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Inflammatory cells, apoptosis and *Chlamydia pneumoniae* infection in atherosclerosis

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Abstract

Chlamydia pneumoniae (CP), chromosomal alterations and apoptosis were suggested as contributing factors in the pathogenesis of atherosclerosis. Early (EP) and unstable plaques (UP) were studied in order to assess infiltrate composition, the apoptotic index, chromosome 7 stability and to investigate the concurrent presence of CP in EP and UP. Paraffin embedded sections of three iliac arteries and four aortas from young donors (EP), and four coronaries and nine carotid arteries (UP) were used. Aside from histological techniques, immunophenotypification for macrophages, T and B cells, smooth muscle and endothelial cells; FISH and DNA nick end labeling were performed. The amplifications with PCR for CP infection were negative in all specimens. In the EP, a focal myointimal thickening with foam cells and scarce smooth muscle cells was observed. Macrophages were most frequent in the intima (10.8%) while T and B cells were found in 2.3 and 1.5%. In the UP a thin cap covering a lipid-rich core with widespread vascularization and with severe luminal obstruction was observed. Macrophages were increased (21%), and T (1.5%) and B cells (3.5%) in the caps and inner areas of the lipid cores. At these sites, the FISH showed trisomy and tetrasomy of chromosome 7 and apoptosis was very frequent (10–30%). Macrophages in intimal lesions is one of the most prominent, consistent and permanent features in EP, and an elevated apoptotic index and chromosome 7 instability might contribute to evolution from stable to complicated plaques, while CP seems to play no role. However, further studies are needed with more cases to confirm this last observation. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Apoptosis; Chromosome 7; Atherosclerosis; *Chlamydia pneumoniae*; Smooth muscle cell; Macrophage

1. Introduction

Atherosclerosis is the main cause of heart attack, stroke and peripheral arterial insufficiency, and because of this accounts for the 50% of all mortality in occidental countries [1]. The atherosclerotic plaque results from an excessive inflammatory fibroproliferative process as a consequence of several forms of damage to endothelial and smooth muscle cells of elastic and large and medium sized muscular arteries [2,3]. A wide number of vasoregulatory molecules, growth factors and cytokines have been implicated in the disease [1]. Moreover, in recent years apoptosis and chromosomal alterations of macrophages and smooth muscle cells (SMC), have been indicated as important contributors. In particular, some infectious agents, such as *Chlamydia pneumoniae*, have been recently implicated in this process [4].

Apoptosis is a vital aspect of normal cell development and dysregulated apoptosis may have an important role in the pathogenesis and progression of

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atherosclerosis [5,6]. Also, a genetic basis for atherosclerosis disease is now recognized [5]. In this connection, our previous observations on unstable atherosclerotic plaques supported the 'monoclonal hypothesis', equating the atherosclerotic plaques to benign tumours [7]. We demonstrated that in unstable plaques the trisomy 7 was the most constant finding, suggesting an increase in platelet-derived growth factor (PDGF) synthesis and, as a consequence, an increase of SMC proliferation [7].

However, the process by which a fibro-fatty plaque evolves into a fibrotic lesion or in an unstable, lipid-rich plaque is poorly understood. It has been suggested that it involves cell deletion, proliferation and migration, and extracellular matrix synthesis and proteolysis [8]. These complex processes are regulated by a wide variety of factors that modulate the growth of vascular SMC and the production of mediators of remodeling of the extracellular compartments of arteries [8,9].

The aims of this study were: (a) to identify, by the use of monoclonal antibodies, the inflammatory and vascular cells present in human early and unstable atherosclerotic plaques, (b) to characterize chromosome 7 instability, (c) and the apoptotic index in the inflammatory infiltrate, particularly in SMC and macrophages, and (d) to investigate the presence of *C. pneumoniae* in atheroma lesions by PCR.

2. Material and methods

Early lesions, expressed as myointimal thickening, were studied in five epicardiac coronary arteries, obtained from infants of 13–18 months of age and seven cases (four abdominal aortas and three iliac arteries) obtained from young donors. All these individuals died in transit accidents.

The unstable plaques were studied from four coronaries obtained from autopsy of patients with myocardial infarction and nine carotids obtained by endarterectomy. Specimens were fixed in 10% buffered formalin, and slowly decalcified in 2.5% nitric acid. The whole specimens were paraffin embedded and horizontally sliced into 1-mm segments. Paraffin blocks were serially cut at 3–5 μm thickness, and every 20th section stained with hematoxylin and eosin and Azan. The following 15 serial sections were mounted on poly-L-lysine-coated slides and used for immunostaining techniques, fluorescence in situ hybridization (FISH) and DNA nick end labeling (TUNEL method) in order to characterize the inflammatory cellular composition, to visualize genomic alterations, and to detect apoptotic cells respectively.

