Apoptosis bcl-2 and nitrotyrosine expression in an angioplasty-restenosis rabbit: An experimental model

Ioannis K. Toumpoulis, Vassiliki D. Malamou-Mitsi, Lampros K. Michalis, Christos Katsouras, Georgia Gloustianou, Dimitrios Galaris, Mary Bai, Dimitrios Vardakas, Niki J. Agnantis, Dimitrios A. Sideris

a Department of Cardiac Surgery, University of Athens School of Medicine, Attikon Hospital Center, Georgiou Sisini 31, 11528 Athens, Greece
b Department of Pathology, University of Ioannina, Ioannina, Greece
c Department of Cardiology, University of Ioannina, Ioannina, Greece
d Department of Biochemistry, University of Ioannina, Ioannina, Greece

KEYWORDS
Angioplasty; Apoptosis; Bcl-2; Nitrotyrosines; Rabbit iliac model; Restenosis

Abstract
Apoptosis has been suggested to have an important role in the pathogenesis of restenosis in addition to cell migration and proliferation. The aim of the present study was to investigate in an experimental in vivo model the occurrence of apoptosis postangioplasty and its relation to bcl-2 and peroxynitrite detection.

Eighteen hypercholesterolemic rabbits underwent transluminal angioplasty of the right iliac artery. The rabbits were sacrificed on the 1st, 2nd, 3rd, 7th, 15th, and 28th day postangioplasty (3 animals per time point) and both the angioplasted and non-injured arteries were studied. Apoptosis was assessed by the terminal uridine nick-end labeling method (TUNEL). Bcl-2 and peroxynitrite were detected by immunohistochemistry using anti-bcl-2 and anti-nitrotyrosine antibodies.

In the angioplasted arteries the number of apoptotic cells was ≤1% of the total cell population in both media and neointima at any of the postangioplasty time points examined. Bcl-2 and nitrotyrosines were detected at all time points in the angioplasted arteries (vs. non-injured, P < 0.001), showed similar localization and had the same peaks of expression both in the media (7th day: Bcl-2 66% and nitrotyrosines 74%) and neointima (15th day: Bcl-2 67% and nitrotyrosines 61%).

In this experimental model we observed low apoptotic rates while bcl-2 and peroxynitrite products were detected. We can hypothesize that the detection of nitrotyrosines is related...
with reduced levels of nitric oxide resulting in increased expression of the bcl-2, preventing thus cell death due to either apoptosis or necrosis. Further studies are needed in order to elucidate their role in the restenosis process.

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Introduction

Restenosis rates, after percutaneous transluminal angioplasty, range from 16--42% with neointimal formation being one of the main mechanisms.1--3 Previous studies have emphasized the pivotal roles of vascular smooth muscle cells (VSMCs) proliferation and migration as well as extracellular matrix accumulation in the postangioplasty neointimal formation.4--8 In addition to these, apoptosis of VSMCs has been suggested to be an important factor in the pathogenesis of restenosis, although the triggering mechanisms are currently unknown.9--13 It should be also noted that the apoptosis rates in the published reports vary widely and range from <1% to >50%. It is also known that bcl-2 is involved in regulating VSMCs apoptosis and more specifically its overexpression can inhibit apoptosis of VSMCs.14,15

There has been considerable interest regarding the role of nitric oxide (NO) in postangioplasty restenosis.16,17 NO can both induce and inhibit apoptosis of VSMCs depending upon the different redox state of the NO once released from the cells.16,17 The anti-apoptotic activity of NO has been related with the increase of the bcl-2 expression, the inhibition of bax overexpression and the S-nitrosylation of caspases. It has also been found that NO can upregulate bax, possibly via p53 upregulation resulting thus in apoptosis. Superoxide anion (O2−) is another factor that is involved in the process of vascular cell proliferation and neointimal growth.15,20 The reaction of NO with O2− forms peroxynitrite (ONOO−) and nitrated tyrosine residues (nitrotyrosines) represent an in vivo footprint marker of ONOO−.21,22

In the present study we used a hypercholesterolemic iliac rabbit model to investigate the expression of bcl-2 in relation to nitrotyrosine formation in order to elicit the evidence for the anti-apoptotic activity of NO in the restenosis process. We also investigated the occurrence of apoptosis using the TUNEL method in order to correlate it with bcl-2 expression.

