

Biphasic pattern of cell turnover characterizes the progression from fatty streaks to ruptured human atherosclerotic plaques

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Abstract

Objective: To study the amount and phenotype of DNA-synthesizing and apoptotic cells during atherogenesis. **Methods:** Atherosclerotic lesions ($n=76$), obtained at autopsy ($N=6$) or during vascular surgery ($N=8$), were classified [type I–VI; American Heart Association (AHA) classification], immunolabeled with MIB 1 or the TUNEL technique and double stained with cell-type-specific antibodies. Subsequently, the labeled fractions were quantified. **Results:** In type II–VI lesions, intimal DNA synthesis was increased compared to that of the non-diseased (ND) arterial wall. DNA synthesis peaked in early type II lesions (2.7 ± 0.5 vs. $0.02\pm 0.02\%$ in ND; $p<0.05$), and declined to $0.7\pm 0.2\%$ in type V lesions ($p<0.05$). Interestingly, a second peak of DNA synthesis of $1.7\pm 0.1\%$, was observed in type VI (ruptured plaque) lesions. Double staining revealed that DNA synthesis was mostly confined to the macrophage-derived foam cell (51.9%). In type II lesions, 100.0% of all DNA-synthesizing cells were present in the intimal foam cell-rich area, while in advanced type III, IV and V lesions, DNA synthesis had shifted to the shoulder region (74.8, 78.5 and 68.1%, respectively). In type VI lesions, DNA synthesis was present in the area underlying the plaque rupture (52.7%). Apoptosis was only elevated in advanced type IV, V and VI lesions (0.8 ± 0.1 , 0.8 ± 0.1 and $1.1\pm 0.1\%$, respectively, vs. $0.0\pm 0.0\%$ in ND) and was predominant in the lipid core (90.5% in type IV lesions; 54.2% in type V lesions) or equally divided between the lipid core and the region underlying the plaque rupture (31.8 and 34.6% in type VI lesions). In type III–VI lesions, 50.0, 38.9, 42.6 and 42.8% of the TUNEL-positive cells were macrophages. **Conclusions:** In stable atherosclerotic lesions, DNA synthesis is an early event, while apoptosis is a late event. Ruptured plaques show a second peak of cell turnover. Lastly, cell turnover is mostly confined to the macrophage-derived foam cell. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Apoptosis; Proliferation; Macrophage; Smooth muscle

1. Introduction

From previous studies, it is known that both DNA synthesis and apoptosis are present in human atherosclerotic lesions [1–7], and may play an important role in lesion development and in the conversion from stable to unstable plaques [5,6,8,9].

The data published so far show that DNA synthesis in

primary human atherosclerotic lesions is low (0–3.5%) [1,5,7,10,11]. This low level of DNA synthesis might be expected in lesions that take many years to become clinically manifest. Topographical analysis in ruptured plaques reveals that DNA synthesis is tightly correlated with areas of structural repair and foam cell infiltration [5]. Cell phenotypes exhibiting DNA synthesis in atherosclerotic lesions are either the macrophage-derived foam cell [1,5,11] or the vascular smooth muscle cell [10].

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Enhanced rates of apoptotic cell death are also found in human atherosclerotic lesions [3,4,6,12,13]. Apoptotic labeling indices of 0–40% have been reported and apoptotic nuclei are either mostly confined to macrophage-derived foam cells in the lipid core and in regions adjacent to the lipid core [3,4,12] or to vascular smooth muscle cells in the fibrous cap [8,9,14]. Enhanced rates of apoptosis in the lipid core and/or fibrous cap are postulated to be major risk factors for the transition from stable to unstable plaques [8,9,14].

There are several limitations to the majority of these human studies. First of all, they almost invariably focus on advanced lesion types and therefore may have missed an early peak in cell turnover. Second, most studies have used atherectomy specimens. In these small samples, the morphology of the atherosclerotic lesion has been disrupted and, therefore, the topographical localization of both DNA synthesis and apoptosis is hard to determine [15].

