Experimental Studies

Time Courses of Apoptosis and Cell Proliferation and Their Relationship to Arterial Remodeling and Restenosis After Angioplasty in an Atherosclerotic Rabbit Model

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Objectives

We sought to evaluate whether cellular mass changes (including apoptosis and proliferation) after arterial injury could interact with restenosis and arterial remodeling.

Background

The mechanisms controlling arterial remodeling after angioplasty remain poorly understood. Apoptosis and cell proliferation have been previously described after balloon angioplasty. However, their importance in the occurrence of arterial remodeling and restenosis is unknown.

Methods

Atherosclerosis was induced in 48 femoral arteries of New Zealand White rabbits by air-desiccation and a high-cholesterol diet. One month later, angioplasty was performed in 40 arteries. Apoptosis, cell proliferation, residual stenosis and arterial remodeling were evaluated at 2 h and 3, 7, 14, 21 and 28 days after angioplasty.

Results

Cell proliferation and apoptosis profiles were similar, but the peak in cell proliferation occurred approximately four days earlier than the peak in apoptosis in the neointima and media. Apoptosis density was positively correlated with arterial remodeling in the neointima and media (r = 0.69, p = 0.005 and r = 0.50, p = 0.05, respectively). Moreover, residual stenosis was inversely correlated with apoptosis density in the neointima and media (r = -0.62, p = 0.008 and r = -0.52, p = 0.04, respectively). In contrast, cell proliferation was independent of restenosis and arterial remodeling.

Conclusions

In this model, cell proliferation preceded apoptosis throughout the four weeks after angioplasty. Apoptosis was inversely correlated with restenosis. Interestingly, apoptosis was also related to enlargement remodeling after balloon angioplasty.

Restenosis remains the principal limitation of coronary angioplasty (1). Neointimal hyperplasia was originally considered to be the primary mechanism of restenosis after balloon angioplasty (2). However, antiproliferative therapies have failed to prevent restenosis in humans (3). The respective roles of neointimal hyperplasia and arterial remodeling were evaluated in various animal models of restenosis (4,5). It has been shown that neointimal hyperplasia was independent of the severity of restenosis, whereas constrictive remodeling was closely correlated with restenosis after angioplasty. Interestingly, similar concepts were shown to pertain to humans studied by intravascular ultrasonography (6,7).

Currently, little is known about the mechanisms controlling arterial remodeling after arterial injury. A better understanding could help to define new strategies. A cellular mass at the arterial injury site depends on the balance between cell death, including apoptosis, and cell proliferation. Although apoptosis and cell proliferation have been extensively studied after balloon angioplasty, the relationships between apoptosis, cell proliferation, arterial remodeling and restenosis have not been evaluated (8–15). Moreover, this injury model could give access to the information relative to the healing process after plaque rupture. In humans, data on apoptosis, cell proliferation and restenosis are discordant: apoptosis and cell proliferation were either decreased or increased in restenotic arteries, as compared with primary atherosclerotic arteries (16–19). Because apoptosis and cell proliferation of restenotic lesions cannot be compared with non-restenotic arteries in humans, experimental models must be used to evaluate this relationship. Our study was designed to simultaneously evaluate the time courses of apoptosis and cell proliferation, as well as their relationship with arterial remodeling and restenosis after balloon angioplasty in an atherosclerotic femoral rabbit model.

Methods

Animal model. The investigation conforms with the “Guide for the Care and Use of Laboratory Animals,” published by the National Institutes of Health (NIH publication no. 85-23, revised 1985).

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Manuscript received December 7, 2000; revised manuscript received February 13, 2002, accepted February 27, 2002.
Atherosclerotic-like lesions were induced in New Zealand White rabbits \( (n = 24) \) by the combination of air-desiccation and a high-cholesterol diet, as previously described (5).