2.1. Immunohistochemistry

Immunophenotypification was performed for macrophages (CD68), T (CD45RO) and B cells (CD20), SMC (HHF35) and endothelial cells (CD31–CD34). The monoclonal antibodies were used at 1:100 dilution, revealed by the immunoperoxidase method (ABC complex) with light hematoxylin counterstaining.

Contiguous, non-overlapping microscopic fields were analyzed covering the whole tissue on the slide; each field was scored for positive nuclei and their location in intima or media.

2.2. DNA nick end labeling

Apoptotic cells were visualized by the TUNEL method (TdT-mediated dUTP-biotin nick end labeling), which identifies early DNA fragmentation in the nucleus on the basis of specific binding of terminal deoxynucleotidyl transferase (TdT) to 3′-OH ends of DNA. Deparaffinized sections were incubated in 20 μg/ml proteinase K (Sigma) for 15 min at room temperature (RT), and thereafter washed four times with deionized distilled water. After inactivating endogenous peroxidase by covering sections with 2% H_2O_2 for 5 min at RT, the specimens were rinsed twice with PBS. Each section was incubated with TdT, dUTP digoxigenin conjugated in TdT buffer in a humid chamber at 37°C for 60 min.

In the negative controls, distilled water was used instead of TdT. Transferring the slides to TB buffer for 30 min at 37°C finished the reaction.

An antidigoxigenin antibody conjugated with peroxidase, which generates a brown-colored product from the chromogen diaminobenzidine, detected the signal of TdT-mediated dUTP nick end labeling. Counterstaining was performed by immersing the slides in 1% methyl green in 0.1 M sodium acetate solution (pH 4) for 10 min at RT. Slides were washed

in deionized water, 100% butanol and finally, in xylene. Cell counting was performed under a light microscope. Cells TUNEL+ showed dark brown nuclei. Sections were first examined at ×100, allowing estimation of the percentage of apoptotic cells present. Afterwards, 10 random fields per section from these sections were examined at ×500 in order to calculate the apoptotic index.

$$\text{Apoptotic index} = (\text{number of tunel positive cell nuclei} / \text{number of total cell nuclei}) \times 100$$

2.3. Fluorescence in situ hybridization (FISH)

The α-satellite DNA probe specific for the centromeric region of chromosome 7 labeled with biotin (ONCOR, Gaithersburg, MD, USA) was used. Three- to 5-μm thick paraffin-embedded sections were deparaffinized by two changes of xylene for 10 min each. Followed by two changes of 100% ethanol for 5 min each. The pretreatment solution (ONCOR) preheated at 45°C was prepared before incubating the slides for 15 min. Slides were rinsed in 2×SSC and then protein digestion with a solution of proteinase K 0.25 mg/ml at 45°C for 15 min was performed. Slides were rinsed in 2×SSC for several seconds and dehydrated in 70, 80 and 95% ethanol for 1 min each and allowed to air dry.

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.

The probe and target DNA on the slide underwent denaturation on a 67±2°C hot plate for 5 min, and then overnight incubation in a pre-warmed humidified chamber at 37°C.

The hybridized signals were detected by using a commercial kit (FITC-avidin detection kit, ONCOR). Propidium iodide 2.5 μg/ml in antifade was used for counterstaining.

The α-satellite DNA probe specific for the centromeric region of chromosome 3, that is not involved in atherosclerotic progression, was also used to ensure that these cells have two FISH-positive signals in each nuclei.

For scoring, a Leitz Orthoplan with Ploemopak incident-light fluorescence microscope, equipped with ×40, ×100 and oil immersion objectives and ultraviolet excitation filter sets were used.

Only interphase cell nuclei with intact morphology were scored, and only cell nuclei with three or more hybridization spots were considered.

The FISH method was also applied to five fragments of the walls of normal arteries, which were used as controls.

2.4. Polymerase chain reaction

For *C. pneumoniae* DNA detection the same protocol reported elsewhere was followed [10]. Only slight modifications were performed: samples were amplified for 35 cycles instead of 40 cycles, a nested PCR was used, where the layer fragment produced by the first round of PCR (where HL-1 and HR-1 primers were employed) was used as the template for the second PCR. Of each sample, 2 μl of amplification product were amplified with HM-1 and HR-1 primers. The DNA was extracted from paraffin-embedded and fresh samples incubated with proteinase K and then purified by phenol/chloroform extraction and ethanol precipitation. Amplifications were performed in a 50 μl volume and the products were separated on 1.5% agarose gel. With the nested PCR an intense band of 229 bp was obtained in the control infected cells. Positive and negative controls were employed, and sequences of β-globin served as internal control of the PCR.

