Apoptosis and Bcl-xs in the intimal thickening of balloon-injured carotid arteries

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ABSTRACT

We performed balloon injury in the rat carotid artery and identified intimal thickening after injury. Balloon-injured carotid arteries showed maximum thickness of the neointima on the 14th day before complete endothelial cell regeneration. In this lesion we identified apoptosis of vascular smooth muscle cells (VSMCs) by in situ DNA labelling and electron microscopy in the neointima on the 14th day after injury. mRNA expression levels of bcl-2, bax, bcl-x, p53 and caspase-1 were determined by the reverse transcriptase–polymerase chain reaction method both in injured and uninjured carotid arteries. Neither bcl-2 nor bcl-xl mRNA expression was detected in either injured or uninjured arteries, whereas bax and p53 mRNA expression was identified and their mRNA levels were not altered after balloon injury. In contrast, both bcl-xs and caspase-1 mRNA was detected and was markedly induced only in the injured carotid artery. Positive staining for immunoreactive Bcl-x was observed specifically in the injured arterial wall and co-localized with positive staining of nuclei identified by in situ DNA labelling. We conclude that two opposite cellular responses, VSMC proliferation and apoptosis, exist together in the neointima of the rat carotid artery after balloon injury, and selective induction of Bcl-xs expression is a key regulator of VSMC apoptosis in the process of vascular remodelling.

INTRODUCTION

Apoptosis plays an important role not only in normal cellular development but also in many pathological processes. Induction of apoptosis has been demonstrated in a variety of diseases including cancer, neuronal disorders and cardiovascular disease [1–4]. In cardiovascular disease, apoptosis of vascular smooth muscle cells (VSMCs) has been identified in the atherosclerotic and restenotic lesions after percutaneous transluminal coronary angioplasty, suggesting that VSMC apoptosis as well as VSMC proliferation is involved in the aetiology or pathogenesis of vascular remodelling.

Although many regulatory factors including humoral growth factors, cytokines and matrix components are well characterized in atherosclerotic and restenotic lesions [5], the major determinants that actually regulate apoptosis in the vasculature are not fully elucidated.

In this study we performed balloon injury in the rat carotid artery and obtained evidence that VSMC apoptosis as well as VSMC proliferation plays a pivotal role in the pathogenesis of neointimal formation after injury. Furthermore, we also identified important apoptosis-related molecules of VSMC apoptosis in the process of vascular remodelling.

METHODS

Balloon catheter injury

Male Sprague–Dawley rats (Charles River, Kanagawa, Japan), weighing 380 to 400 g, were used in this experiment, and all surgical procedures were performed using general anaesthesia with an intraperitoneal injection of sodium pentobarbitone (Nembutal®, 65 mg/kg body weight; Abbott Laboratories, North Chicago, IL, U.S.A.). Balloon injury was performed in the right common carotid artery by the method described pre-
viously [6]. Briefly, the right external carotid artery was surgically exposed, and a 2F Fogarty catheter (Baxter Healthcare Corp., Irvine, CA, U.S.A.) was inserted. When the catheter was advanced to the aortic arch, the balloon was inflated at moderate pressure and the endothelium was stripped three times by the passage of the balloon to the bifurcation of the carotid artery. The external carotid artery was then ligated after removal of the catheter, the blood flow was restored, and the wound was closed.

**Tissue preparation and morphological evaluation**

At each selected time point, rats were killed with an overdose of pentobarbitone. Arteries were infused with ice-cold saline at physiological pressure from the left ventricle, and blood was drained from the right atrium. When the drained solution cleared, the arteries were fixed for 1 h at 4 °C, embedded in mounting medium (Historesin, Leica Instruments, Heidelberg, Germany) and serially sectioned at a thickness of 2 μm. To confirm the establishment of neointimal formation in the injured artery, two or three sections were immediately subjected to staining with haematoxylin–eosin.

**In situ DNA labelling**

*In situ* DNA labelling was performed with the *In Situ* Apoptosis Detection Kit (ApopTaq™ Plus, Oncor, Gaithersburg, MD, U.S.A.) according to the manufacturer’s specifications. Tissue sections were pretreated with PBS containing 0.01% trypsin for 10 min at 37 °C, and then incubated for 10 min at room temperature in PBS containing 0.3% H₂O₂ to inactivate endogenous peroxidase activity. They were then treated with proteinase K (20 μg/ml solution in PBS) for 30 min at 37 °C and covered with a solution containing digoxigenin-conjugated dUTP in the presence of terminal deoxynucleotidyl transferase for 1 h at 37 °C. After exposure to a solution containing diluted anti-digoxigenin monoclonal antibodies conjugated with peroxidase for 60 min at room temperature, nuclei were visualized with a substrate solution of 3′,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan). Tissue sections were counterstained with haematoxylin, dehydrated with ethanol and xylene, and finally mounted in mounting medium (Mount-Quick, Daido Sangyo Co. Ltd, Tokyo, Japan).