Four weeks later, angioplasty was performed in 40 femoral arteries. Eight arteries in four animals did not undergo angioplasty, serving as the control group. A baseline iliofemoral angiogram was performed. After a bolus of 100 UI/kg heparin, angioplasty consisted of three inflations at 6 atm for 60 s, with an angioplasty balloon catheter (balloon/artery ratio between 1.0 and 1.2). Angiography was performed 10 min after the last inflation and an intra-arterial injection of nitrates (250 \( \mu \)g) to minimize post-angioplasty spasm. After angioplasty, a high-cholesterol diet was replaced by normal rabbit chow.

Angiography was repeated before sacrifice—at 2 h \( (n = 4) \) and 3 \( (n = 4) \), 7 \( (n = 8) \), 14 \( (n = 8) \), 21 \( (n = 8) \) and 28 days \( (n = 8) \) after angioplasty—and 8 weeks after air-desiccation for nondilated arteries. Arteries were retrieved after in vivo fixation by 10% buffered formaldehyde solution perfused at 100 mm Hg for 15 min.

**Angiographic analysis.** The minimal lumen diameter was measured by two independent physicians using electronic calipers, and the final results were obtained by averaging the two separate measurements.

**Histomorphometric analysis.** Each femoral artery was cut in serial sections \( (5 \mu m) \) at sites 1 to 2 mm apart, from the proximal to distal end, embedded in paraffin and stained with orceine for morphometric analysis. Each artery was evaluated at the lesion and reference sites, as previously described (5).

“Residual stenosis” was defined as the difference between the luminal areas of the reference and lesion sites, normalized by the luminal area of the reference site. The “remodeling index” was defined as the ratio of the area circumscribed by the external elastic lamina of the lesion site to the same area of the reference site. “Neointimal medial growth” was defined as the difference between the area of intimamedia at the lesion site and the proximal reference site, normalized by the same area of the reference site.

**Immunohistochemistry.** Cell proliferation was detected with a monoclonal rabbit antibody against human Ki-67 proliferation antigen (dilution of 1/50; Dako SA, Trappes, France), after antigen retrieval by boiling in 10 mmol/liter citrate buffer for 10 min (15). The sections were then incubated with avidin and biotin horseradish peroxidase complex for 30 min. The sections were stained with diaminobenzidine and counterstained with hematoxylin.

In situ detection of apoptotic cells was performed simultaneously with double-stranded labeled deoxyribonucleic acid (DNA) fragments ligated to the DNA ends, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) and single-stranded DNA in paraffin-embedded tissue samples, as previously described (20,21). Briefly, double-stranded DNA fragments were prepared by polymerase chain reaction with *Taq* polymerase, and digoxigenin-labeled fragments were ligated to DNA in tissue sections, using T4 DNA ligase (20). For the TUNEL technique, the sections were pretreated with proteinase K \( (20 \mu g/ml) \) for 15 min. Endogenous peroxidase activity was blocked with 3% \( H_2O_2 \). An apoptosis detection kit (Dako) that included the chromagen diaminobenzidine was used. For single-stranded DNA, the sections were stained with Mab F7-26 (Apostain, Alexis, San Diego, California) with the chromagen 3-amino-9-ethyl-carbazone.

Ribonucleic acid (RNA) splicing and DNA repair were evaluated on adjacent sections, with mouse monoclonal antibodies against the splicing factor (SC-35, dilution of 1/200; Sigma, Saint Louis, Missouri) and a poly(ADP-ribose) polymerase-1 (PARP-1; dilution of 1/100; Pharmingen, San Diego, California), as previously described (22,23). Splicing of RNA was evaluated to improve the specificity of the TUNEL technique (22). Single-stranded DNA, TUNEL and T4 DNA ligase were combined with PARP-1 detection, because this enzyme is known to be a target of the caspase protease activity associated with apoptosis (23).

For co-localization of apoptotic and proliferating cells with specific cell type markers, immunostaining was performed on arterial sections adjacent to those used for apoptosis and cell proliferation (i.e., 5 \( \mu m \)). Smooth muscle cells (SMCs) and macrophages were detected by monoclonal mouse anti-human alpha-smooth muscle actin (dilution of 1/100; Dako) and monoclonal mouse anti-rabbit macrophages (RAM11, dilution of 1/50; Dako), respectively.

