Incidence of intimal proliferation and apoptosis following balloon angioplasty in an atherosclerotic rabbit model

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Abstract

Objective: The aim of this study was to determine the occurrence of apoptosis in relation to the proliferative response in the intimal layer after experimental balloon angioplasty of a pre-existing plaque. Methods: After induction of an intimal plaque in the right carotid artery by electrical stimulation, 26 rabbits underwent balloon angioplasty. Twelve animals served as a control group without performance of angioplasty after plaque induction. To study the time course of intimal apoptosis and cell proliferation the vessels were excised on day 7, 14 and 28 after balloon angioplasty. For in situ detection of apoptosis, the TUNEL-technique (TdT-mediated d-UTP fluorescein nick end labeling) was used. In addition, bromodeoxyuridine labeling in all animals allowed the determination of the percentage of cells undergoing DNA synthesis in the neointimal area. Additionally, smooth muscle cells were detected by immunostaining of α-actin and macrophages by a specific antibody (RAM 11). Results: Within 28 days of balloon angioplasty, the number of cells undergoing apoptosis remained at a very low level and was not significantly different to the control group without interventional treatment (controls: 0.1±0.15%; 7 days: 0.44±0.68%; 14 days: 0.13±0.11%; 28 days: 0.1±0.1%). In contrast, the number of cells undergoing DNA synthesis was significantly increased at day 7 after angioplasty (3.72±2.0% vs. 0.51±0.29% in controls), resulting in an increase of the total intimal area from 0.088±0.037 mm² in the control animals up to 0.256±0.172 mm² at day 28 following balloon dilatation. Conclusions: Our data showed that significant changes in the occurrence of apoptosis are not involved in the regulation of cellular turnover during the examined time period after vessel wall injury. The lacking up-regulation of apoptosis in comparison to the increased cell proliferation in order to maintain the tissue balance is perhaps an important regulatory mechanism leading to intimal hyperplasia after vascular injury in this animal model. Overall, we suggest that there may be a delicate balance between cell proliferation and apoptosis in smooth muscle cells of the vessel wall, and only small shifts in this balance could account for both cellular accumulation in restenotic lesions as well as cell death in mature atheroma.

Keywords: Restenosis; Apoptosis; Angioplasty; Macrophages; Smooth muscle

1. Introduction

Proliferation and migration of smooth muscle cells have for a long time been emphasized in the development of atherosclerotic plaques as well as in the process of restenosis following angioplasty [1–3]. The pivotal role of proliferation in vascular disease has become even more evident with the development of balloon angioplasty [1,3,4,7]. After vascular injury excessive accumulation of cells can be observed with a subsequent intimal thickening causing restenotic lesion formation which is largely composed of smooth muscle cells having migrated and proliferated in response to wall injury [1,2].

Based on these observations, therapies for restenosis have sought to inhibit the proliferation of smooth muscle cells which is believed to be fundamental to the disease process [1,3].

However, studies of balloon injured rat carotid arteries by Clowes et al. [4] showed that the increase in the neointimal cell number is much lower as calculated on the...
level of ongoing cellular proliferation. In 1983, they proposed that cell death probably accounts for the lack of smooth muscle cell accumulation at later time points. Accordingly, there is evidence in experimental observations in animal models as well as in angiographic studies in humans that the process of restenosis often is limited after a certain time interval following angioplasty [5–8]. There is rather a tendency to regression and decrease in cell number in the ongoing process. Fibrosis also occurs as atherosclerosis progresses, often yielding a lesion containing dense extracellular matrix with a relatively low density of cells in advanced atheromatous plaques [9,10].

Cell death has been recognized as a part of the athrogenic process for a number of years [11–16]. Apoptosis, a form of cell death physiologically involved in tissue morphogenesis and homeostasis, has been suggested to play an important role in the pathogenesis of restenosis and atherosclerosis in addition to cell migration and proliferation [11,13,14]. Apoptosis features a distinct biochemical and morphological program [17,18]. It is a tightly regulated, active process induced by physiological stimuli that requires energy, de novo gene expression and protein synthesis. Unlike necrosis, apoptosis is a noninflammatory mechanism of cell elimination which involves cytoplasmatic shrinkage, maintenance of membrane integrity, DNA fragmentation and condensation, and formation of apoptotic bodies which are phagocytosed by adjacent cells.