**Electron microscopy**

Balloon-injured carotid arteries were fixed for 2 h at 4 °C in a buffer of 2.5% glutaraldehyde and 0.1 mol/l sodium cacodylate, pH 7.4. They were then fixed again for 1 h at room temperature in 1% OsO₄, dehydrated and finally embedded in epoxy resin (Epon 812, TAAB Laboratories Equipment, Ltd., Berks, U.K.). Thin sections with a thickness of 80 nm were made with a Reichert ultramicrotome (Leica Instruments) and viewed with a Hitachi H-800 electron microscope (Hitachi, Tokyo, Japan) at 100 kV.

**Measurement of bcl-2, bax, bcl-x, p53 and caspase-1 mRNA**

To extract total cellular RNA, the balloon-injured rats were killed with an overdose of pentobarbitone and the carotid arteries on both sides were excised separately. After immediate freezing in liquid nitrogen, the excised arteries were put in a plastic tube and stored at −80 °C. Total cellular RNA was extracted with ISOGEN (Wako Pure Chemical Industries). To measure the mRNA expression level of bcl-2, bax, bcl-x, p53 and caspase-1, reverse transcriptase–polymerase chain reaction (RT–PCR) was performed as described previously [7,8]. The expression level of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was also determined to standardize the amount of RNA used for RT–PCR. Extracted RNA (1 μg) was reverse-transcribed to single-stranded cDNA with oligo-dT primers in the presence of avian myeloblastosis virus reverse transcriptase (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.) in a volume of 20 μl, and 1 μl of the reaction mixture was directly subjected to PCR. PCR was carried out with a Perkin–Elmer DNA Thermal Cycler 9600 in a volume of 50 μl, and reaction conditions were 94 °C for 45 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 60 s (elongation) for 25 cycles for all target genes. Sequences of the specific oligonucleotide primers used in this study were as follows. Forward and reverse primers for rat *bcl-2* were: 5'-ATGCCGCAAGCCGGGAGA-3' and 5'-TCA-TTGTGGCCCAGGTATG-3';

for rat *bax*,

5'-GACACCTAGCTGACCTTGGG-3' and 5'-GAG-GAAATTCAGTGTCAGC-3';

for rat *bcl-x*,

5'-ATGTCTCAGAGAACCCGGA-3' and 5'-TCAC-TTCCGACTGAAGAGTG-3';

for rat *p53*,

5'-ATGAGAGATTCCACAGTCGGGA-3' and 5'-TCAC-CTTCCGACTGAAGAGTG-3';

for rat *caspase-1*,

5'-GATCACCATCCTCAATGAAGGCACCAACCCA-3';

and for G3PDH,

5'-TGAGCTTAGCTGCTGCGATGGTTC-3' and 5'-CAAGGTGGAATGGGAGGC-3'.

Specific primers for *bcl-x* used in this experiment enabled us to recognize both *bcl-X* (518 nucleotides) and
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bcl-xl (707 nucleotides) mRNA. PCR products were finally analysed by electrophoresis on a 1.4% agarose gel, and visualized by staining with ethidium bromide.

**Immunohistochemistry**

Tissue sections used for immunohistochemistry were treated by the same procedure described for in situ DNA labelling to inactivate endogenous peroxidase activity, and the following steps were carried out according to the manufacturer’s specifications with a VECTASTAIN Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.). After blocking for 30 min at 37°C with diluted normal goat serum, tissue sections were incubated for 1 h at room temperature with diluted primary antibodies against human Bcl-2, human Bax or human Bcl-x (can detect both Bcl-xl and Bcl-xs) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). We confirmed that these polyclonal antibodies against human proteins were readily cross-reactive with the corresponding rat proteins. After exposure to a solution containing diluted biotinylated secondary antibodies, the tissue sections were treated with VECTASTAIN Elite ABC Reagent. Positive staining of cells was finally visualized with 3,3′-diaminobenzidine tetrahydrochloride solution, and the tissue sections were counterstained, dehydrated and mounted as described for in situ DNA labelling.

**RESULTS**

**Morphological evaluation and in situ DNA labelling**

Rats were killed 1, 4, 7, 14 and 28 days after balloon injury, and the carotid arteries were subjected to morphological evaluation. The injured carotid artery revealed thick neointimal formation (Figure 1). Balloon-injured carotid arteries showed maximum thickness of the neointima on the 14th day before complete endothelial cell regeneration. In situ DNA labelling of the balloon-injured carotid artery clearly demonstrated positive staining of nuclei only in the neointima, exclusively in the innermost layer of the lesion (Figure 2A). In contrast,
there was no positive staining in the uninjured carotid artery on the opposite side (Figure 2B). The frequency of the positive cells was about 20%. Morphological evaluation with an electron microscope was also performed on cells of the injured lesion. The VSMCs in the neointimal lesion displayed condensed chromatin (Figure 3A) or an irregular nuclear membrane (Figure 3B), whereas organelle membranes were almost intact. The frequency of cells showing these morphological characteristics in the nuclei was approximately 2.9% by electron microscopy.

