Apoptosis and Cell Proliferation After Porcine Coronary Angioplasty

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Background—Angioplasty initiates a number of responses in the vessel wall including cellular migration, proliferation, and matrix accumulation, all of which contribute to neointima formation and restenosis. Cellular homeostasis within a tissue depends on the balance between cell proliferation and apoptosis.

Methods and Results—Profiles of apoptosis and proliferation were therefore examined in a porcine PTCA injury model over a 28-day period. Forty-two arteries from 21 pigs, harvested at the site of maximal injury at 1, 6, and 18 hours, and 3, 7, 14, and 28 days after PTCA, were examined (n=3 animals per time point). Uninjured arteries were used as controls.

Apoptosis was demonstrated by the terminal uridine nick-end labeling (TUNEL) method, transmission electron microscopy (TEM), and DNA fragmentation. Cells traversing the cell cycle were identified by immunostaining for proliferating cell nuclear antigen (PCNA). Apoptosis was not detected in control vessels at all time points nor at 28 days after PTCA. Apoptotic cells were identified at all early time points with a peak at 6 hours (5.1±0.26%; compared to uninjured artery, P<0.001) and confirmed by characteristic DNA ladders and TEM findings. Regional analysis showed apoptosis within the media, adventitia, and neointima peaked at 18 hours, 6 hours, and 7 days after PTCA, respectively. In comparison, PCNA staining peaked at 3 days after PTCA (7.16±0.29%; compared to 1.78±0.08% PCNA-positive cells in the uninjured artery, P<0.001). Profiles of apoptosis and cell proliferation after PTCA were discordant in all layers of the artery except the neointima. These profiles also differed between traumatized and nontraumatized regions of the arterial wall. Immunostaining with cell-type specific markers and TEM analysis revealed that apoptotic cells included vascular smooth muscle cells (VSMCs), inflammatory cells, and adventitial fibroblasts.

Conclusions—These results suggest that the profile of apoptosis and proliferation after PTCA is regional and cell specific, and attempts to modulate either of these events for therapeutic benefit requires recognition of these differences. (Circulation. 1998;98:1657-1665.)

Key Words: angioplasty • apoptosis • cell proliferation • restenosis

Cell death may occur by engaging a specific program (apoptosis) or necrosis. First identified in embryonic tissue,1 apoptosis is now implicated in many processes.2,3 Unlike necrosis, apoptosis is an active process requiring protein synthesis and gene transcription.4 It is associated with characteristic morphologic changes involving both the nucleus and cytoplasm.4,5 Apoptotic cells do not cause an inflammatory response, and cell integrity is maintained until removal by phagocytosis.7,9 A hallmark of apoptosis is internucleosomal cleavage of genomic DNA into 200- to 300- and/or 30- to 50-kb pieces, and eventual oligonucleosomal fractions consisting of 180 to 200 base pairs.6,10 This generates characteristic ladders on DNA electrophoresis and is the basis of in situ detection of DNA fragments with the use of the terminal uridine nick-end labeling (TUNEL) method.10

Arterial injury during PTCA initiates vascular healing including vascular smooth muscle cell (VSMC) migration and proliferation and connective tissue remodeling.12-14 This may lead to restenosis.15-17 Because the total cell number within any tissue depends on the rate of cell death and proliferation, we hypothesize that apoptosis may be involved in the genesis and/or modulation of restenosis. Apoptosis peaks synchronously with proliferation in a rat carotid injury model 9 to 10 days after injury, but early time points after injury were not examined.18 In a rat aortic endothelial injury model, apoptosis is involved in the regulation of intimal thickening.19 Apoptosis is seen in medial VSMCs as early as 30 minutes after balloon injury in both rat and rabbit arterial models with rapid elimination by 4 hours after PTCA.20 We therefore investigated a complete time course of apoptosis in the vascular response to injury and its relationship to cell proliferation using a porcine coronary balloon injury model.

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Methods

Porcine Coronary Angioplasty Model
Twenty-one Yorkshire white pigs weighing 20 to 25 kg were premedicated with aspirin 150 mg by mouth, and we performed coronary angioplasty as described previously. The animals were killed with phenobarbital (10 mL of 200 mg/mL, Vetdrug) at 1, 6, and 18 hours and at 3, 7, 14, and 28 days after the procedure with 3 animals per time point. All experiments conformed within UK Home Office regulations.