3. Results

3.1. Early plaques

In the early plaques ($n=6$), a focal myointimal thickening with foam cells and scarce smooth muscle cells was observed. Macrophages were most frequent in the intima (10.8%) (Fig. 1) while T and B cells (Figs. 2 and 3) were found in 2.3 and 1.5%, respectively (Table 1). SMC were scarce, showing normal ploidy. Accordingly, normal arterial walls showed only cells with two signals per nucleus.

These plaques showed scarce or absent apoptosis (0.5±0.01%) as compared to unstable plaques ($P<0.01$).

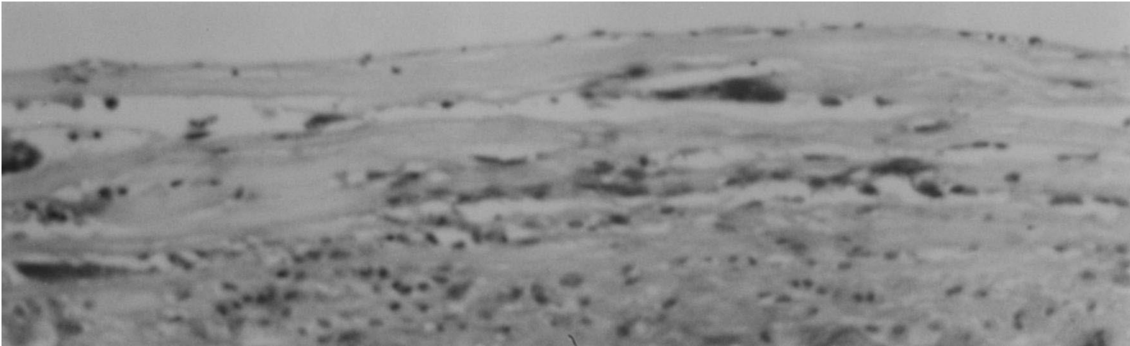

Fig. 1. Atherosclerotic early plaque: immunocytochemical detection of macrophages (CD68+) (×400).

3.2. Unstable plaques

In the unstable plaques ($n=14$), a thin cap covering a lipid-rich core with widespread vascularization and with severe luminal obstruction was observed. Because of the richness of the cellular and vascular components of the plaques, histological and immunohistochemical findings are described from the lumen to the media:

3.2.1. Fibrous cap

The continuous, undamaged row of endothelial cells stained with anti-VIII factor, anti-CD31 and anti-CD34 was interrupted in the site of plaque rupture. Inflammatory cells consisted of macrophages (CD68+), two-thirds of the total infiltration and one-third of T-lymphocytes and scarce B-lymphocytes. CD8+ cells predominated among T lymphocytes, whilst CD4+ cells were very sparse. SMC in different quantities were also observed. Macrophages were increased (21%) (Fig. 4) and T (1.5%) and B cells (3.5%) (Figs. 5 and 6) as compared to early plaques (Table 1).

