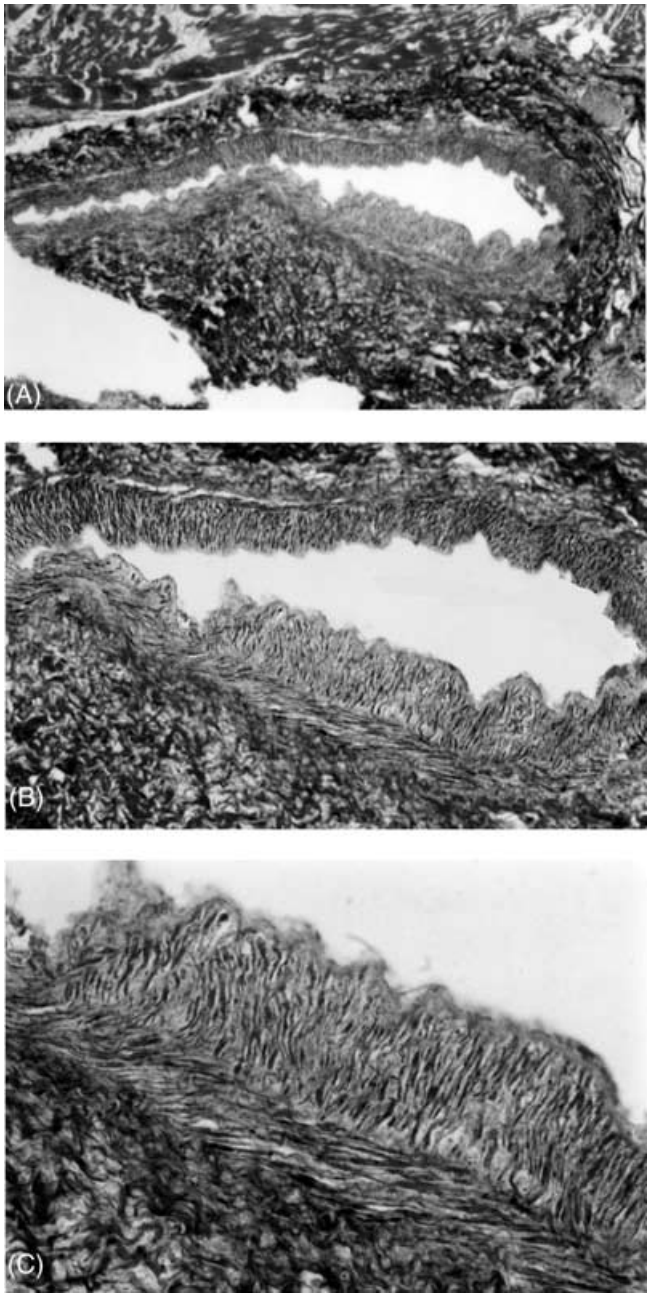

Fig. 2. Epicardial coronary artery (left anterior descending branch) of a fetus of 39 gestational weeks (case no.3). The myointimal thickness shows increased amounts of mucoid ground substance in the subendothelial connective tissue. Azan stain. Original magnifications: A, × 20; B, × 100; C, × 400.

Discussion

In this study we have analyzed the morphological pattern of preatherosclerotic coronary artery lesions in human fetuses and the possible atherogenic role of maternal cigarette smoking before and during pregnancy.

In the literature, it has been reported that initial atherosclerotic or preatherosclerotic damage is detectable even in infancy. Many of these studies [1–10] show that such lesions are initially characterized by thickening of the intima, disruption of the internal elastic lamina, and proliferation of the medial SMCs. As first reported by one of the present authors [1,2], the intimal lesions are characterized by an accumulation of glycosaminoglycans in the fundamental substance of the intimal connective tissue. Only subsequently can lipids be discerned.

The possible etiologic factors of early atherosclerotic lesions include mechanical causes [15,16], high serologic levels of lipids [17,18], infections [19], and genetic susceptibility [20,21].