Since DNA synthesis and apoptosis are thought to be important features in atherogenesis, and since human data on cell turnover in all of the different stages of atherosclerotic lesion development, including ruptured plaques, are not available, we investigated the amount, phenotype and topographical localization of both DNA synthesis and apoptosis in type I–VI lesions.

2. Methods

2.1. Patients' characteristics and tissue preparation

In total, 76 atherosclerotic segments of non-branching parts of the descending aorta were obtained during autopsy (Department of Pathology, Academic hospital Maastricht) ($N=6$) or vascular surgery (Department of General Surgery, Academic hospital Maastricht) ($N=8$). Seven non-atherosclerotic segments of non-branching parts of the descending aorta of four of the six autopsy patients served as control tissue. All tissue samples were obtained at comparable hemodynamic locations. Autopsy specimens were obtained from three adult male patients (58.3 ± 1.8 years old, range 55–61 years) and three adult female patients (74.3 ± 11.2 years old, range 52–86 years). The causes of death of the autopsy patients were diverse (cardiovascular disease, cancer). Surgical specimens were obtained from six male patients (62.6 ± 1.8 years old, range 56–68 years) and two female patients (66.5 ± 0.5 years old, range 66–67 years). Patients undergoing vascular surgery all had symptoms of vascular occlusion. The investigation conforms with the principles outlined in the 'Declaration of Helsinki'.

Immediately after harvesting, the samples were fixed in 10% phosphate buffered formalin (pH 7.4) for 24 h and processed for paraffin embedding. Sections (4 μm) were cut and stained with haematoxylin and eosin. Only sections with an intact lesion morphology were selected. These

selected lesions were classified I–VI according to the recommendations of the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association [16].

2.2. Immunohistochemistry

2.2.1. DNA synthesis

DNA synthesis was detected using the antibody MIB 1 (Immunotech, France), targeting the Ki-67 antigen, which is expressed in all stages of the cell cycle except G_0 and the early phase of G_1 . The Ki-67 antigen is mostly upregulated in the G_2 and M phases. Therefore, Ki-67 expression only correlates with DNA synthesis and does not reflect DNA repair [17,18].

After dehydration and blocking of endogenous peroxidase activity with 0.3% H_2O_2 , the sections were placed in a 0.01 mol/l citrate buffer, pH 6.0, and boiled for 2×3 min in a microwave. After subsequent washings in H_2O and Tris-buffered saline (TBS), the slides were incubated with the MIB 1 monoclonal mouse antibody for 60 min at room temperature at a dilution of 1:50, followed by incubation with a biotinylated sheep anti-mouse IgG (1:250, Amersham, Life Science, UK) for 30 min at room temperature. Sections were then labeled for 30 min with an avidin–biotin peroxidase complex (Brunschiwig, USA). 3,3'-Diaminobenzidine (DAB) was used as the chromogen. The sections were counterstained with haematoxylin and mounted with coverslips.

2.2.2. Phenotyping of DNA-synthesizing cells

To assess the phenotype of DNA-synthesizing cells, parallel sections were double labeled with MIB 1 and cell-type-specific antibodies for macrophages (CD68), vascular smooth muscle cells (VSMCs) (α -smooth muscle actin), endothelial cells (CD34) and T-lymphocytes (CD3). After performing the MIB 1 staining as described above, sections were washed with H_2O and TBS and incubated with one of the antibodies [CD68: mouse monoclonal, 1:500, Dako, Denmark; α -smooth muscle actin (ASMA): 1:500, mouse monoclonal, Dako and CD3, 1:200, rabbit polyclonal, Dako) for 45 min at room temperature. Subsequently, sections were labeled for 30 min with a biotin-labeled sheep anti-mouse IgG (1:250, Amersham Life Science) (CD68 and ASMA) or sheep anti-rabbit IgG (1:1000, Dako) (CD3), followed by a 30-min incubation with an avidin–biotin alkaline phosphatase (1:200, Dako) complex. Fast blue (Sigma, St. Louis, MO, USA) was used as the chromogen. CD34 immunostaining (1:400; Becton Dickinson, CA, USA) preceded the MIB 1 staining. No counterstaining was performed and sections were mounted with immunomount (Shandon, PA, USA).