Varying percentages of cells with three fluores-

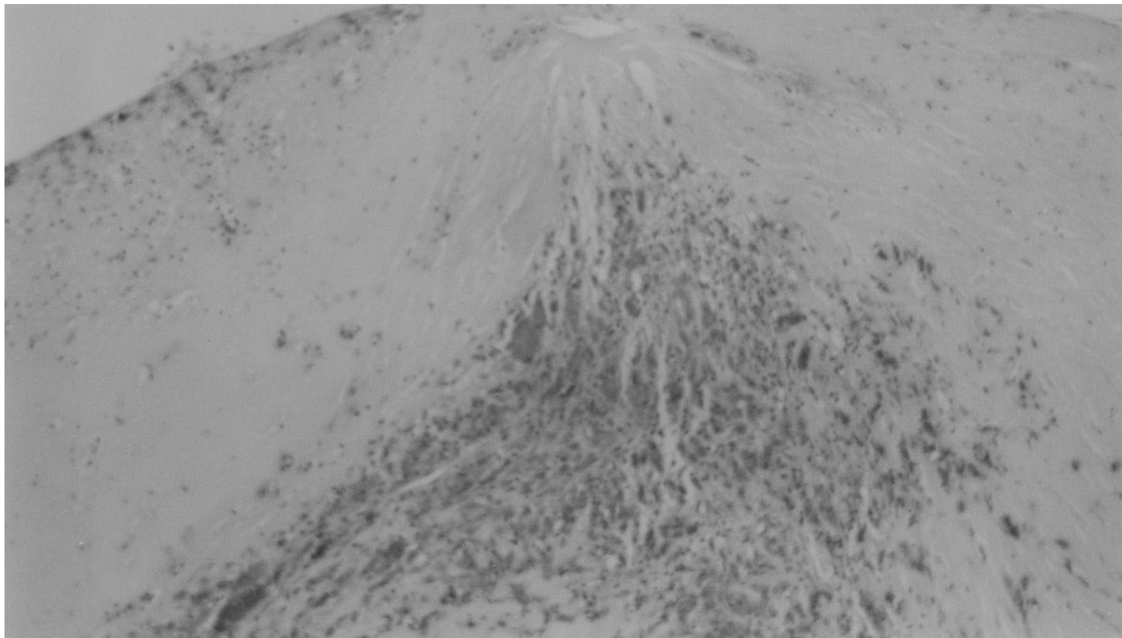

Fig. 2. Atherosclerotic early plaque: immunocytochemical detection of T-cells (CD45RO+) (×400).

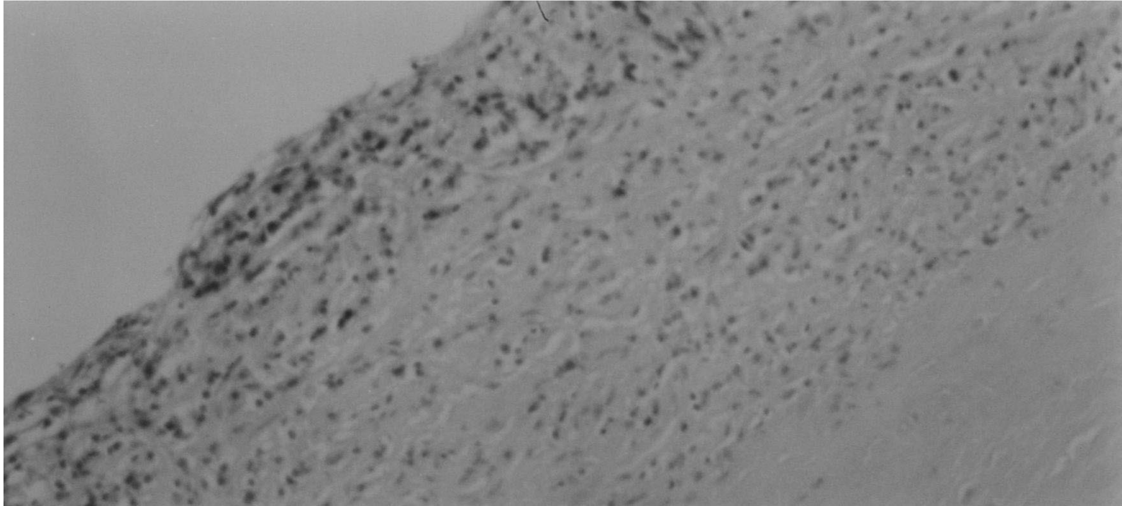

Fig. 3. Atherosclerotic unstable plaque: immunocytochemical detection of B-cells (CD20+) (×400).

Table 1

Cell types	Early plaques ($n=6$)		Unstable plaques ($n=14$)	
	Intima (%)	Media (%)	Caps (%)	Core (%)
Macrophages	10.8	0.8	21.0	4.50
T-lymphocytes	2.3	0	1.5	0.72
B-lymphocytes	1.5	0	3.5	0.60

cence signals indicative of trisomy of chromosome 7 were found in 80% in the regions of high cellularity (Fig. 7). Tetrasomy and/or polysomy of chromosome 7 could be found at a lower extent.

Apoptosis was very frequent (10.8±1%) in the fibrous caps and in the internal layers of the lipid cores, where an outstanding hyperplasia of SMC and macrophages was evident.