Materials and methods

Animals

Eighteen (male) New Zealand white rabbits (mean body mass 3.0 kg) housed in individual cages constituted the study material. Animals were fed a chow diet with cholesterol supplementation (92% chow, 2% cholesterol and 6% peanut oil) over a five week period, leading to high serum cholesterol levels (mean ± SD: 1050 ± 540 mg/dl). All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health 5377-3, (Washington: National Academy Press; 1996) and the animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Ioannina, Greece.

Angioplasty

Balloon angioplasty was carried out at the right iliac artery of all eighteen rabbits during the first day of the sixth week period. Under general anesthesia (ketamine HCl: 35 mg/kg, midazolam 5 mg/kg and atropine 150 μg/kg) the right femoral artery was surgically exposed in each animal and a transverse arteriotomy was performed to its distal part. A modified Baumgartner technique was used for balloon angioplasty. More specifically, a 2.5 mm diameter centering balloon, 20 mm in length, was inserted into the lumen of the artery and advanced into the right iliac artery. The balloon was then inflated at 10 Atm over a 2 min long period. Clips were positioned at the muscles adjacent to the proximal and distal injury sites. The balloon catheter was then deflated and withdrawn, the artery was repaired surgically and the subcutaneous tissue was closed. After angioplasty a standard diet without cholesterol supplementation was fed to the animals.

Tissue preparation and analysis

The animals were euthanized on the 1st, 2nd, 3rd, 7th, 15th and 28th day after angioplasty (3 animals per time point). The balloon dilated right iliac arteries (angioplasty group) and the left non-injured iliac arteries (control group) were studied in each animal. Sixty minutes before perfusion-fixation the rabbits were infused intravenously with 60 mg/kg of Evans blue dye in PBS.

After application of a lethal dose of sodium phenobarbital, a thoracotomy was performed and the entire vasculature was fixed in situ with perfusion of 500 ml of 10% neutral buffered formalin solution via a catheter inserted into the left ventricle. After 15 min of perfusion-fixation the right (angioplasted) and the left (non-injured) arteries were excised and fixed in the same fixative for 4 to 6 h. Two to three cross sections were taken from the central de-endothelialized region of each right iliac artery stained blue which probably represented the middle of the denuded and diluted arterial segment. Cross sections at 2 mm intervals were also taken from the non-injured left iliac arteries (control group).

The samples were routinely processed in grading ethanol and embedded to paraffin. Serial tissue sections (5 μm thick) were then cut. Two slides (2--3 sections/slide) from each block were stained with hematoxylin-and-eosin (H&E) and Masson trichrome stain respectively for the initial morphological evaluation and the others were used for further immunohistochemical and apoptosis analyses.

Immunohistochemical analysis

The indirect avidin-biotin horseradish peroxidase complex method was applied for all the immunohistochemical stains, as it has been described previously. As primary
antibodies, the monoclonal antibodies smooth muscle actin (1A4, DAKO), macrophage CD68 (KP1, DAKO) Von Willebrand Factor (F8/86, DAKO) and CD34 (Becton-Dickinson) were used for the identification of VSMCs, macrophages and endothelial cells respectively. Bcl-2 protein expression and peroxynitrite generation were demonstrated by using the bcl-2 (124, DAKO) and anti-nitrotyrosine (ZUO5-233, TC5) monoclonal antibodies respectively. Positive control slides were included in all tests and negative control slides were prepared by omitting the primary antibodies.

Bcl-2 expression was also determined by the percentage of the ratio of positive cells with cytoplasmic staining to the total number of cells counted. Nitrotyrosines expression was assessed by the percentage of the ratio of positive cells with intracellular staining (mainly cytoplasmic and rarely nuclear) to the total number of cells counted.

**TUNEL assay**

The in-situ visualization of DNA fragmentation was performed on paraffin sections (5 μm thick) placed on super-frost slides using the commercial end-labeling Apop Tag Peroxidase Kit (Oncor). After deparaffinization and dehydration, slides were rinsed in phosphate buffer solution (PBS, pH = 7.2). Tissue sections were then digested by incubation for 20 min with proteinase K (20 μg/ml, Oncor) at room temperature and then were rinsed in distilled water.

The peroxidase activity was blocked by incubation for 5 min in 2% hydrogen peroxide in PBS. After the application of an equilibration buffer, slides were incubated in working-strength TdT enzymes that contained dUTP-digoxigenin for 1 h at 37 °C. The reaction was stopped by a pre-warmed at 37 °C working-strength stop/wash buffer and then slides were rinsed in PBS. The incorporated nucleotides were identified by adding peroxidase conjugated anti-digoxigenin antibody and visualized with 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Oncor). Counterstaining was performed with Harris’ hematoxylin. Negative control slides were prepared by omitting the TdT reaction step. Germinal centers of human lymph nodes served as positive controls.