Tissue Collection and Preparation
The porcine heart was explanted and coronary arteries dissected with a minimum of surrounding tissue. After they were flushed with saline, arteries were sectioned into 3-mm blocks and alternate segments that were either snap-frozen for DNA analysis or processed for histology and transmission electron microscopy (TEM). Histology specimens were preserved in buffered formalin for 24 hours and embedded in paraffin. Sequential 3- to 4-μm-thick transverse sections were cut and affixed to aminopropyltriethoxysilane-coated slides. Representative sections from each block were stained with hematoxylin and eosin and Van Gieson-elastin to determine location and extent of injury. Arterial blocks with maximum balloon injury, defined as maximum disruption in internal elastic lamina (IEL), were selected for investigation of apoptosis and proliferation.

TUNEL and PCNA Immunostaining
The TUNEL method was used with minor modifications. Specifically, optimal proteolysis required 100 μg/mL proteinase K (Pro-mega) for 30 minutes. An apoptosis detection kit (Appligene Oncor) was used with the chromogen diaminobenzidine (DAB) (Sigma). The counterstain was 0.5% methyl green. Biochemical controls were performed with positive control slides treated with DNase-1 (Pharmacia Biotech) and with staining in the absence of terminal deoxynucleotidyl transferase enzyme as negative control. Cell proliferation was detected immunohistochemically with proliferating cell nuclear antigen (PCNA) labeling on arterial sections immediately adjacent to those used for TUNEL. For colocalization of apoptotic and proliferating cells with specific cell-type markers, immunostaining was combined with TUNEL and PCNA staining, respectively. TUNEL and PCNA staining were performed up to DAB stage. Sections were then blocked with 10% (vol/vol) normal horse serum (DAKO) and incubated overnight at 4°C with primary antibodies, a monoclonal mouse anti-human α-SM actin (Sigma) at 1:150 dilution, and a monococyte/granulocyte (MAC 387, DAKO) at 1:100 dilution. MAC 387 was not used with PCNA. A biotinylated horse anti-mouse IgG (H+L) secondary antibody (Vector laboratories, 1:200 dilution) was added for 30 minutes and detected by streptavidin alkaline phosphatase (DAKO) with New Fuchsin Red (DAKO). The counterstain in both cases was methyl green. Appropriate negative controls (without primary and/or secondary antibodies) were also performed.

Quantification of TUNEL and PCNA Staining
When viewed with a ×40 objective, cells showing morphologic features characteristic of apoptosis in addition to positive TUNEL reaction were considered to be apoptotic. Nonspecific cytoplasmic staining without nuclear involvement was considered negative. PCNA-positive cells were also quantified with a ×40 objective. The apoptotic and proliferating cells per high-power field (HPF) were represented as a percentage of total cells per HPF×100. The TUNEL and PCNA indices for each layer of artery were plotted against time. Results are expressed as mean ± SEM of 3 animals per time point. Cells that did not colocalize with either antibody were classed as “other cells.”

Electrophoretic Internucleosomal DNA Fragmentation
Arterial segments immediately adjacent to site of maximum injury were frozen in liquid nitrogen. Samples were homogenized under liquid nitrogen and suspended in equal volumes of 2× TNE (200 mmol/L NaCl, 20 mmol/L Tris, 2 mmol/L EDTA, pH 8.0) and 1% sodium dodecyl sulfate. DNA was extracted. The DNA was resuspended in 10 μL of distilled water with 5 μL of loading dye (0.25% bromphenol blue, 40% sucrose), and subjected to electrophoresis on a 1% agarose gel.
phoresis through a 2% agarose gel (containing ethidium bromide) at 80 V for 1 hour. The gel was visualized under UV transillumination and photographed with a Polaroid camera.

**Statistical Analysis**

TUNEL and PCNA results are expressed as mean±SEM (n=3 animals per time point). The comparisons of TUNEL and PCNA indices at each time point with their respective controls (uninjured arteries) were performed by use of a 1-way ANOVA. A value of P<0.05 was regarded as significant. All statistical work was done with SPSS software (Microsoft).

**Results**

**TUNEL and PCNA Staining**

The profiles of TUNEL and PCNA positivity over time were different according to the area analyzed. When analyzed for the whole arterial section, TUNEL-positive cells were undetectable in control sections and at 28 days after PTCA. They were detectable at 1 hour (4.47±0.30%; compared to uninjured artery, P<0.001), maximal at 6 hours (5.39±0.23%, P<0.001) and at low levels (0.32±0.09%, P<0.21) at 14 days.
days after PTCA. In contrast, PCNA staining was maximal at 3 days (7.33±0.28%, compared with 1.78±0.08% in the uninjured artery, \(P<0.001\)) and lower than baseline level (\(P<0.01\)) at 28 days after PTCA.