The specificity of the immunohistochemical reactions was checked by omitting the primary antibody and substituting the antibody with an unrelated antibody at the same concentration.

**Quantification of apoptosis and cell proliferation.** Apoptosis and cell proliferation were manually identified in the neointima, media and adventitia at \( \times100 \) magnification. Apoptosis density was defined as the number of positive nuclei by in situ DNA ligation at the lesion site, normalized by the total number of nuclei at the lesion site. Cell proliferation density was defined as the number of Ki-67-positive nuclei at the lesion site, normalized by the total number of nuclei at the lesion site.

**Statistical analysis.** Results are expressed as the mean value \( \pm SD \). Occluded arteries \( (n = 3) \) were excluded from the study. Correlations were evaluated by the Spearman
The statistical threshold was set at $p < 0.05$.

**RESULTS**

Apoptosis was quantified using the T4 DNA ligase technique, because the TUNEL technique was nonspecific, as previously described (i.e., some cells stained positive by both TUNEL and the proliferation marker Ki-67 or SC-35, indicating false positivity, in contrast to T4 DNA ligase) (Fig. 1) (22). Indeed, the stains with T4 DNA ligase, single-stranded DNA or PARP-1 were very similar, which was not always the case with TUNEL (Fig. 2).

**Time courses of apoptosis and cell proliferation.** The apoptosis density was low in control arteries and dilated arteries 2 h after angioplasty, at the levels of the neointima, media and adventitia (Fig. 3A). The apoptosis density reached a maximum at day 7 in the neointima and media, remained high at 14 days, decreased by 21 days and returned to baseline at 28 days (Fig. 3A). The apoptosis density peaked earlier in the adventitia (day 3) and followed the same decay until day 28 (Fig. 3A). The apoptosis density was particularly high in SMCs, but was also increased in macrophages (Table 1). The time courses of apoptosis in SMCs and macrophages were similar (data not shown).

After angioplasty, cell proliferation was predominantly detected in the neointima, but it also occurred in the media and adventitia. Proliferation of SMCs reached a maximum at three days and progressively returned to baseline (Fig. 3B). Cell proliferation was nearly undetectable in the control arteries (Fig. 3B).

In the neointima and media, profiles of cell proliferation and apoptosis were similar, but the peak in cell proliferation occurred four days earlier than the peak in apoptosis. The apoptosis density remained high for a longer period than did cell proliferation. In contrast, apoptosis and cell proliferation were approximately synchronous in the adventitia.

**Apoptosis, arterial remodeling and restenosis.** Residual stenosis (days 14 to 21) was inversely correlated with the density of apoptosis in the neointima ($r = -0.62, p = 0.008$) (Fig. 4A) and media ($r = -0.52, p = 0.04$) (Fig. 4B), but not in the adventitia ($r = -0.2, p = NS$).

The apoptosis density was positively correlated with
arterial remodeling at the level of the neointima ($r = 0.69$, $p = 0.005$) (Fig. 4C) and media ($r = 0.50$, $p = 0.05$) (Fig. 4D), but not in the adventitia ($r = -0.2$, $p = NS$).

**Cell proliferation, arterial remodeling and restenosis.** Residual stenosis was independent of cell proliferation in the neointima ($r = -0.03$, $p = NS$), media ($r = -0.2$, $p = NS$) and adventitia ($r = 0.14$, $p = NS$). Cell proliferation was independent of arterial remodeling after angioplasty at each layer of the arterial wall (neointima: $r = 0.2$, $p = NS$; media: $r = 0.02$, $p = NS$; adventitia: $r = 0.04$, $p = NS$).

**Arterial remodeling, neointimal medial growth and restenosis.** The minimal lumen diameter increased after angioplasty (1.56 ± 0.04 vs. 1.76 ± 0.04 mm; $p = 0.01$) and decreased at 28 days (1.15 ± 0.09 mm, $p = 0.01$).