**mRNA levels of bcl-2, bax, bcl-x, p53 and caspase-1**

mRNA levels of bcl-2, bax, bcl-x, p53 and caspase-1 were evaluated by RT–PCR (Figure 4). Bands were detected by staining with ethidium bromide for all PCR products except bcl-2 and bcl-xl mRNA, and each detected band corresponded to its expected size. Neither bcl-2 nor bcl-xl mRNA expression was identified in either injured (lanes 4 and 8) or control (lanes 3 and 7) arteries. bax and p53 mRNA expression was clearly identified both in injured (lanes 6 and 10) and in uninjured (lanes 5 and 9) arteries, and the corresponding mRNA level was not altered after balloon injury. In contrast, mRNA of bcl-xs (lane 8) and caspase-1 (lane 12) was detected and was markedly induced only in the injured arteries. There was no significant difference in the mRNA level of G3PDH between injured and uninjured arteries (lanes 1 and 2).

**Immunohistochemical evaluation of Bcl-2, Bax and Bcl-x**

Protein levels of Bcl-2, Bax and Bcl-x were evaluated by an immunohistochemical technique using specific anti-

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**Figure 3** Ultrastructure of apoptotic VSMCs in superficial portion of neointima in balloon-injured carotid artery

Apoptotic VSMCs reveal (A) condensed chromatin or (B) irregular nuclear membrane. Magnification, ×14 400.

**Figure 4** mRNA expression levels of bcl-2, bax, bcl-x, p53 and caspase-1 in both injured and uninjured carotid arteries

RT–PCR products for G3PDH, bcl-2, bax, bcl-x, p53 and caspase-1 were analysed by electrophoresis on a 1.4% agarose gel. Lane 1, 3, 5, 7, 9 and 11 indicate the corresponding mRNA in uninjured arteries, and lanes 2, 4, 6, 8, 10 and 12 the mRNA in injured arteries. Reference markers are shown at both ends of the gel.
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**DISCUSSION**

In atherosclerosis, excessive accumulation of cells in the intima is believed to be the major cause of disease progression [9,10]. This accumulation is attributed to increased migration and/or proliferation of cells including VSMCs, monocytes/macrophages and T-lymphocytes [11,12]. On the other hand, many recent studies have demonstrated that dysregulated apoptosis also has a pivotal role in the pathogenesis and progression of atherosclerotic lesions in cardiovascular diseases [13,14]. Han et al. [15] examined 35 human atherosclerotic lesion samples and identified a substantial number of cells undergoing apoptosis in 25 of the samples (approximately 70%). Furthermore, they also reported that apoptotic cells were specifically identified in the neointima of rat balloon-injured carotid artery, and approximately 20% of the cells in the lesion were undergoing apoptosis 14 days after injury as shown by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) assay. In our experiments, balloon-injured carotid arteries showed maximum thickness of the neointima on the 14th day before endothelial cell regeneration. Therefore, we carried out morphological and biochemical evaluations of the neointima 14 days after injury. We also demonstrated by in situ DNA labelling that positive staining of nuclei was detected only in the neointima at the same time after injury. The frequency of cells showing positive staining by in situ DNA labelling was about 20% of neointimal cells as in Han et al. [15].

Electron microscopy was also performed on the neointima and confirmed the result obtained by in situ DNA labelling. The initial description of apoptotic changes is based on morphological features, especially nuclear or cytoplasmic condensation, nuclear membrane budding, cell fragmentation and ensuing phagocytosis of apoptotic bodies [16,17]. In the present study, electron microscopic evaluation indicated that VSMCs in the superficial portion of the neointima showed chromatin condensation or irregularity of the nuclear membrane, typically observed in the early phase of apoptosis. However, by transmission electron microscopy, the frequency of the cells showing typical apoptotic morphology was 2.9% of the neointimal cells. Bauriedel et al. [18] has demonstrated that the frequency of the apoptotic cell was 3.2% in atherectomy-derived restenotic lesions from human coronary arteries. The frequency of apoptotic cells by electron microscopy in their study is in agreement with our report. However, there is a dis-
crepancy between the apoptotic cell number obtained by in situ DNA labelling and that by electron microscopy. Kockx et al. [19,20] demonstrated that in addition to apoptotic nuclei, non-apoptotic nuclei that show signs of active gene transcription are also labelled by the in situ DNA labelling technique. Because VSMCs undergo high rates of proliferation and apoptosis in restenotic lesions after injury, in situ DNA labelling would overestimate the number of apoptotic cells. The correction estimation of in situ DNA labelling is fundamental for understanding cell kinetics in neointima.