Since injury is an important trigger for the development of vascular lesions, the balance of proliferation and apoptosis during the healing response and aberrations of this process may be important factors in lesion progression.

Recently, apoptosis has been detected in various atherosclerotic and restenotic lesions [12–16] reporting very different rates of apoptosis from <2% up to 50%. So far the influence of the balance between cell proliferation and apoptosis on the time course of the development of a restenotic lesion following balloon angioplasty still remains unclear.

The aim of the present study therefore was to determine the occurrence of apoptosis in relation to the proliferative response in the intimal layer following experimental balloon angioplasty in an established animal model of restenosis [3]. This is to our knowledge the first report on the time course of apoptosis after experimental balloon angioplasty of a pre-existing plaque.

2. Methods

2.1. Animal model

2.1.1. Plaque-induction in carotid arteries of rabbits

For the present study, fibromuscular plaques were produced in 38 male New Zealand White rabbits weighting 2.5±0.2 kg by using the electrostimulation method by Betz and Schlote as previously described in detail [3,19]. Briefly, this method is based on the implantation of two graphite-coated gold electrodes in the adventitia of the right carotid artery under general anaesthesia (15 mg ketamine and 0.8 mg xylazine/kg body weight intravenously in combination with inhalation anaesthesia using 3.5% Halothane). Subcutaneously placed leads from the electrodes (anode and cathode) were connected to a small plastic socket which was attached to the skull.

Constant-current DC impulses (15 ms/impulse, 0.1 mA, 10 Hz) were applied twice per day for 30 and 15 min with a time interval of 8 to 10 h between the stimulation cycles in each animal for a period of 28 days using an external stimulation unit. Long thin leads were connected to the skull socket so that the animals could move freely during the stimulation procedure. To produce atheromatous plaques by electrical stimulation, all animals additionally received a 0.2% cholesterol diet (Altromin, Lage, Germany). After 28 days of electrical stimulation, microscopic evaluation showed plaques with a maximum size located beneath the anode of the electrode (Fig. 1). The electrostimulation method is of distinct advantage because fibromuscular atherosclerotic plaques with comparable size under standardized conditions can be produced by maintaining the integrity of the endothelial lining. By this method, however, it is impossible to induce a >50% luminal stenosis.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Balloon angioplasty

After 28 days of electrical stimulation and 7 days of additional cholesterol feeding balloon angioplasty was performed in 26 rabbits at the site of the produced atherosclerotic plaque as described before [3]. The right carotid artery was again surgically prepared under general anaesthesia. The exposed vessel was temporarily ligated at least 3 cm distal to the implanted electrodes and a balloon catheter (2.5-mm diameter, Medtronic Minneapolis, USA) was introduced by direct arteriotomy under stereo-optic control. Dilatation of the preformed plaque was performed twice with 6 atmospheres for 30 s with a time interval of 30 s in between. After removal of the catheter, the small incision was closed with a 7-0 polypropylene suture under stereo-optic control and arterial blood flow was restored. None of the animals received therapy with heparin or drugs to inhibit platelet aggregation during or after intervention.

2.3. Study protocol

After 28 days of electrical stimulation, the animals were split into five different groups. Twelve rabbits served as
Fig. 1. Histological cross section of a rabbit carotid artery after 28 days of electrical stimulation. Intimal proliferation of smooth muscle cells resulted in a fibromuscular plaque with a maximum beneath the anode (Elastica-van-Gieson staining, original magnification 10×).

two control groups of six rabbits each. One group was histologically examined directly after the period of plaque development by electrical stimulation, the other group received the cholesterol diet for 7 more days and was then sacrificed and histologically examined.

In 26 rabbits balloon angioplasty was performed at the site of the produced atherosclerotic plaque using a 2.5-mm standard balloon catheter as described above.

To study the time course of intimal apoptosis, cell proliferation and accumulation of macrophages after balloon angioplasty, the animals were divided into three different groups (at least six rabbits each) and sacrificed 7, 14 and 28 days after balloon angioplasty (Table 1). The cholesterol diet was continued in all groups until the end of the experiment.