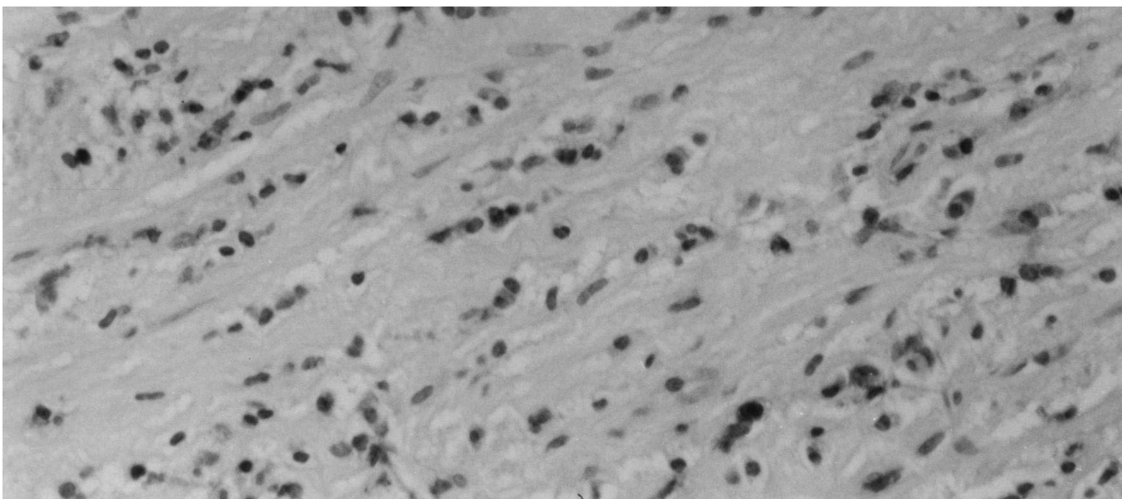

Fig. 4. Atherosclerotic unstable plaque: immunocytochemical detection of macrophages (CD68+) (×100).

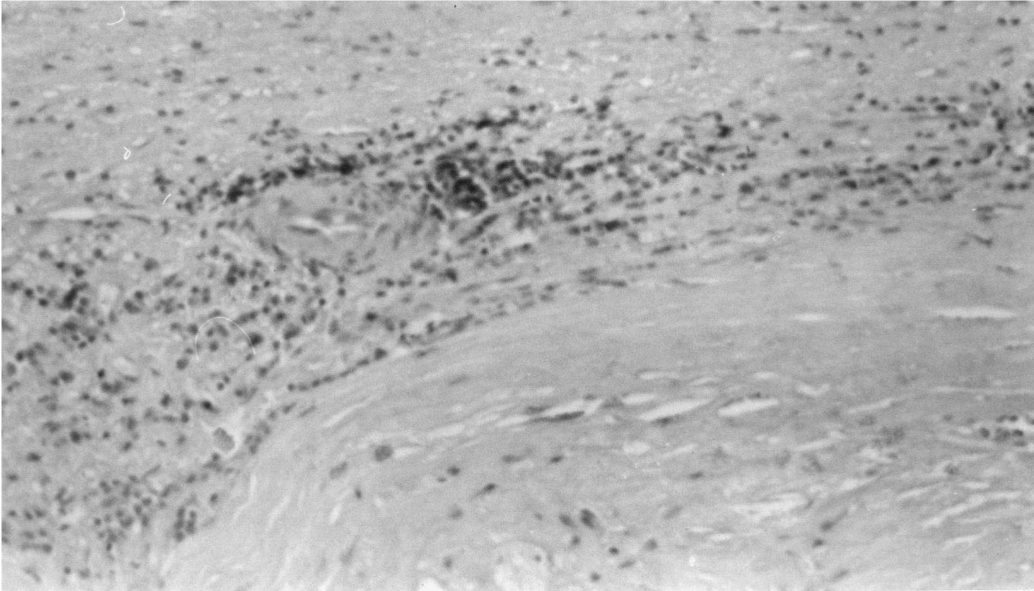

Fig. 5. Atherosclerotic unstable plaque: immunocytochemical detection of T-cells (CD45RO+) (×100).