2.2.3. Apoptosis

For the detection of apoptosis, the TUNEL technique was used [6,19].

After dehydration, proteinase K was applied (Boehringer Mannheim, Germany; 20 $\mu\text{g}/\text{ml}$) for 15 min at room temperature. After washing, sections were placed for 2 min in triton X-100 (0.1%)/sodium citrate (0.1%) on ice, rinsed in phosphate-buffered saline (PBS, pH 7.2), incubated for 15 min in PBS containing 0.3% H_2O_2 , and incubated for 1 h in 3% citric acid (pH 7.2) at room temperature. After 10 min of equilibration, sections were incubated with enzyme mix (0.03 U/ μl TdT, 0.04 nmol/ μl dig-dUTP, 1.5 mM CoCl_2 ; Boehringer Mannheim) for 60 min at 37°C. After blocking the reaction in stop/wash buffer and rinsing in PBS, sections were incubated with peroxidase-labeled anti-digoxigenin (1:450, Boehringer Mannheim) for 30 min. DAB was used as the chromogen. The sections were counterstained with haematoxylin and mounted with coverslips.

2.2.4. Phenotyping of apoptotic cells

For the detection of the phenotype of apoptotic cells, double labeling with the TUNEL technique and antibodies against CD68, ASMA, CD34 and CD3 was performed on parallel sections. After the TUNEL technique, sections were incubated with one of the above-described antibodies for 45 min at room temperature. After washing in TBS, sections were labeled for 30 min with a biotin-labeled sheep anti-mouse IgG (1:250, Amersham Life Science; CD68 and CD34) or sheep anti-rabbit IgG (1:1000, Dako; CD3), followed by a 30-min incubation with an avidin–biotin alkaline phosphatase complex (1:200, Dako). The alkaline phosphatase substrate kit (Brunschwig, Netherlands) was used as the chromogen. ASMA immunolabeling preceded the TUNEL technique. No counterstaining was performed and sections were mounted with immunomount.

2.2.5. Cell counting

Tissue sections were investigated by light microscopy at $\times 400$ magnification using a standard field size. All intimal nuclei (± 600 –5000 nuclei) of the respective lesion type, and ten fields of the underlying media (± 400 nuclei) were counted. Cells containing nuclear MIB 1 staining were considered to be DNA-synthesizing cells. TUNEL-positive nuclei were only considered to be apoptotic nuclei when the cells also showed at least one of the morphological features of apoptotic cell death (cell shrinkage, aggregation of chromatin into dense masses and cell fragmentation) [20–22].

The intimal labeling index, defined as the total number of intimal-positive nuclei divided by the total nuclear intimal number, was calculated for the entire atherosclerotic lesion. The medial labeling index was calculated as the number of medial-positive nuclei divided by the total number of positive nuclei present in ten standard medial fields. The labeling index was calculated for both MIB 1- and TUNEL-stained sections. To obtain further insight into the distribution pattern of DNA-synthesizing and apoptotic cells, labeling indices were also determined

at different sites within the lesion: the endothelial coverage (ec), foam cell-rich area (fcr), shoulder region (sh), the lipid core and regions adjacent to the lipid core (lc), the fibrous cap (fc) and the area underlying the plaque rupture (rupt). Double labeling of parallel sections was used to assess the phenotype of DNA-synthesizing and apoptotic cells. To calculate the different fractions of phenotypes, the number of MIB 1-positive or TUNEL-positive nuclei, also positive for one of the phenotype-specific antibodies, was divided by the total number of MIB 1- or TUNEL-positive cells. With the above-described panel of antibodies (CD68, ASMA, CD34, CD3), the immunophenotype of 72.1% of the DNA-synthesizing and 59.9% of the apoptotic cells could be determined. All countings were performed by one investigator. The intra-observer variation was less than 10%.