2.4. Tissue analysis

2.4.1. Bromodeoxyuridine labeling

To determine the extent of SMCs undergoing DNA synthesis of mitosis, bromodeoxyuridine (BrdU), a thymidine analogue, was given to each animal 18 and 12 h before excision of the vessels. As described previously [3] 100 mg/kg body wt. BrdU and 75 mg/kg body wt. deoxycytidine (both from Sigma, Deisenhofen, Germany) were given as subcutaneous neck depot 18 h before the

Table 1
Morphometric and immunohistological findings after balloon angioplasty

<table>
<thead>
<tr>
<th>Study group</th>
<th>Total intimal area (mm²)</th>
<th>Intimal cell number</th>
<th>Intimal cell density (cells/mm²)</th>
<th>Intimal apoptotic cells (%)</th>
<th>Intimal proliferating cells (%)</th>
<th>Intimal macrophages (%)</th>
<th>Serum cholesterol levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I</td>
<td>0.102±0.060</td>
<td>612±204</td>
<td>7734±4112</td>
<td>0.30±0.30</td>
<td>0.53±0.47</td>
<td>0.4±0.4</td>
<td>295±104</td>
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<tr>
<td>Control II</td>
<td>0.088±0.037</td>
<td>470±138</td>
<td>5761±2315</td>
<td>0.10±0.15</td>
<td>0.51±0.29</td>
<td>0.6±0.9</td>
<td>307±136</td>
</tr>
<tr>
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<tr>
<td>7 days after BAL</td>
<td>0.168±0.090</td>
<td>930±507b</td>
<td>5815±1529</td>
<td>0.44±0.68</td>
<td>3.72±2.0a</td>
<td>1.5±0.7</td>
<td>478±182</td>
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<tr>
<td>14 days after BAL</td>
<td>0.154±0.142</td>
<td>983±614</td>
<td>8990±4445</td>
<td>0.13±0.11</td>
<td>1.69±2.46</td>
<td>0.3±0.4</td>
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<tr>
<td>28 days after BAL</td>
<td>0.256±0.172c</td>
<td>1573±934b</td>
<td>5871±6715</td>
<td>0.10±0.10</td>
<td>0.20±0.18</td>
<td>1.9±3.0</td>
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</table>

Results are expressed as mean±S.D., BAL=balloon dilatation.

Statistically significant to the control group (P<0.05 Wilcoxon signed-rank test).
animals were killed. In addition 30 mg/kg body wt. BrdU and 25 mg/kg body wt. deoxycytidine were injected 18 and 12 h before perfusion fixation.

2.4.2. Tissue fixation and histological preparation

The rabbits were killed by CO₂ intoxication. All carotid arteries were perfused with a 0.1 M cacodylate-buffered 2% paraformaldehyde solution, then excised and immersed in paraformaldehyde for a maximum of 24 h. A sample of small intestine was excised to control the incorporation of BrdU into replicating cells.

After removing the implanted electrodes under stereoptical control, the dilated arterial segments were embedded in paraffin. The arterial segments below the PTFE cuff were used for histological analysis. The embedded vessels were cut into 4-μm thick cross-sections beginning at the distal end of the dilated region up to the maximal plaque size within the dilated area.

2.5. Morphometric analysis

For morphometric evaluation the sections were stained with standard Elastica van Gieson. The sections were then projected onto a digital image analyzer (Nikon/software from Bilaney Consulting, Düsseldorf, Germany) and the total intimal area as well as the maximal intimal thickness were calculated.

For determination of the neointimal cell density per area the sections were stained with standard hematoxylin and eosin. Cells were counted in the total intimal area and neointimal cell density was calculated in relation to the morphometrically determined total intimal area.

2.6. Immunohistochemistry

2.6.1. Quantification of proliferating neointimal cells

To determine the extent of cells undergoing DNA synthesis, BrdU was given to each animal 18 and 12 h before excision of the vessels as described above. A monoclonal antibody against BrdU (Bio Cell Consulting, Grellingen, Switzerland) allowed the identification of all proliferating cells, which had entered the S-phase during labeling period [20]. Using the avidin–biotin–peroxidase method, BrdU-labeled cells were visualized washed three times in PBS for 5 min. The sections were

18 h of the labeling period [20]. Using the avidin–biotin–peroxidase method, BrdU-labeled cells were visualized washed three times in PBS for 5 min. The sections were

identi®ed of all fragments with ¯uorescein-conjugated d-UTP. The end-

monoclonal antibody against BrdU (Bio Cell Consulting, the enzymatic labeling of the free 3

9

2.6.2. Quantification of macrophages

The rabbit macrophage-specific monoclonal antibody

RAM 11 [21] was used to identify macrophages in the

2.6.3. Identification of smooth muscle cells (SMCs)

To identify neointimal cells as SMCs, additional immunohistological staining (avidin–biotin method) with a monoclonal antibody against α-actin (Renner, Dannstadt, Germany) was performed. The α-actin isoform is known to be a highly specific marker for SMCs [22].