Arterial remodeling was undetectable before day 14, after angioplasty. As expected, residual stenosis was correlated with arterial remodeling ($r = 0.70$, $p = 0.004$), but was independent of neointimal medial growth ($r = -0.16$, $p = NS$).

**DISCUSSION**

The purpose of this study was to evaluate the time courses of apoptosis and cell proliferation, as well as the relation-
ships between apoptosis, cell proliferation, arterial remodeling and restenosis, after balloon angioplasty in the atherosclerotic rabbit model. We found that cell proliferation preceded apoptosis throughout the four weeks after angioplasty. Furthermore, apoptosis, but not cell proliferation, was associated with enlargement remodeling and the absence of restenosis.

Profiles of apoptosis and cell proliferation after angioplasty. The balance between SMC death and proliferation determines the number of SMCs in the arterial wall after balloon angioplasty. Their time courses after angioplasty have been extensively evaluated in experimental models in which dilation was often performed in normal arteries, which cannot simulate dilation of existing atherosclerotic plaque (8–15). We found that the SMC proliferation profile was similar to those profiles previously found in models with dilation of normal arteries (8,9). In contrast, the profile of apoptosis differed. First, apoptosis was not detected in the first hours after angioplasty, as previously reported by Perlman et al. (12). Second, apoptosis was detected in the neointima, media and adventitia after injury (day 3), and it persisted subsequently (day 21).

Simultaneous evaluation of apoptosis and cell proliferation profiles showed that proliferation preceded apoptosis throughout the four weeks after angioplasty, at the level of the neointima and media. Further studies are needed to evaluate the effect of antiproliferative therapies on the occurrence of apoptosis.

Apoptosis, arterial remodeling and restenosis. The other purpose of this study was to evaluate the relationship between apoptosis and arterial remodeling after angioplasty, because it has been shown that constrictive remodeling was related to apoptosis in neonatal arteries and human atherosclerotic arteries (24,25). We found that apoptosis dramatically increased in the neointima and media of arteries with enlargement remodeling, as compared with those with constrictive remodeling. Apoptosis was inversely correlated with restenosis. In contrast, we did not find any relationship between apoptosis and remodeling at the level of the adventitia. This does not eliminate the possible role of adventitial cells in the remodeling process, because it has been reported that myofibroblasts proliferate and migrate into the media and neointima, where apoptosis could occur (26). We do not know whether apoptosis promotes enlargement remodeling. However, we reported that the collagen content was significantly lower in arteries with enlargement remodeling, as compared with those with constrictive remodeling (27). A loss of SMCs by apoptosis could lead to a decreased synthesis of collagen.

Study limitations. As previously reported, RNA splicing and cell proliferation reduces the specificity of the TUNEL technique under our experimental conditions (22). In con-
trast, T4 DNA ligase and single-stranded DNA were more specific for the detection of apoptosis and closely correlated with PARP-1. Transmission electron microscopy and DNA electrophoresis could not be used, because our tissue samples were needed for histomorphometric analysis to evaluate arterial remodeling and apoptosis.

Restenosis has usually been evaluated in the atherosclerotic rabbit model 28 days after balloon angioplasty (4,5). However, we attempted to compare the levels of apoptosis and cell proliferation with arterial remodeling and the severity of restenosis at days 14 and 21, but not at day 28, because apoptosis and cell proliferation were nearly undetectable at day 28. Furthermore, we did not evaluate this relationship at day 7, because restenosis and arterial remodeling did not occur yet.

In clinical practice, the majority of patients undergo coronary stenting. However, ~20% still undergo balloon angioplasty, especially in small arteries (i.e., reference diameter <2.5 mm) in which the systematic benefit of stenting is controversial. Therefore, our results are only applicable to restenosis after balloon angioplasty.

Conclusions. Cell proliferation preceded apoptosis. Apoptosis, but not cell proliferation, was associated with enlargement remodeling and inversely correlated with restenosis. These results raise questions related to whether strategies aimed at enhancing apoptosis might promote enlargement remodeling and prevent restenosis after angioplasty.

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