The few studies carried out on the fetal coronaries are predominantly referred to experimental animals. Bolande *et al*. [22] observed that proliferative modifications of the

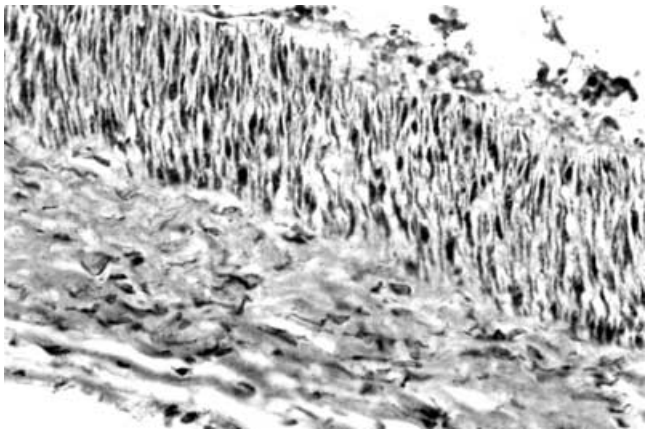

Fig. 3. Epicardial coronary artery (left anterior descending branch) of a fetus of 38 gestational weeks (case no.1). Coronary lesion shows high positivity for the c-*fos* gene. c-Fos immunohistochemistry: original magnification × 500.

coronary arteries are present in 100% of piglet fetuses and regress after birth, concluding that such lesions represent a physiological developmental process and therefore cannot be related to coronary atherogenesis in the adult.

The rare studies of the coronary arteries of human fetuses reported in the literature yielded controversial findings and date back to over 30 years ago. In a 1957 study [23], Moon did not observe any pathological process, while a few years later, Robertson [8] and Neufeld *et al*. [24] reported structural subversion of the coronary walls only in fetuses at term.

The results of our histopathologic study performed on the four major epicardial coronary arteries, serially sectioned, from 22 late stillborns, have enabled us to demonstrate the incidence and characteristics of intimal preatherosclerotic lesions of proliferative aspect and the possible role of maternal cigarette smoking. In particular, our findings show that at first the intima appears to be infiltrated by SMCs that, due to loss of polarity, seem to be arranged in a column perpendicular to the main axis of the media. The longitudinally oriented SMCs probably originate from the media but they are intimal structures. Only later does the intimal thickening contain acid mucopolysaccharide deposits, probably synthesized by SMCs, that give an edematous aspect of the subendothelial connective tissue. Few monocytes are present. These intimal preatherosclerotic lesions of proliferative aspect were observed in 12 stillborns, 10 of whose mothers were smokers. Maternal smoking has been discussed as a possible etiologic factor of abnormal intimal development of coronary arteries in infants dying during the first month of life [25].

Molecular biology analyses in our study have made it possible to clarify the biological nature of the observed coronary wall alterations. These studies revealed intense activation of the proto-oncogene c-*fos*, while proliferative PCNA expression and trisomy of chromosome 7, previously reported by us in over 50% of unstable plaques in adults and concluded to be indicative of a marked proliferative process, were not observed [11–13].

The c-*fos* gene belongs to the family of immediate early genes, so defined for their ability to be rapidly activated in many tissues in response to various injuries, because they do not require protein synthesis. Subsequently, the increased expression of such genes has a mitogenic effect on the cells [26–28]. Therefore, c-Fos positivity in fetal coronary lesions represents the first biological reaction, caused by the gaseous products of nicotine combustion. By crossing the cellular membrane, particularly of the SMCs, the nicotine products can directly modify