2.6.4. Identification of apoptosis in situ

For the detection of apoptotic cell death in situ, the specific labeling of nuclear DNA fragmentation first described by Gavrieli et al. [23], and modified by Gold et al. [24] was used. Briefly, the paraffin sections were deparaffinized by immersing the slides in xylene twice for 10 min and then rehydrated in 100, 96, 70 and 0% ethanol for 5 min each. After rehydration, the sections were incubated with proteinase K (20 μg/ml in 10 mM Tris–HCl, pH 7.4–8.0) for 15 min. This proteolytic digestion enzymatic incorporation of nucleotides. Slides were then washed in PBS twice for 5 min. To block endogenous peroxidase, the sections were incubated with 0.3% H₂O₂ in methanol for 15 min at room temperature. After washing, the slides were immersed in TdT (terminal deoxynucleotidyl transferase) buffer (Boehringer Mannheim). TdT and fluorescein-dUTP in TdT buffer (Boehringer Mannheim) were then added to cover the tissue sections and incubated in humid atmosphere at 37°C for 1 h. In principle, the enzyme terminal deoxynucleotidyl transferase, which is able to blunt 3’OH ends of double stranded DNA breaks independent of a template, catalyzes the enzymatic labeling of the free 3’-OH ends of the DNA fragments with fluorescein-conjugated d-UTP. The end-labeling method has also been called TUNEL (TdT-mediated X-dUTP nick end labeling). The slides were then washed three times in PBS for 5 min. The sections were covered with 2% aqueous solution of bovine serum albumin (BSA) for 10 min, rinsed in distilled H₂O and immersed in PBS for 5 min. Next, the sections were incubated with converter-AP [anti-fluorescein antibody conjugated with alkaline phosphatase (Boehringer Mannheim)] to detect the incorporated nucleotides in a humidified chamber for 30 min at 37°C, then washed in PBS and visualized by a substrate reaction with Fast Red (Boehringer Mannheim). Counterstaining was performed with hemalaune.

For each arterial specimen, at least five sections were examined. The cells with clear nuclear labeling were
defined as TUNEL-positive cells and and the apoptotic index was calculated as the percentage of TUNEL-positive cells in relation to the total number of cell nuclei in the intimal area.

2.6.5. Controls

In each experimental set-up negative controls as well as four different positive controls were included. As positive controls rabbit small intestine, human tonsils and human intestine, which are known to contain a lot of apoptotic cells, were used. Furthermore, one section of each tissue was processed as a positive control by pre-treatment with DNase I [1 μg/ml (Sigma)] for 10 min as described before [23] to induce DNA strand breaks. After extensive washing, slides were processed through the nick end labeling procedure as described above.

One negative control slide per tissue was incubated in the absence of the TdT enzyme.

2.6.6. Double-immunostaining procedures

To identify cell types undergoing apoptosis, double staining was performed by combining TUNEL and immunohistochemistry with antibodies against α-actin (SMCs) and RAM 11 (macrophages). After deparaffinization and rehydration, the slides were stained for detection of apoptotic cells by TUNEL and then immunostained as described above with the exception of using the chromogen diaminobenzidine [DAB (Boehringer Mannheim)] instead of AEC-staining complex for visualization, causing a black precipitate.

2.7. Statistical evaluation

Results are expressed as mean±S.D. The statistical significance of differences between control and balloon dilated carotid arteries was determined using the Wilcoxon matched-pairs signed-ranks test. Analysis of variance was used to determine the significance of differences comparing dilated arteries. Differences were considered to be significant at a value of \( P<0.05 \).

3. Results

3.1. Serum cholesterol levels

Within 28 days of feeding a 0.2% cholesterol diet, an increase of serum cholesterol concentration up to 295±104 mg/dl was found (control group I). A further 7 days of cholesterol feeding (control group II) showed no significant increase of the serum levels (307±136 mg/dl). Measurement of serum cholesterol levels in the intervention groups up to 28 days did not differ significantly from the controls (Table 1).