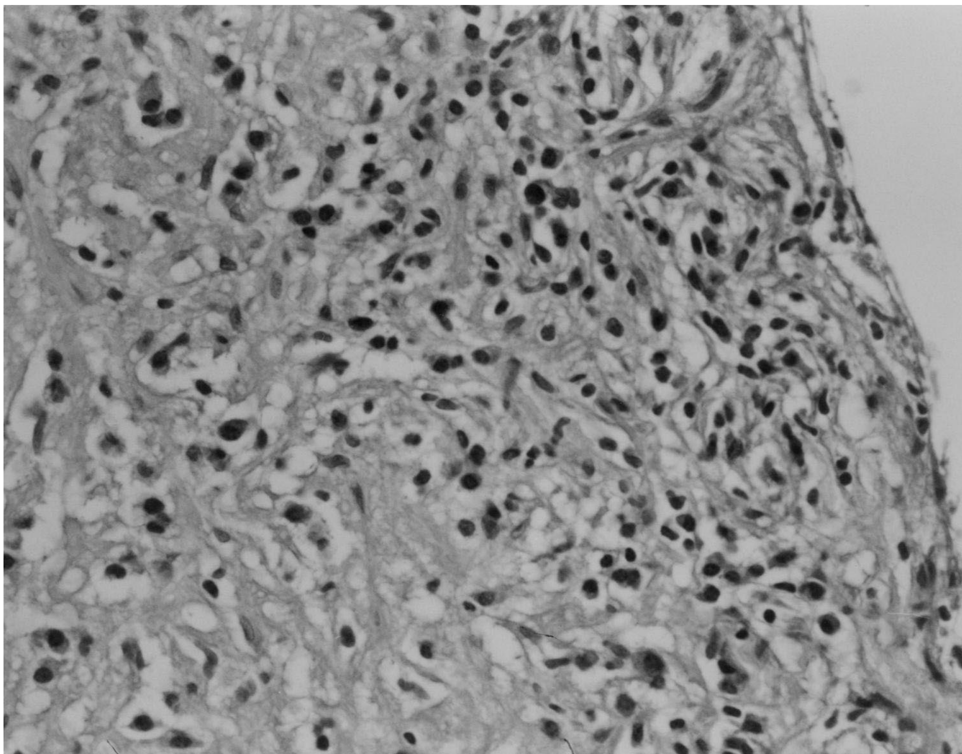

Fig. 6. Atherosclerotic early plaque: immunocytochemical detection of B-cells (CD20+) (×100).

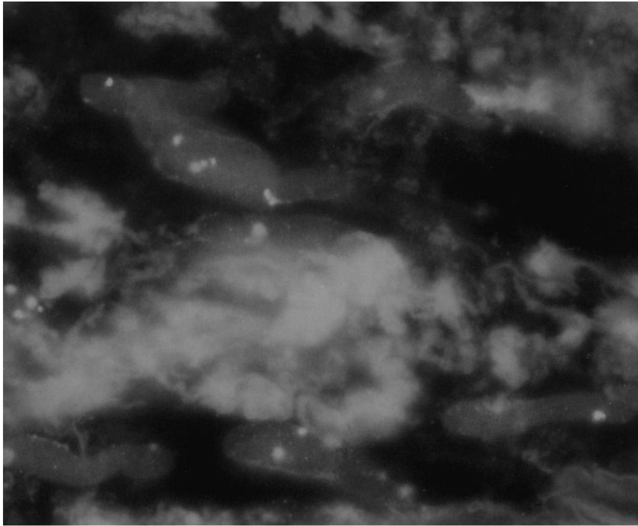

Fig. 7. Atherosclerotic unstable plaque: FISH detection of trisomy and polysomy of chromosome 7 (×1000).

Conversely, in distant 'stable plaques' only scarce cellular and vascular components were observed.

3.2.2. Lipid cores

Lipid cores were lipid rich and highly vascularized, with neoformed vessels stained with CD34 and CD3. These vessels differed from proliferated small, thin-walled blood vessels to bigger ones. Macrophages and T-lymphocytes were found to be in close contact to neoformed vessels. In the borders of the lipid cores numerous SMC could be observed migrating from media to intima showing alteration of the chromosome 7.

3.2.3. Deeper layers of the plaques

The base and the shoulders of the unstable plaques showed neoformed vessels, with thin or thick vessel walls, CD34+, surrounded by extensive mononuclear infiltrates (macrophages, and T-lymphocytes, mainly CD8+).

The amplifications with PCR for CP infection were negative in all specimens.

4. Discussion

In this paper early plaques and unstable plaques were studied in order to assess a significant difference in infiltrate composition, apoptotic index and to investigate the concurrent presence of *Chlamydia pneumoniae*.

The findings will be discussed in the context of the plaque type.

4.1. Early plaques

Early plaques showed moderate intimal thickening, foam cells, mononuclear infiltration and deposits of amorphous lipids and/or lipoproteins. In these lesions, SMCs were very low or absent.

The finding of a great number of monocytes and lymphocytes in early plaques suggests that these inflammatory cells are probably important and necessary contributors to the progression of atherosclerotic lesion formation [11], and that they may be responding to antigens that are expressed by cells within the intima.

Macrophages in intimal lesions are one of the most prominent, consistent and permanent features in early plaques. In fact, these cells are the main inflammatory mediators in the atheromatous plaque microenvironment.

The pathological accumulation of cells may be due to migration of macrophages and T lymphocytes [5]. It is now well established that artery injury triggers excessive cell migration and proliferation, and these events are considered to be a major cause of plaque lesions [8].

4.2. Unstable plaques

Extensive SMC proliferation is considered a hallmark of progression of atherosclerosis [1]. This proliferation is stimulated by different factors like cytokines and growth factors including transforming growth factor-β, PDGF and fibroblast growth factor produced by injured endothelial cells and macrophages or released from thrombus, which play an important role in the progression of the disease [12,13].

The increase in proliferative activity in the unstable plaques could be associated with a tendency to chromosome instability, as indicated by the numerical alterations found with the FISH technique in chromosome 7. Then, the activation and/or reactivation of the atherosclerotic process where the complications

could occur might be related to chromosome instability.