Table 1 Results of fetal analyses

Case no	Gestational age (weeks)	Sex	Body weight (g)	Preatherosclerotic lesions*	Biological markers			Maternal smoking
					PCNA	c-fos	+cr 7	
1	38	F	3200	1	−	+	−	+
2	39	M	3580	−	−	−	−	−
3	39	M	2050	2	−	+	−	−
4	40	F	3100	1	−	+	−	+
5	40	M	3200	2	−	+	−	+
6	36	F	2200	−	−	−	−	−
7	38	F	3530	−	−	−	−	−
8	32	F	1680	−	−	+	−	−
9	36	M	2200	−	−	−	−	−
10	36	F	1840	1	−	+	−	+
11	34	M	2350	−	−	−	−	−
12	38	M	3160	1	−	+	−	+
13	39	M	2330	1	−	+	−	−
14	38	M	3530	2	−	+	−	+
15	33	F	1840	−	−	−	−	−
16	32	M	1540	−	−	−	−	+
17	38	M	3210	2	−	+	−	+
18	39	M	3250	1	−	+	−	+
19	40	F	3060	−	−	−	−	−
20	35	M	2480	2	−	+	−	+
21	36	M	2650	1	−	+	−	+
22	40	F	3100	−	−	−	−	+

*1, media alterations; 2, media alterations and myo-intimal thickness.

the expression of the c-*fos* gene, interacting with nuclear receptors.

The SMCs so activated reacquire the primordial characteristic of ameboid movement and leave the media, which appears subverted and thinned, to move through the intima towards the lumen without proliferation. This is confirmed by the lack of PCNA-positive SMCs in the fetal coronary arteries. Only subsequently does the c-*fos* gene exert its mitogenic effect.

Thus the triad of events, often reported in the literature and universally recognized as the first sign of coronary lesions [1–10], represented by (i) thickening of the intima, (ii) fragmentation of the internal elastic lamina, and (iii) proliferation of the SMCs of the media, is anticipated, at least in the fetuses we observed, by a phase characterized by gene activation and disturbance of the biological homeostasis of the SMCs, morphologically represented by structural disorder. SMC proliferation may occur only subsequently.

A similar hypothesis has already been made by some authors in experimental studies [29–31], who have suggested that proliferation of SMCs in the coronary artery walls is an early event, but subsequent to the activation of mitogenic factors during the course of the injury itself.

In conclusion, in the present study we have demonstrated that very early preatherosclerotic alterations of the coronary arteries are already detectable in the prenatal period and do not represent a physiological process, as suggested in an experimental study [22], but are significantly associated with maternal cigarette smoking even before the beginning of pregnancy. Thus, they are probably a consequence of the action of nicotine combustion products.

Therefore, the strong atherogenic effect of cigarette smoking on the human coronary arteries already begins in fetal life, probably in genetically predisposed individuals. This knowledge has important inherent consequences for the prevention of atherosclerosis and of its sequelae.