3.2. Morphological and morphometric results

After 28 days of electrical stimulation an intimal fibromuscular plaque with a maximum beneath the anode of the electrodes could be observed (Fig. 1). Morphometric quantification revealed a maximal intimal thickness of 128±92 μm and a total intimal area of 0.102±0.060 mm². The endothelial lining was intact. Staining of α-actin showed that plaques predominantly consist of SMCs with a stronger expression in the abluminal area. RAM 11-staining revealed 0.4±0.4% of macrophages. The mean neointimal cell density was 773±4112 cells/μm². After 7 more days of cholesterol feeding (control group II) no significant differences were found. For further statistical evaluation served control group II.

After balloon angioplasty a continuous increase of the intimal area was observed from 0.168±0.090 mm²; at day 7 to 0.256±0.172 mm²; \( (P<0.05) \) at day 28 after intervention (Fig. 2A). The total neointimal cell number had accordingly risen from 470±139 in the control group to 1573±943 \( (P<0.05) \) 28 days after balloon angioplasty whereas the neointimal cell density showed no significant differences (Fig. 2B). Elastica-van-Giessen staining showed a mainly intact internal elastic lamina (IEL) with only minor disruptions in nine of twenty-four rabbits. Interestingly, a bilayered configuration of the intima could be observed with a possible distinction between a luminal layer potentially caused by balloon dilatation with a mainly circular orientation of the SMCs around the lumen and a deep (abluminal) layer with more longitudinal orientation of the cells which represents the original plaque produced by electrical stimulation (Fig. 3). Seven days after angioplasty the abluminal layer of the plaque consisted mainly of cells immunoreactive for α-actin in contrast to the luminal part where many cells had a decreased α-SMC actin content (Fig. 3). After 28 days more than 80% of the neointimal cells were found to be α-actin positive. Immunohistological staining of macrophages in the balloon-treated animals revealed an increase in the number of intimal macrophages up to 1.85±2.9% 28 days after balloon angioplasty, which was not significant in comparison to the control group.

By observation of the macrophages we mainly localized them in the abluminal area of the plaques in the intervention groups as well as in the control groups.

3.3. Quantification of neointimal cell proliferation and apoptosis

In control animals without angioplasty determination of cells undergoing DNA synthesis by BrdU-labeling showed 0.5±0.3% in control group II (0.8±0.3% in control group I/not significant). Apoptotic cell nuclei detected by TUNEL were present in 0.1±0.2% in control group II (0.3±0.3% in control group I).

Seven days after balloon angioplasty quantification of
intimal cell proliferation showed a significantly increased proliferative activity (3.72±2.0%, \( P<0.05 \)) (Fig. 4). Already 14 days after intervention intimal cell proliferation was not significantly increased (1.7±2.5%) and returned to the level of the control group at day 28 post intervention (0.2±0.18%) (Fig. 2C). Differentiation of the intima in a luminal and abluminal area showed that about 70% of the proliferating cells were located in the luminal region of the intima.

The number of TUNEL-positive cells was less than 1%
of the total neointimal cells in all the specimens examined. No significant difference was found in the number of apoptotic cells detected by TUNEL in the three intervention groups and the control group. The frequency of TUNEL positive cells was highest at day 7 after dilatation (0.44±0.68%) (Figs. 2C, 5 and 6).

The number of apoptotic nuclei was on a very low level in both the luminal and the abluminal layer of the plaques.

Fig. 3. Right carotid artery, 7 days following balloon angioplasty (original magnification 10×), immunohistological staining of α-actin (avidin–biotin) for smooth muscle cells combined with hemalaune. Interestingly, the plaque shows a distinction between a luminal layer potentially caused by balloon dilatation with a mainly circular orientation of the smooth muscle cells around the lumen and an abluminal (deep) layer with more longitudinal orientation of the cells which represents the original plaque produced by electrical stimulation.

Fig. 4. Cross-section of carotid artery 7 days after balloon angioplasty. Avidin–biotin staining of bromodeoxyuridine shows some labeled cells in the intimal proliferate mainly in the luminal part (original magnification ×40).
Fig. 5. Serial cross-section of a rabbit carotid artery 7 days after balloon dilatation using the double-staining method, a combination of TUNEL (apoptosis) and immunohistochemistry for α-actin (SMCs). Using the avidin–biotin peroxidase method, SMCs were visualized with the DAB substrate (black dye). One cell was stained positively with TUNEL (red dye) which also expresses α-actin, suggesting an apoptotic SMC (original magnification ×60).