To determine the apoptotic index light microscopic examination of each artery in both groups (angioplasty and control) at a magnification of ×400 was carried out by two experienced pathologists. Separate calculations were done for neointima and media in each artery of the angioplasty group of animals. The apoptotic index was determined from the percentage of the ratio of TUNEL positive nuclei to the total number of cells counted (at least 1000 cells per artery were counted).

**Statistical analysis**

The results were expressed as mean values ± SD. The comparisons of TUNEL, Bcl-2 and nitrotyrosines expression at each time point with their representative controls (non-injured arteries) were performed by non-parametric Mann–Whitney U-test. Differences were considered to be significant at a value of P < 0.05 as determined with SPSS 11.0 Software (SPSS, Inc, Chicago, IL).
Results

Morphological results

1st postangioplasty day
In the angioplasted arteries extensive loss of endothelial cells was observed in H&E stained sections and confirmed using immunohistochemical stains for VWF and CD34 antigen. Also extensive damage with reduction of VSMCs was seen in the media by H&E, Masson and smooth muscle actin (SMA)-immunostain.

2nd and 3rd postangioplasty days
The loss of endothelial cells remained as a consistent finding, whereas restoration of media started with an increasing number of VSMCs.

7th postangioplasty day
Neointima formation consisted of VSMCs with a small amount of extracellular matrix was demonstrated by Masson and SMA-immunostain (Fig. 1B).

15th postangioplasty day
Increase of neointima thickening and reendothelization was noted. The increase of neointima thickening was composed of VSMCs, macrophages and extracellular matrix (H&E, Masson, CD34, CD68 and SMA-immunostains; Fig. 1C).

28th postangioplasty day
Further increase of neointimal thickening was seen. All arteries were atherosclerotic at the time of angioplasty. The left iliac arteries (control group) did not show any significant morphological changes at 28th postangioplasty day except for few foam cells occasionally found in the media or subendothelially (Fig. 1A).

Quantification of apoptosis

In non-injured arteries apoptotic cell nuclei detected by TUNEL in 0.2 ± 0.2%. In the angioplasted arteries the TUNEL-positive cells remained at a very low level (<1%) in both media and neointima at any of the postangioplasty time points examined (Table 1, Figs. 2, 4 and 5). The Bcl-2 staining was cytoplasmic and was seen mainly in the neointima and media (Figs. 3A,C).

In non-injured arteries nitrotyrosines were present in 0.2 ± 0.3%. In angioplasted arteries were also observed during all studied time points (Table 1) with the peak of expression in the media and neointima been at the 7th (74.0 ± 22.6%, P < 0.001) and at the 15th postangioplasty day (61.3 ± 9.3, P < 0.001; Figs. 4 and 5) respectively. Nitrotyrosines was mainly seen in the cytoplasm and rarely intranuclear. Extracellular positive staining was observed only in the more damaged segments of the vascular wall (Figs. 3B,D).

Discussion

There is increasing evidence that apoptosis is involved in the restenotic process. In previous experimental studies the percentages of apoptotic cells in the restenotic lesions vary from <1% to >50%9,11,12,23e25 with the higher percentages seen when tissue was analyzed during the first hour postangioplasty.13,26 Although, there is a controversy on the apoptosis' rate, most studies conclude that both responses (VSMC proliferation and apoptosis) co-exist in the neointima. The variance in the apoptotic rates reported in the literature is possibly due to the fact that tissue analysis occurred at different postangioplasty time points. In our study the number of apoptotic cells was <1% of the total cell population in both media and neointima at any of the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Bcl-2, TUNEL and Nitrotyrosines expression at time points examined in media and neointima (data are expressed as mean (SD) %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bcl-2 media</td>
</tr>
<tr>
<td>Day 1</td>
<td>8.0 (0.4)</td>
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<tr>
<td>Day 2</td>
<td>13.8 (8.8)</td>
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<tr>
<td>Day 3</td>
<td>50.0 (14.1)</td>
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<tr>
<td>Day 7</td>
<td>65.9 (17.2)</td>
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<tr>
<td>Day 15</td>
<td>64.0 (8.0)</td>
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<tr>
<td>Day 28</td>
<td>56.2 (6.5)</td>
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Figure 2  TUNEL-positive cells in the media (M) and in the secondary thrombus (2nd postangioplasty day, original magnification ×660).
postangioplasty time points been examined, although it should be noted that the first tissue analysis occurred 24 h postangioplasty.