3. Statistics

To test whether lesions of the same type from different patients could be pooled, a one-way ANOVA was performed. This was performed separately for MIB 1- and TUNEL-stained sections. Since no statistical significance was found ($p > 0.05$), lesions of one type, but from different patients, were assumed to be comparable.

A Mann-Whitney U test was used for comparisons between the lesion types. For comparisons within the lesion types, the Wilcoxon signed ranks test was used. In all tests, the level of statistical significance was assumed to be at $p < 0.05$. Data are expressed as mean \pm SEM.

4. Results

In total, 76 lesions ($n = 8$ –12 per lesion type) and seven non-atherosclerotic segments (controls) of the non-branching parts of the descending aorta were investigated.

4.1. DNA synthesis

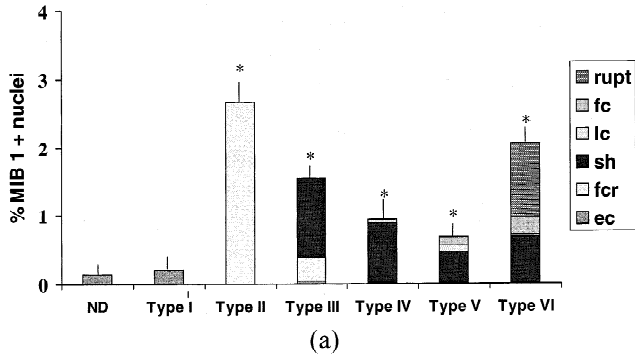
4.1.1. Media

Medial DNA synthesis in all lesion types was low ($0.06 \pm 0.03\%$), and not elevated in any of the lesion types compared to the non-diseased (ND) arterial wall ($p > 0.05$). However, a small peak, of $0.19 \pm 0.10\%$ (compared to $0.02 \pm 0.01\%$ ND), was observed in type I lesions ($p = 0.31$). Medial DNA synthesis was confined to the vascular smooth muscle cells (93.2%).

4.1.2. Intima

In all lesion types, except type I lesions, intimal DNA synthesis was significantly elevated (Fig. 1a), compared to the ND arterial wall. The highest MIB 1-labeling was found in type II (fatty streak) lesions (2.7 ± 0.5 vs. $0.02 \pm 0.02\%$ in the ND arterial wall; $p < 0.05$; Fig. 1a).

DNA-synthesis in human atherosclerotic lesions



Phenotype of DNA-synthesizing cells

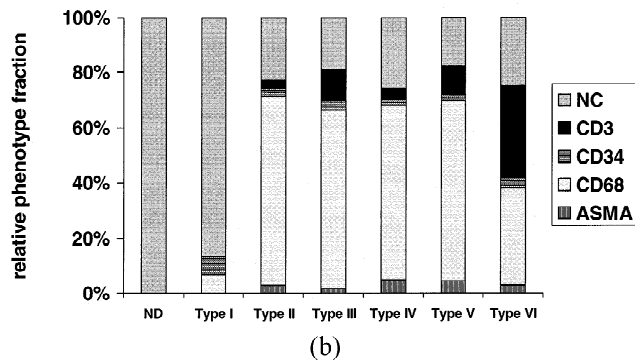


Fig. 1. (A) Level and localisation of DNA synthesis in human atherosclerotic lesions. Abbreviations: ec=endothelial coverage; fcr=foam cell-rich area; sh=shoulder region; lc=lipid core; fc=fibrous cap; rupt=rupture and ND=non-diseased arterial wall. $*=p<0.05$. (B) Phenotype of DNA-synthesizing cells in human atherosclerotic lesions. Abbreviations: ASMA= α smooth muscle actin; CD68=macrophages; CD34=endothelial cells; CD3=T-lymphocytes and NC=not classified.