The percentages of labeled nuclei in the abluminal layer were not significantly different from the percentages in the superficial regions.

In double immunostaining, TUNEL positive cells showed a simultaneous α-SMC actin expression in about 50% (Fig. 5), but only about 5% showed a simultaneous RAM 11 expression for macrophages. The origin of the rest of the TUNEL-positive cell could not be determined. There was also no correlation between the occurrence of intimal macrophages and apoptotic cells.

Fig. 6. Photomicrograph of a cross-section of a carotid artery 14 days following balloon angioplasty (original magnification ×40). In situ detection of apoptotic cells (red dye) by TUNEL assay using AP substrate Fast Red, counterstained with hemalaune.
4. Discussion

Apoptosis has been examined in various atherosclerotic lesions including human atherectomy specimens from primary and restenotic lesions [13–16,25], injured rat carotid arteries and aorta [16,26,27], cholesterol-fed rabbits [12] and during vascular development [28]. However, the reported percentages of apoptotic cells present in atherosclerotic plaques and restenotic lesions showed remarkable differences, and the role of apoptosis especially in the process of restenosis after angioplasty is so far not well defined. In the present study the occurrence of apoptosis in relation to the proliferative response in the intimal layer following experimental balloon angioplasty of a pre-existing plaque in the rabbit carotid artery has been determined. Using the TUNEL assay for detecting internucleosomal DNA fragmentation as a marker for apoptosis, we found a very low number of apoptotic cells — not exceeding 1% of the total neointimal cell number in the majority of specimens examined — without any significant change of the apoptosis rate in the different groups. Taking into consideration that apoptosis is a physiological process that maintains the tissue balance, the lacking up-regulation of apoptosis in comparison to the increased cell proliferation potentially represents a pathophysiological phenomenon that gives a possible explanation for the continuous increase of the neointimal cell number, and subsequently of the intimal area after vascular injury in this animal model. By day 28, a homeostasis between cell proliferation and apoptosis seemed to be reached, possibly resulting in a growth retardation of the restenotic lesion. In later stages, which were not examined in this study, a decrease of cell density has been reported due to both an increased production of extracellular matrix as well as an absolute cell loss [2,7,10]. SMC loss by apoptosis could be a possible explanation for the transformation of a smooth muscle rich myointimal thickening towards a fibrous, cell-poor intimal thickening. Previous reports emphasized that even a small proportion of apoptotic cells can represent a considerable magnitude of cell loss [29], especially with regard to the limited (maybe shorter) appearance of apoptotic cells in comparison to the expression of proliferative markers.

For methodological reasons, a direct comparison of the percentages of intimal cell proliferation with apoptosis has to be made cautiously, because estimates of rates of cell death are difficult to derive from the frequency of TUNEL-positive cells as we do not have a reliable method to estimate the period during which a cell undergoing apoptosis will display a positive TUNEL reaction [12,30]. Other variables are the duration of the apoptotic process itself and the persistence of apoptotic bodies in the tissue which seems to depend on the specific turnover of the tissue [31]. The BrdU-labeling used in this study allowed the identification of all proliferating cells which had entered the S-phase within 18 h of the labeling period. The TUNEL assay detects cells undergoing apoptosis for an unknown period of time which is probably far less than 18 h. Taking this into account, the apoptosis rate detected by TUNEL is probably too low if directly compared with the proliferation rate detected by BrdU.

Another limitation of the present study is inherent in the use of the TUNEL method. Exceptions have been described where morphological features of apoptosis were not accompanied by DNA fragmentation [17,32]. On the other hand, it has been reported that late stages of necrosis can be stained by TUNEL, but less intensely than apoptotic cells [33]. Han et al. [16] obtained a good agreement between TUNEL and propidium iodine for the frequency of apoptosis in different areas of human plaques. The data obtained by electron microscopy also seem to confirm results of the TUNEL assay [14,15]. Overall, the TUNEL assay seems to give a good orientation concerning the frequency of apoptosis in atherosclerotic and restenotic lesions.