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References

1 Giordano A, Matturri L. Le role des mucopolysaccharides dans l'athérosclérose. *Acta Cardiol* 1966; **11**: 109–29.
2 Giordano A, Masini T, Matturri L. Istochimica dei primi stadi della coronarosclerosi umana. *Cardiol Prat* 1961; **8**: 237–72.
3 Stary HC. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 1989; **9**: 119–32.
4 Pesonen E. Preliminary and early stages of atherosclerosis in childhood. *Zentralbl Allg Pathol* 1989; **135**: 545–8.
5 Meyer WW, Lind J, Yao AC, Kauffman SL. Early arterial lesions in infancy and childhood and ways of prevention. *Paediatrician* 1982; **11**: 136–56.
6 Davies H. Atherogenesis and the coronary arteries of childhood. *Int J Cardiol* 1990; **28**: 283–92.
7 Schornagel HE. Intimal thickening in the coronary arteries in infants. *Arch Pathol* 1956; **62**: 427–32.
8 Robertson JH. The significance of intimal thickening in the arteries of the newborn. *Arch Dis Child* 1960; **35**: 588–90.
9 Velican D, Velican C. Study of fibrous plaques occurring in the coronary arteries of children. *Atherosclerosis* 1979; **3**: 201–15.
10 Minkowski WL. The coronary arteries of infants. *Am J Med Sci* 1947; **214**: 623–9.
11 Matturri L, Cazzullo A, Turconi P, Lavezzi AM. Cytogenetic aspects of cell proliferation in atherosclerotic plaques. *Cardiologia* 1997; **42**: 833–6.
12 Matturri L, Cazzullo A, Turconi P, Lavezzi AM, Vandone PL, Gabrielli L, Fernandez Alonso G, Grana D, Milei J. Chromosomal alterations in atherosclerotic plaques. *Atherosclerosis* 2001; **154**: 755–61.
13 Lavezzi AM, Milei J, Grana DR, Flenda F, Basellini A, Matturri L. Expression of c-fos, p53 and PCNA in the unstable atherosclerotic plaque. *Int J Cardiol* 2003; in press.
14 Roberts W, Jones FA. Quantitation of coronary arterial narrowing at necroscopy in sudden coronary death. Analysis of 31 patients and comparison with 25 control subjects. *Am J Cardiol* 1979; **44**: 39–45.
15 Glagov S, Zarins CK, Massawa N, Xu CP, Bassiouny H, Giddens DP. Mechanical functional role of non-atherosclerotic intimal thickening. *Frontiers Med Biol Engl* 1993; **5**: 37–43.
16 Nereim RM. Hemodynamics and the vascular endothelium. *ASME J Biochen Eng (B)* 1993; **115**: 510–4.
17 Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W Jr, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis: American Heart Association. *Circulation* 1994; **89**: 2462–78.
18 Koschinsky T, Bunting CE, Rutter R, Gries FA. Increased growth stimulation of human vascular cells by serum from patients with primary hyper-LDL-cholesterolemia. *Atherosclerosis* 1987; **63**: 7–13.
19 Pesonen E. Infection and intimal thickening: evidence from coronary arteries in children. *Eur Heart J* 1994; **15**: 57–61.
20 Kaprio J, Norio R, Pesonen E, Sarna S. Intimal thickening of the coronary arteries in infants in relation to family history of coronary artery disease. *Circulation* 1993; **87**: 1960–8.
21 Pesonen E, Norio R, Sama S. Thickenings in the coronary arteries in infancy as an indication of genetic factors in coronary heart disease. *Circulation* 1975; **51**: 218–25.
22 Bolande RP, Leistikow EA, Wartman F, Louis T. The development of preatherosclerotic coronary artery lesions in perinatal piglets. *Biol Neonate* 1996; **69**: 109–18.
23 Moon HD. Coronary arteries in fetuses, infants and juveniles. *Circulation* 1957; **16**: 263–7.
24 Neufeld HN, Wagenfoort CA, Edwards JE. Coronary arteries in fetuses, infants, juveniles and young adults. *Lab Invest* 1962; **11**: 837–44.
25 Lehtovirta P, Pesonen E, Sarna A. Effect of smoking on the fetal coronary arteries. *Acta Pathol Macrobiol Immunol Scand (A)* 1984; **92**: 189–93.
26 Herrera DG, Robertson HA. Activation of c-*fos* in the brain. *Prog Neurobiol* 1996; **50**: 83–107.
27 Dony C, Gruss P. Proto-oncogene c-*fos* expression in growth regions of fetal bone and mesodermal tissue. *Nature* 1987; **299**: 711–4.
28 Muchnok JP, Klishis ML, Roberts JR, Antonini JM, Whong WZ, Ong T. Cadmium-induced cell transformation and tumorigenesis are associated with transcriptional activation of c-*fos*, c-*jun* and c-*myc* proto-oncogenes: role of cellular calcium and reactive oxygen species. *Toxicol Sci* 2001; **61**: 295–303.
29 Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. *Lab Invest* 1983; **49**: 327–33.
30 Taylor AJ, Farb AA, Angello DA, Burwell LR, Virmani R. Proliferative activity in coronary atherectomy tissue. Clinical, histopathologic and immunohistochemical correlates. *Chest* 1995; **108**: 815–20.
31 Resnick N, Collins T, Atkinson W, Bonthrom DT, Dewey CF, Gimbrone MA. Platelet-derived growth factor B chain promoter contains a *cis*-acting shear-stress-responsive element. *Proc Natl Acad Sci USA* 1993; **15**: 4591–5.