With progression of lesion severity, DNA synthesis decreased to $0.7\pm 0.2\%$ in advanced type V lesions ($p<0.05$). Interestingly, a second peak of DNA synthesis of $1.7\pm 0.1\%$, was observed in ruptured plaques (type VI lesions).

Not only the level, but also the site of DNA synthesis changed with progression of the lesion (Fig. 1a). In the early type II lesions, 100.0% of all DNA-synthesizing cells were found in the foam cell-rich intimal area. With the development of a shoulder region in type III and IV lesions, DNA synthesis shifted to that region (74.8 and 83.9%, respectively; $p<0.05$). In type V lesions, the majority of DNA-synthesizing cells (68.1%) was still present in the shoulder region, although the number had significantly decreased compared to that found in type III and IV lesions. This decrease is probably due to a shift of DNA synthesis from the shoulder region to the fibrous cap in which 20.3% of all DNA-synthesizing cells was located.

Type VI lesions showed a different pattern. The majority of DNA-synthesizing cells (52.7%) in the ruptured plaques was found in the area underlying the rupture, while only 33.7% of all DNA-synthesizing cells was located in the shoulder region and 15.1% in the fibrous cap. Interestingly, DNA synthesis was low in the lipid core of type IV, V and VI lesions (5.4, 11.6 and 1.8% of all DNA-synthesizing cells, respectively).

Double staining of all lesions revealed that $51.9\pm 4.3\%$ of the DNA-synthesizing cells were macrophage-derived foam cells, $3.0\pm 0.9\%$ were vascular smooth muscle cells, $3.2\pm 1.0\%$ were endothelial cells and $12.7\pm 2.6\%$ were T-lymphocytes (Fig. 1b). In all stable lesion types (type II–V), the majority of DNA-synthesizing cells were macrophage-derived foam cells [68.6% (type II), 64.7% (type III), 63.6% (type IV) and 68.9% (type V)]. However, in ruptured plaques, the majority of DNA synthesis was almost equally divided between macrophages and T-lymphocytes (35.2 ± 3.6 and $33.3\pm 5.8\%$).

4.2. Apoptosis

4.2.1. Media

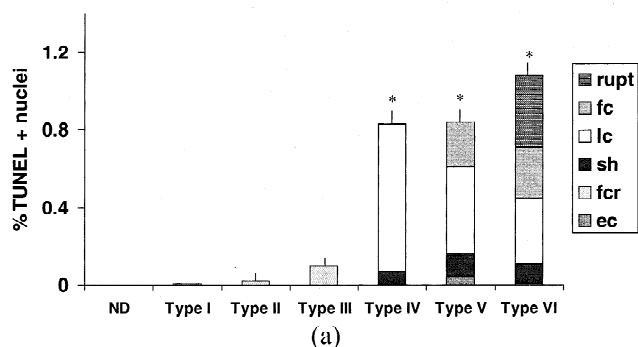
Medial apoptosis was a rare phenomenon. In fact, the medial apoptotic labeling fraction was low in all lesion types ($0.01\pm 0.01\%$) and did not increase during atherogenesis.

4.2.2. Intima

While intimal DNA synthesis was an early event, apoptosis predominantly occurred in the late stages of the disease (Fig. 2a). In type I, II and III lesions, the fraction of apoptotic cells in the intima was not different from that in the non-diseased arterial wall. However, the apoptotic labeling fraction was significantly elevated in advanced type IV, V and VI lesions ($0.8\pm 0.1\%$ in type IV, $0.8\pm 0.1\%$ in type V and $1.1\pm 0.1\%$ in type VI vs. $0.0\pm 0.0\%$ in ND). In type IV lesions, apoptosis was mostly confined to the lipid core (90.5% of all apoptotic cells; Fig. 2a). In type V lesions, the apoptotic labeling fraction in the lipid core significantly decreased to 54.2% ($p<0.05$ compared to type IV lesions), while 27.7% of all apoptotic cells were found in the fibrous cap. In ruptured plaques (type VI lesions), apoptosis was equally divided between the lipid core (31.8%), fibrous cap (24.3%) and the area underlying the rupture (34.6%).