In this study, we found that immunophenotypified SMCs of unstable plaques showed chromosome 7 trisomy and tetrasomy, thus identifying a clonal expansion corresponding to hyperplastic SMC [14–16]. This is an interesting feature since it can be correlated with overexpression of PDGF coded in 'pter-7q22' [12,14]. Also the prevalence of a +7 genotype in plaque SMC involves the gene(s) located in this chromosome. Some of its products are PDGF, tropoelastin, AQP1/CHIP28, nitric oxide synthase and EGF receptor [17].

Moreover, the finding of a clonal expansion, which may be considered a biological characteristic of the lesion is the mechanism involved in benign tumor production. Some authors [14,18,19] suggested a similarity between these two entities. Conversely, stable plaques do not present these chromosomal abnormalities.

In the early plaques, as well as in the unstable plaques, the cellular reaction is similar. The differences could be found in the complexity of the latter, characterized by the reactivation of the atherosclerotic process in the arterial wall where the early lesions have occurred.

Different authors [5,7,20] reported involvement of these mechanisms in cardiovascular diseases, such as hypertension, vascular injury and progression of human atherosclerosis.

Normal tissues show a certain balance between cell growth and apoptosis. Alterations of this balance account for different lesions on which cell hyperplasia is present.

Cultured SMC and endothelial cells from atheroma plaques undergo apoptosis independently of the presence of proliferating factors in culture media, while their normal counterparts suffer apoptosis only when deprived of growth factors [21].

Also, removal of endothelium has been associated with SMC proliferation, due to the release of growth factors (PDGF and FGF) by platelets leading to SMC migration and proliferation [13,21]. PDGF influences vascular SMC migration, and together with bFGF and other factors, cell proliferation [21].

Geng et al. [22] showed that apoptosis of SMCs is induced by cytokines and triggered by activated macrophages.

Our finding of an apoptotic index between 10 and 30% is in agreement with previous reports of apoptotic indexes between 10 and 46% for human coronary and peripheral plaque tissue [5,23]. We found a high rate of apoptosis in cells immunophenotypified as SMCs and as macrophages in unstable plaques. Of note, stable plaques and normal artery walls did not show this feature.

Apoptosis is recognized as a mechanism of foam cell death in plaques. Cell death leads to the spill of lipids and, hence, the enlargement of the soft tissue core [24]. Cholesterol accumulated in the vessel wall activates T-cells, possibly through autoimmune responses to modified lipoproteins [25]. It was recently demonstrated that oxidized LDLs induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway [26,27].

Taking into account that apoptosis could directly influence aspects of arterial structure important for plaque stability [28–30], apoptosis may act as an important independent contributing factor of the pathological changes of the unstable plaques. They are the generation of hypocellular fibrotic regions of the plaque, formation of the cytogenic lipid core and interference with normal maintenance and repair of the extracellular matrix of the lesion.

4.3. Does a Chlamydia pneumoniae–atherosclerosis relationship exist?

The majority of serological studies have shown an association between *C. pneumoniae* and atherosclerosis. However, this is not true when strict criteria for chronic infection are used [31]. Moreover, association does not prove causality, and serological distinction between past and current infection is difficult. Herein is the relevance of *C. pneumoniae* detection from the atherosclerotic lesions. Preliminarily, some animal studies [32–35] and antibiotic trials [36,37] suggest that *C. pneumoniae* may exacerbate atherosclerosis.

But there is no conclusive evidence that *C. pneumoniae* causes atherosclerosis [31] (Table 2). Many of the studies listed have no appropriate controls, and the number of cases reporting a positive association between *C. pneumoniae* and atherosclerosis is similar to the number of cases in studies which found no association, when using strict criteria for chronic infection [31].

Table 2
Studies investigating *Chlamydia pneumoniae* presence in blood vessels by PCR or immunocytochemistry (ICC) (modified from Ref. [28])