The occurrence of apoptosis after vessel wall injury has been examined experimentally in detail using the rat carotid injury model [16,26,27]. In this model, maximal apoptosis occurred during maximal proliferation within the first week of injury. Investigations have demonstrated that up to 40% of cells in the proliferating neointima underwent apoptosis 9 days post injury and declined over time to approximately 10% at 4 weeks which interestingly exceeded the number of proliferating cells [16]. Perlman et al. [27] reported very high apoptosis rates up to 70% of medial SMCs at very early time points after balloon injury; this was not examined in our experimental setting representing a limitation of the study. As previously described for the rat model [4], the reaction to vascular injury is more intense resulting in a higher proliferation, as well as apoptosis rates, in comparison to the rabbit model used in the present study. Balloon denudation was also performed on a native vessel without pre-existing plaque formation. Regarding the proliferation rate of only 3.72±2.0% on day 7 post angioplasty in our study such a high apoptosis rate does not seem to be realistic in this animal model and would not be compensated by the following cell replication and migration. Overall, cellular turnover after balloon angioplasty in this animal model appears to be on a lower level in comparison with other results reported [15,16,26,27].

Furthermore, the restenotic lesions should be differentiated from primary atherosclerotic lesions examined in different studies, because they display a very large morphological variability. Results from restenotic lesions obtained by atherectomy are diverging regarding the absolute frequency of apoptotic cells in comparison to primary lesions. Han et al. [16] and Geng et al. [15] examined human primary atherosclerotic lesions (coronary and peripheral) and found up to 30–40% of all cells to be apoptotic. Isner et al. [13] examined coronary and peripheral human primary lesions as well as restenotic
lesions by TUNEL and described a frequency of apoptotic cells less than 2%. Interestingly, this frequency was found to be greater in restenotic lesions than in primary atheromas, especially in those with evidence of more extensive proliferative activity. Restenotic lesions characteristically have a higher proliferative activity than primary lesions and a higher cell turnover rate is reasonable. Also the factors that control apoptosis and cell proliferation are not necessarily different and in vitro results showed that the regulation of apoptosis and proliferation are mediated by closely linked mechanisms [17,34]. For evaluation of the growth dynamic of a plaque, the direct comparison of the proliferative activity and apoptosis rate over a certain time interval seems to be more important than their absolute frequency in the tissue. In the present study, apoptosis rate 7 days after balloon dilatation was slightly higher in accordance to the cell proliferation rate, but did not reach significance in comparison to the control group. Differentiation of the plaque in a superficial and deep layer after balloon angioplasty demonstrated that cell replication is mainly present in the superficial layer whereas apoptotic cells, however, showed no distinct localization. These findings did not suggest a direct relationship between apoptosis and cell proliferation in this model.

The morphology of the lesion also seems to have an experimental influence on the frequency of apoptosis. In this experimental setting the produced plaques showed a fibromuscular structure mainly composed of smooth muscle cells and a low number of macrophages in comparison to former studies due to a cholesterol diet of only 0.2% in order to obtain lower serum cholesterol levels [35]. It has been shown that lipids are cytotoxic, especially when oxidized, and that they can induce apoptosis [36,37]. Immunological activation of macrophages by certain cytokines such as interferon-γ may also induce apoptosis [38]. Consistently, apoptotic cells were often observed in close proximity to the lipid core of plaques [12,15,16,37]. It is therefore conceivable that lesions with a small lipid content and small inflammatory cell components might display lower apoptotic frequencies. In the present study, double immunostaining displayed no correlation between the occurrence of TUNEL positive cells and macrophages. In accordance with the fibromuscular composition of the neointima the majority of TUNEL-positive cells expressed markers of SMCs. A reasonable number of the TUNEL-positive cells were negative for markers of macrophages as well as for SMCs. Loss of α-SMC actin expression is probably a marker of cytoplasmatic changes preceding cellular disintegration and DNA fragmentation in the apoptotic process [39].

In conclusion, we suggest from the presented data that there may be a delicate balance between cell proliferation and apoptosis in smooth muscle cells of the vessel wall. Shifts in this balance could account for both cellular accumulation in restenotic lesions and cell death in mature atheroma. Therefore, the absolute values of cell replication and apoptosis which seem to vary widely depending on the lesions examined are probably not as important as their balance. However, the question of the definitive function of apoptotic cell death and its regulation during the process of restenosis could not be answered with this study. Further studies will be necessary examining earlier and later time points after injury and using other restenosis models.

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References