The profiles of TUNEL and PCNA positivity were also characteristic for individual layers of artery. Within the adherent thrombus, levels of TUNEL and PCNA staining were low, with peak for TUNEL at 6 hours and for PCNA at 3 days (Figure 2A). The neointima was distinguishable from 7 days. Maximal PCNA and TUNEL positivities were concordant at 7 days within this layer and returned to control artery levels at 28 days (Figure 2B). Within the media (Figure 2C), TUNEL was maximal at 18 hours with a decline to baseline at 14 days, whereas PCNA positivity was maximal at 3 days, returning to baseline at 28 days. Within the adventitia, TUNEL positivity was maximal at 6 hours and basal at 14 days, whereas PCNA was maximal at 3 days and basal at 28 days (Figure 2D). In loose connective tissue (Figure 2E), relatively high levels of PCNA-positive cells were found, whereas TUNEL positivity was low. In this layer, TUNEL was maximal at 18 hours and basal at 14 days, whereas PCNA was maximal at 3 days and basal at 28 days.

Within vessel wall, response to injury was different between sites of damage, as defined by a breach in IEL, and the rest of the arterial wall (undamaged region). TUNEL within the damaged region of the arterial wall (Figure 3A) was maximal at 18 hours, remained so until 7 days, and returned to baseline at 28 days. The peak in PCNA positivity was at 3 days and basal at 28 days. In the undamaged region of the arterial wall, the region immediately adjacent to the fractured IEL (Figure 3B) showed higher levels of TUNEL and PCNA positivity than the region opposite to the site of injury (Figure 3C). In both subregions of the undamaged wall, TUNEL peaked at 6 hours and PCNA at 3 days.

**Verification of Apoptosis**

**Light Microscopic Features**
The characteristic light microscopic features of apoptosis were frequently associated with TUNEL staining in our arterial sections. The features identified with a \(\times100\) objective included cell shrinkage, a preserved cell membrane, and a small rounded nucleus (Figure 4D).

**TEM Features**
Apoptotic cells with characteristic nuclear morphology were seen frequently at early time points, notably at 6 hours after PTCA. These included VSMCs and inflammatory cells. Only a few apoptotic cells were seen at 3 days. The ultrastructural features of apoptotic VSMCs identified included cell shrinkage, membrane blebbing, and chromatin condensation with collapse against the nuclear membrane and apoptotic bodies (Figure 5A and 5B).

**DNA Fragmentation by Gel Electrophoresis**
Electrophoresis of genomic DNA showed characteristic 180 to 200 bp DNA fragmentation at 6 hours after PTCA, consistent with peak level of apoptosis from our in situ data and TEM analysis. The evidence for similar DNA cleavage at 1 and 18 hours was less definite. Only high-molecular-weight
DNA was seen 3 days after PTCA, similar to control (uninjured) arteries (Figure 6).

**Immunocolocalization of VSMC and Inflammatory Cell Antigens With Apoptotic Nuclei**

Colocalization data are summarized in the Table. Figure 7A shows colocalization of TUNEL with \( \alpha \) SM actin. The TUNEL-positive VSMCs at 1, 6, and 18 hours after PTCA constituted 52.0 ± 7.9%, 43.0 ± 8.4%, and 35.0 ± 8.8% of total TUNEL-positive cells, respectively. At 1 hour, these were mostly at the site of damage (Figure 4A), whereas at 6 and 18 hours they were distant from the damage, including cells in deeper parts of the media (Figure 4B and 4C). The proportion of TUNEL-positive VSMCs remained fairly constant at 7 days (32.5 ± 2.0%), 14 days (41.4 ± 9.8%), and 28 days (33.0 ± 16.7%). Other TUNEL-positive cells, mainly in the adventitia and loose connective tissue of the artery, which did not colocalize with either antibody used were classified as “other cells” (Table).