Study	Cases/controls (tissue studied)	Positive cases for CP (%)	
		PCR	ICC
Kuo (Proc. Natl. Acad. Sci. USA 1995;92)	18/31 (coronary)	17/0	39/0
Maass (Angiology 1997;48)	61/39 (carotid)	15/0	
Petersen (Eur. J. Vasc. Endovasc. Surg. 1998;15)	40/40 (aorta)	35/5	
Grayston (Circulation 1995;92)	5/0 (carotid)	60	100
	56/6 (carotid)		57/0
Ong (J. Clin. Pathol. 1996;49)	32/6 (aorta)	44/50	38
Jackson (Am. J. Pathol. 1997; 150)	38/38 (vascular and non-vascular tissues)	16/0–8	24/5–11
Juvonen (J. Vasc. Surg. 1997;25)	12/9 (aorta)	100/0	100/0
Kuo (J. Vasc. Surg. 1997;26)	23/8 (vascular)	48/0	
Maass (JACC 1998;31)	70/17 (coronary and vascular)	30/0	
Wong (JACC 1998;31)	58/58 (coronary)	39/12–30	
Shor (S. Afr. Med. J. 1992;82)	10/5 (coronary)		71/0
Kuo (Arterioscler. Thromb. 1993;13)	20/4 (aorta)		30/0
Chiu (Circulation 1997;96)	76/20 (carotid and aorta)		71/0
Kuo (J. Infect. Dis. 1993;167)	36/0 (coronary)	43	42
Campbell (J. Infect. Dis. 1995;172)	37/0 (coronary)	32	45
Ramírez (Ann. Intern. Med. 1996;125)	12/0 (coronary)	41.7	41.7
Jackson (J. Infect. Dis. 1997;176)	25/0 (carotid)	24	50
Blasi (J. Clin. Microbiol. 1996;34)	51/0 (aorta)	51	
Weiss (J. Infect. Dis. 1996;173)	72/0 (coronary)	2	
Davidson (Circulation 1998;98)	60/0 (coronary)	23.3	33.3
Bauriedel (JACC 1998;31)	32/0 (carotid, coronary)		47
Lindholt (Eur. J. Vasc. Endovasc. Surg. 1998;15)	20/0 (aorta)	0	
Paterson (Pathology 1998;30)	30/0 (carotid, coronary)	0	
Saldeen (JACC 1998;31)	60/0 (coronary)		62
Burke (JACC 1999:876-5	81/0 (coronary, carotid)	0	
Daus (Cardiology 1998;90)	29/0 (coronary)	0	
Yamashita (Stroke 1998;29)	20/0 (carotid)		55

However, Davidson et al. [38] identified that different pathological grades of coronary plaques correlated with positive identification (by immunocytochemistry and PCR) of *C. pneumoniae* within plaque and with serological markers of infection. This retrospective study is of particular interest as it was performed on previously stored (8.8 years premortem) serum specimens and autopsy tissues from 60 Alaska Natives which have a lower mortality rate from coronary heart disease compared with whites. Thus, they had paired samples in a 'closed population'. They identified *C. pneumoniae* with PCR or immunocytochemistry staining in coronary arteries of 37% of all subjects, the latter method being more often positive than PCR (91 vs. 64%, respectively) [37].

Sessa et al. [39] demonstrated that high titers of *C. pneumoniae* antibodies are associated with elevated triglyceride and lowered HDL cholesterol levels. These organisms may favor atherosclerosis by changes in the antithrombotic properties of the endothelium, induction of cytokines with direct penetration into the arterial wall and effects on lipid metabolism [39].

In this study, it was found that young infants exhibited PCR-negative stable plaques. The fact that those early plaques were negative to *C. pneumoniae*, could be explained by the fact that they belonged to children who may not have suffered a primary infection by the time of their deaths. Also, one may speculate that these are still stable plaques not complicated by a primary infection, or still it could be said that *C. pneumoniae* has no association with atherosclerosis.

Bearing in mind that Davidson et al. [38] found a 64% of positiveness by PCR, our small sample size should contribute to the PCR-negative results.

We must also take into account that at present, the evidence of an association of *C. pneumoniae* and atherosclerosis does not constitute causation [38].

It is also possible that the inflammatory changes and apoptotic processes noted in unstable lesions maybe secondary (at least in part) to a 'hit and run' phenomenon with infective agents, i.e., original infection provoking cellular changes and damage, but these microorganisms no longer persisting (or being detectable). Deep-sited infections and presence of inhibitors may also contribute to the negative PCR results.

5. Conclusion

The therapeutic goals are now being focussed on the reduction of plaque progression and preventing plaque instability and rupture. The molecular understanding of these processes related to cell migration, proliferation and death, opens new strategies for the preventive treatment of these lesions. Interruption of growth factor activity or other regulative mechanisms further downstream may prevent the onset and progression, including the development of further complications, and may even induce plaque regression [40].

So far, pathological findings suggest that plaque evolution is a complex process in which gene alterations, immune-mediated cell changes, alterations of growth and proliferation pathways are involved, due to different factors.

Regarding *C. pneumoniae*, further studies are needed with more cases to evaluate its real role in the atherosclerotic process.

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Editorial comment

Apoptosis, infection and atherosclerosis: partners in crime?

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Exactly what is going on in atherosclerosis? The composition and cellular 'activity' of atherosclerotic lesions continue to bewilder and previously unrecognised pathological features continue to emerge. What, for example, are we to make of the recent demonstration of apoptosis — genetically programmed death — within atheromatous plaques?

When a cell undergoes apoptosis, it shrinks, its chromatin condenses, the nucleus fragments and the cell disintegrates into small bodies [1]. These apop-

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