Double staining failed to identify the phenotype of 41.8% of the apoptotic nuclei. It showed however, that $37.8\pm 4.4\%$ of all apoptotic nuclei were macrophages, $17.0\pm 3.3\%$ were vascular smooth muscle cells, $1.6\pm 0.8\%$ were endothelial cells and $1.8\pm 0.9\%$ were T-lymphocytes (Fig. 2b). In advanced lesion types (type III–VI), the majority of identified TUNEL-positive cells were macrophages (50.0, 38.9, 42.6 and 42.8% of all apoptotic cells, respectively). The vascular smooth muscle cells were another prominent TUNEL-positive cell type (23.6, 19.2

Apoptosis in human atherosclerotic lesions



Phenotype of apoptotic cells

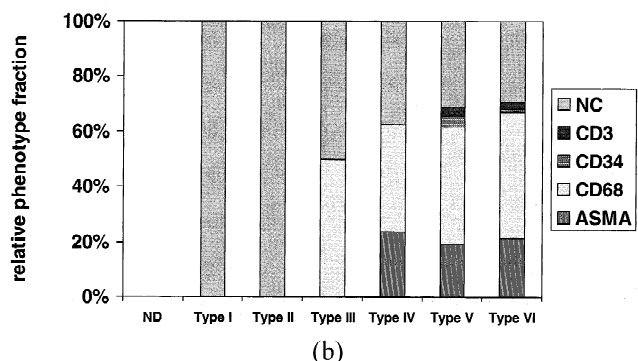


Fig. 2. (A) Level and localisation of apoptosis in human atherosclerotic lesions. Abbreviations: ec=endothelial coverage; fcr=foam cell-rich area; sh=shoulder region; lc=lipid core; fc=fibrous cap; rupt=rupture and ND=non-diseased arterial wall. $*=p<0.05$. (B) Phenotype of apoptotic cells in human atherosclerotic lesions. Abbreviations: ASMA= α smooth muscle actin; CD68=macrophages; CD34=endothelial cells; CD3=T-lymphocytes and NC=not classified.

and 20.1% of all apoptotic cells in type IV, V and VI lesions, respectively).

5. Discussion

This is the first study describing DNA synthesis and apoptosis during several stages of atherosclerosis in humans. The low, but significantly elevated levels of DNA synthesis that we found in advanced stable human atheromata are in accordance with other published data [1,5,7,10,11,23]. As described in the present study, the presence of a peak in fatty streaks (type II) is a remarkable feature of the pattern of DNA synthesis during atherogenesis in humans. This early peak is in accordance with the peak in DNA synthesis that has been observed in human *in vitro* studies [24] and in several hypercholesterolemic animal models [25–27]. Recently, Orekhov et al.

[7] also described ‘enhanced proliferation in lipid rich lesions’, while much lower levels of DNA synthesis were observed in fibrous lesions. A possible explanation for the occurrence of this early peak is the mitogenic effect of shortly oxidized low density lipoprotein (LDL) [28], which is present in macrophage-derived foam cells in these early lesions.

While DNA synthesis is an early phenomenon in atherogenesis, apoptosis shows the opposite. As our results show, apoptosis is confined to the advanced stages of atherosclerosis and is not elevated in early type I, II and III lesions. Apoptosis in the intima of advanced lesion types has already been reported previously, but its levels show remarkable variation (0–40%) [3,4,12–14,29]. The high levels of apoptosis might reflect non-specific staining of calcium-containing vesicles [30], the detection of both apoptosis and oncosis [14], and staining of RNA-synthesizing nuclei [22] by the TUNEL technique. In the present study, we therefore applied very strict criteria for the TUNEL method and the detection of apoptotic nuclei (pretreatment with citric acid, optimization of the enzyme concentration and apoptotic morphology) [22,30]. Independent from our study, Kockx et al. [6], using the very same criteria, found comparable levels of apoptosis in human lesions. They also reported that apoptosis was predominantly confined to advanced stages of the disease.