At 1, 6, and 18 hours an inflammatory cell infiltrate was observed in damaged regions and in overlying thrombus. Figure 7B shows colocalization of TUNEL with inflamma-
The proportions of TUNEL-positive cells colocalizing with MAC387 at 1, 6, and 18 hours were 5.1 ± 0.4%, 8.9 ± 1.7%, and 34.1 ± 7.1%, respectively (Table). At 3 days, inflammatory cells were seen in loose connective tissue, especially in tissue adjacent to the breached IEL. The TUNEL-positive inflammatory cells constituted 16.6 ± 2.7% of total TUNEL-positive cells at 3 days, 5.4 ± 1.6% at 7 days, and 4.2% at 14 days. No inflammatory cells were seen at 28 days.

**Immunocolocalization of α-SM Actin With PCNA Staining**

The majority of PCNA-positive cells in the media and neointima were VSMCs. A few PCNA-positive cells in the neoadventitia also colocalized for α-SM actin. PCNA-positive cells in the adventitia and loose connective tissue did not colocalize for α-SM actin, suggesting a different phenotype.

**Discussion**

In this study, we have demonstrated apoptosis in porcine coronary arteries after balloon injury by use of TUNEL assay with corroborating evidence from TEM and DNA cleavage. We have also assessed cell proliferation in immediately adjacent sections using PCNA immunostaining. Apoptotic cells appeared early, reaching a peak at 6 hours and returning to a low level at 14 days. The maximum number of apoptotic cells in the damaged region

<table>
<thead>
<tr>
<th>Time</th>
<th>Apoptotic VSMCs % Mean±SEM</th>
<th>Apoptotic Inflammatory Cells % Mean±SEM</th>
<th>Other Cells % Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 h</td>
<td>52.0±7.9</td>
<td>5.1±0.4</td>
<td>42.9±8.3</td>
</tr>
<tr>
<td>6 h</td>
<td>43.0±8.4</td>
<td>8.9±1.7</td>
<td>48.1±10.1</td>
</tr>
<tr>
<td>18 h</td>
<td>35.0±8.8</td>
<td>34.1±7.1</td>
<td>30.9±15.9</td>
</tr>
<tr>
<td>3 d</td>
<td>42.3±7.9</td>
<td>16.6±2.7</td>
<td>41.1±10.6</td>
</tr>
<tr>
<td>7 d</td>
<td>32.5±2.0</td>
<td>5.4±1.6</td>
<td>62.1±3.6</td>
</tr>
<tr>
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</tr>
<tr>
<td>28 d</td>
<td>33.0±16.7</td>
<td>0</td>
<td>67.0±16.7</td>
</tr>
</tbody>
</table>

Proportion of TUNEL positive cells immunocolocalized as VSMCs, inflammatory cells, and other cells, represented as a percentage of total TUNEL positive cells in whole arterial section. Results are expressed as mean±SEM of 3 animals per time point over a 28-day period. Percentage (%) of other cells was calculated by subtracting the sum of % means of VSMCs and inflammatory cells.
Regional Differences in Apoptosis and Cell Proliferation

We used immunohistochemical methods to assess apoptosis and cell proliferation in adjacent sections within the same model, which allowed comparison of the timing and site of peak activity of these two processes. We found regional differences in levels of apoptosis and cell proliferation, as well as an important influence of trauma on these biological events. Concordance of peak activities of apoptosis and proliferation was seen only within the neo-intima. In all other regions, apoptosis peaked earlier than proliferation. The most plausible explanation for separation in peak activities is that our TUNEL and PCNA data are derived from arterial tissue sections that contain a variety of cell types: VSMCs, inflammatory cells, and fibroblasts. These have different properties and may undergo apoptosis at different rates. For example, neutrophils are terminally differentiated cells that undergo constitutive apoptosis and are removed by macrophage phagocytosis, prompted by specific cell-surface changes. The kinetics of VSMC apoptosis, in contrast, are less clear. Our data confirm the importance of an inflammatory cell influx after PTCA, notably in the outer adventitial and loose connective tissue layers. The discordance in proliferation and apoptosis profiles in different wall regions and layers may be explained by the distribution of apoptotic neutrophils (which cannot divide). Furthermore, cells in the cell cycle display a...
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low but increased level of apoptosis, presumably reflecting incorrect cell division. 26–27 This may explain the concordance of apoptosis and proliferation in the neointima. The influence of apoptotic inflammatory cells on apoptosis in VSMCs is unknown and needs further investigation.