Bcl-2 proto-oncogene inhibits apoptotic deaths being induced by diverse stimuli. The protein products of the bcl-2 gene family are localized in intracellular sites where oxygen free radicals are generated (e.g. mitochondria, endoplasmic reticula and nuclear membranes). In vitro studies have shown that bcl-2 overexpression suppresses membrane lipid peroxidation which represents an early step of the apoptotic cell death being induced by reactive oxidizing factors. Thus, it is suggested that bcl-2 regulates an antioxidant pathway at sites of free radical generation. This antioxidant function of bcl-2 is attributed to its ability to prevent the harmful effects of oxidative stress in the cellular components, although bcl-2 does not stop free radical generation. Currently bcl-2 was detected as early as the 1st postangioplasty day and reached the peak of their expression at day 7 in the media and at day 15 in the neointima. At all time points that bcl-2 was examined was higher at the angioplasted arteries compared with the controls. Our results (increased bcl-2 expression at sites with low apoptosis) are in concordance with the above findings.

It is known that NO is a ubiquitous molecule, that acts as an intracellular and extracellular messenger and has both deleterious and beneficial effects on the target cells. NO regulates the expression of genes involved in the apoptotic cell death and in high concentrations causes apoptosis. NO toxicity is enhanced by producing ONOO⁻ after reacting with O₂⁻. Peroxynitrite (ONOO⁻) not only inhibits the normal homeostatic function of NO (e.g. regulation of vasomotor tone, regulation of proliferation and cell migration, induction of cytoprotective genes and modulation of cytotoxicity by activated macrophages and toxic cytokine production) but also causes cellular damage as it is a strong oxidant. Peroxynitrite can be detected in biological samples indirectly by measuring tyrosine residues nitration (nitrotyrosines). Currently peroxynitrite biological products (nitrotyrosines) were detected as early as the 1st postangioplasty day and reached the peak of their expression at day 7 in the media and at day 15 in the neointima. According to our results the staining for nitrotyrosines and Bcl-2 proteins had analogous peaks of expression in the media and neointima (Figs. 4 and 5) and showed similar localization and distribution in the studied arterial segments (Fig. 3). We can speculate that bcl-2 overexpression in the media and neointima might render them resistant against the toxic effects of peroxynitrite and generally of the oxidative stress preventing thus cell death due to either apoptosis or necrosis. We can also hypothesize that the detection of nitrotyrosines is related with reduced levels of NO (NO is consumed in the reaction with O₂⁻) resulting thus in increased levels of Bcl-2 protein.

Our findings suggest that nitrotyrosines in the 1st postangioplasty day can be formed despite the lack of endothelial cells and therefore of endothelial NO synthase.
Preliminary results from our study (data not shown) suggest that a kind of inducible NO synthase is produced by VSMCs immediately after the angioplasty procedure. These findings are in concordance with recently reported data.31

The present study has several limitations. First, the experimental model been used is a non-stenting one and therefore, the results are only applicable to restenosis after plain balloon angioplasty. Second, for apoptosis detection the TUNEL method was used, which has a reduced specificity in cases with increased cell proliferation rates. Third, currently we used a 4-week time point, starting 24 h postangioplasty. Earlier time course experiments may provide additional information regarding apoptosis rates and detection of bcl-2 and nitrotyrosines. Finally, we could not use DNA electrophoresis or Western blot analysis because our tissue samples were needed for histological and immunohistological analysis in order to evaluate apoptosis and expression of bcl-2 and nitrotyrosines. Forth, the results of the present may be different whether cholesterol supplementation used during the postangioplasty period.

**Conclusion**

Apoptosis as measured with TUNEL method practically does not occur after angioplasty in restenotic lesions. Bcl-2 and peroxynitrite products (nitrotyrosines) are detected at all times points postangioplasty, while their expression and localization in the restenotic lesions are similar. Oxidative stress occurs after angioplasty leading to an increase in peroxynitrite, and that increased bcl-2 expression counteracts this oxidative stress, preventing apoptosis and cell death. Further studies are needed in order to elucidate their role in the restenosis process.
Ethical Statement: All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health 5377-3, (Washington: National Academy Press; 1996) and the animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Ioannina, Greece.

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