Most TUNEL-positive nuclei were located in macrophages and vascular smooth muscle cells, as demonstrated by double labeling with CD68 and α -smooth muscle actin. However, a significant fraction of TUNEL-positive cells could not be identified by the panel of antibodies used. This could reflect a loss of specific markers during apoptosis [3,4,6].

In a recent study, we investigated DNA synthesis and apoptosis in type II–V lesions in APOE*3 Leiden mice. Interestingly, in this atherosclerotic mouse model, we observed the same pattern of cell turnover during atherosclerotic lesion development as in humans [26]. In accordance with the data described here, APOE*3 Leiden mice also show a peak in DNA synthesis in early type II lesions and a decrease with lesion progression. Furthermore, apoptosis in atherosclerotic lesions in APOE*3 Leiden mice is also confined to advanced lesion types [26].

Our data show that both parameters of cell turnover are increased in ruptured plaques (type VI). The majority of both DNA synthesis and apoptosis in these lesions is present at the site of plaque rupture, and cell turnover was mostly confined to inflammatory cell types (macrophages and T-lymphocytes). Thus, cell turnover, and especially apoptosis, may play an important role in the transition from stable to unstable plaques. Morphological characteristics of atherosclerotic lesions prone to rupture are an extensive lipid core, loss of vascular smooth muscle cells of the fibrous cap, macrophage infiltration into the shoulder region of the fibrous cap and thinning of the fibrous cap [8,31]. These phenomena can, at least partially, be

explained by the different patterns of cell turnover we observed during atherogenesis.

In accordance with other publications, we found that the majority of apoptosis in human atheromata occurs in macrophage-derived foam cells in the lipid core and in regions adjacent to the lipid core [3,4,6,12,13,29]. Strongly oxidized LDL, which is abundantly present in the lipid core of atherosclerotic lesions, may be one of the factors that induce apoptosis in this region. Indeed, several *in vitro* studies have reported that strongly oxidized LDL is capable of inducing apoptosis in macrophages. Moreover, macrophages are able to oxidize LDL, which may induce self-inflicted apoptosis [28,32,33]. The loss of cellular mass due to apoptosis in the lipid core and adjacent regions may contribute to further expansion and softening of the lipid core and may make the lesion more prone to rupture [8,9].

The vascular smooth muscle cell is the other cell type that shows elevated levels of apoptosis in advanced atherosclerotic lesions. Elevated levels of apoptosis in vascular smooth muscle cells not only result in a decreased content of vascular smooth muscle cells in the fibrous cap, but also reduce the population of cells that are able to synthesize extracellular matrix proteins. Both of these factors contribute to thinning of the fibrous cap and, therefore, to the decreased stability of the atherosclerotic plaque [14,34,35].

The present study suggests that cell turnover mechanisms may provide targets for therapeutic interventions in atherosclerosis. First of all, inhibition of DNA synthesis may slow the progression of atherosclerosis. According to our findings, inhibitors of DNA synthesis may be most effective when applied early in the disease process (early DNA synthesis peak). Inhibition of DNA synthesis of advanced atherosclerotic lesions may even be undesirable, since it has been postulated that proliferation of vascular smooth muscle cells is part of a repair process and contributes to the maintenance of lesion stability [36,37].

A different approach could be to interfere with the apoptotic process. Stimulation of apoptosis in atherosclerotic lesions may decrease lesion size. However, extensive apoptosis may make the lesion more vulnerable to rupture. Thus, since there appears to be such a delicate balance between the cell turnover mechanisms in atherosclerotic lesions, the effect of interventions, affecting parameters of cell turnover, is rather unpredictable.

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