Levels of Apoptosis and Proliferation After PTCA
A quantitative difference in levels of apoptosis and proliferation after PTCA was seen, with proliferation greater than apoptosis, except at early time points: 1, 6, and 18 hours. This difference was evident within each layer and region of the artery. In the neointima, proliferation was greater than apoptosis but with both maximal at 7 days. Within the media, adventitia, and loose connective tissue, a quantitative difference was present but with a disparity in the timing of their maximal activities (apoptosis peaking earlier than proliferation). The higher levels of TUNEL and PCNA positivity adjacent to fractured ends of IEL, relative to the site opposite to the damage, is evidence of the contribution of this region toward neointima formation. Although some difference in levels of apoptosis and proliferation may be accounted for by the different duration of these two biological events (proliferation requires 14 to 18 hours, whereas apoptosis requires 2 hours), the longer half-life of PCNA positivity, 22,29 and the possibility of different rates of removal of different types of apoptotic cells, it may partly explain the net accumulation of tissue.

Although our PCNA data was not greatly dissimilar from data concerning cell proliferation with bromodeoxyuridine (BrdU), 14 PCNA expression occurs early in the cell cycle, and may identify cells which fail to undergo mitosis but will undergo apoptosis. 26–27 This may explain the observed 1.5-fold increase in PCNA positivity at 1 hour compared with control artery. This possibility is supported by the paucity of cells showing mitotic figures at early time points. Furthermore, PCNA positivity at 6 and 18 hours remained similar to that at 1 hour. This is in agreement with findings of a 1.5-fold increase in PCNA positivity at 1 day after PTCA in the porcine carotid artery. 20–21 In contrast, the 4-fold increase in PCNA positivity at 3 days, when apoptosis has returned to lower levels, may reflect an increase in the number of cells traversing the cell cycle. BrdU labeling would have been more reliable, but it cannot be used on archival tissue.

VSMC Proliferation After PTCA: A Possible Role for In Vivo Apoptosis
In the vascular response to injury, VSMC apoptosis preceded proliferation of remaining cells. This implies that cell death may trigger cell migration and proliferation. The barotrauma of balloon angioplasty may provide the initiating stimulus to propagate a cascade of biochemical apoptotic responses in VSMCs similar to those reported in cardiac myocytes, 32 including gene transcription, cytokines, calcium, platelet activating factor, free radicals, and nitric oxide release. Although stretch may initiate VSMC proliferation, 33–35 we may hypothesize that signals from apoptotic cells (VSMC and inflammatory cells) may induce proliferation in surviving VSMCs. Obviously, this point needs further investigation and is beyond the scope of this article.

Study Limitations
In our study, injury was induced in normal arterial segments. The apoptotic response may differ in atherosclerotic human coronary arteries exposed to PTCA. In vitro studies showing that VSMCs derived from human coronary plaques undergo apoptosis at a higher rate than normal VSMCs support this concept. 36 In situ detection of apoptosis and proliferation within tissue sections concerns the kinetics of cell cycle. Information on kinetics of in vivo apoptosis are less clear than cell proliferation. 24,31,35 There are no data regarding duration of TUNEL positivity in vivo once cells are committed to the apoptotic pathway. Furthermore, the total number of cells exhibiting TUNEL positivity will be determined in part by rate of clearance of apoptotic cells. This appears to be very rapid for apoptotic neutrophils in the presence of primed macrophages. The rate of clearance of apoptotic VSMCs within the arterial wall is less clear. For these reasons, total levels measured must be interpreted with caution. Rates of apoptosis (and proliferation) cannot be measured in tissue sections like we have used and, therefore, this term has been avoided.

Implications
In conclusion, we have demonstrated different frequencies of apoptosis and proliferation in different regions of the arterial wall and involving different cell types after porcine PTCA. Considering the similarities of porcine arterial injury model to human coronary restenotic lesion, 24,37 we believe these findings may provide reliable insights into the mechanisms and interplay of apoptosis and proliferation after balloon injury. A particular strength of this model is the comparison of damaged and undamaged regions of the artery, which may allow novel therapeutic interventions that target these regions to inhibit neointimal hyperplasia. Previous studies have demonstrated potential benefits of antiproliferative therapies to reduction of neointimal hyperplasia with anti-sense oligonucleotides to c-myc 38 and c-myb, 21 and transfection with growth-inhibitory genes 39–40 in a porcine model. Local delivery of an agent to modulate apoptosis after injury may reduce neointimal hyperplasia and thus restenosis after PTCA. However, modulation of apoptosis will require recognition of the fact that apoptosis after injury occurs in a number of different cell types, at different levels, and in different regions of the artery.

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References
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