Bochaton-Piallat et al. [21] demonstrated that VSMC apoptosis becomes important in the pathogenesis of neointimal formation 15 days after injury by endothelial denudation of the thoracic aorta in rats, and is detected exclusively in the superficial portion of intimal thickening. We also demonstrated that apoptotic VSMCs were detected exclusively in the innermost layer of the neointima by in situ DNA labelling and electron microscopy. These results suggest that similarity in the distribution of apoptotic cells is a common feature in the neointima of moderate and large arteries and is thought to be a cellular response to compensate for VSMC migration and/or proliferation. This may improve our understanding of the morphological changes in the process of vascular remodelling.

Recent studies based on molecular cellular biology also lead us to postulate that there exist two opposite cellular responses, cell proliferation and apoptosis, in the pathological process of various disorders. In fact, apoptosis is regulated by a genetic programme requiring both suppressors and inducers. Recent studies have demonstrated that both the caspase family and Bcl-2 family are implicated in the regulation of apoptosis in a variety of cells. Caspases, which belong to cysteine proteases, are key enzymes for induction of DNA damage and ensuing apoptotic cell death, and their function is positively or negatively modified by Bcl-2 family proteins including Bcl-2, Bax and Bcl-x. Miura et al. [22] have reported that overexpression of caspase-1 (also known as IL-1β-converting enzyme), which is a mammalian homologue of the nematode Caenorhabditis elegans cell death gene ced3, induces apoptosis in fibroblasts. Geng et al. [14] have reported that apoptotic cells are seen in the atheroma of human carotid artery and are co-localized with positive staining for immunoreactive caspase-1. In the present study we demonstrated that caspase-1 mRNA expression was detected and induced only in the balloon-injured carotid artery (Figure 4). Caspase-1 is thought to be mainly involved in cytokine activation. In restenotic lesion after balloon injury, a lot of cytokines such as transforming growth factor-β [23], interferon-γ [24] and platelet-derived growth factor [25] are involved in the pathogenesis of neointimal formation. Furthermore, it is reported that caspase-1 activation is associated with apoptosis by tumour necrosis factor-α [26] or interferon-γ [27]. Therefore, caspase-1 is considered to be primarily necessary for the induction of VSMC apoptosis in neointimal lesion [28,29].

The Bcl-2 family revealed a variety of mRNA or protein expression levels in the neointima of the rat carotid artery. Bcl-2, which belongs to integral membrane oncoproteins, is localized on the membranes of organelles including mitochondria, endoplasmic reticulum and nuclear envelope [30], and suppresses the induction of apoptosis by inhibiting caspase activity [31]. On the other hand, Bax, which has been identified as a Bcl-2-associated x protein, acts as an inducer of caspase activity, and the Bcl-2/Bax expression ratio determines cell survival or death after apoptotic stimuli [32]. In this study we demonstrated that both mRNA and protein of Bcl-2 were not detected in the neointima. This result is consistent with other recent reports [33,34].

In our study there was no difference in the mRNA level of bax between injured and uninjured carotid arteries, and immunohistochemical staining demonstrated that Bax protein was detected homogeneously in the neointimal lesion as well as in the intact media. We also measured the mRNA level of p53, which is thought to be a pivotal regulatory factor of Bax, and there was no significant difference in its expression between injured and uninjured carotid arteries. These results indicated that Bax is not directly involved in rat VSMC apoptosis in neointimal formation after balloon injury. Perlman et al. [35] also reported that Bax was homogeneously expressed in medial VSMCs in both injured and uninjured rat carotid arteries. However, Kockx et al. [33,34] demonstrated that Bax was up-regulated in both human and rabbit experimental atherosclerotic plaques. Our study and Perlman’s study were evaluating the neointimal lesion as a restenosis model, whereas Kockx’s investigations were performed in the atherosclerotic lesions. The role of Bax in induction of VSMC apoptosis may be different in the neointimal and atherosclerotic lesions.

Bcl-x, which is another member of the Bcl-2 family, is composed of two isoforms, Bcl-xs and Bcl-xl, translated respectively by a short-form (518 nucleotides) or a long-form (707 nucleotides) mRNA according to its alternative splicing from a single gene [36]. Furthermore, members of the Bcl-2 family are functionally divided into two categories, Bax/Bcl-xs and Bcl-2/Bcl-xl, on the basis of their ability to promote or suppress the apoptosis induced by caspases respectively. We previously demonstrated that the induction of apoptosis in rat cultured VSMCs was associated with induction of bcl-xs mRNA expression [37]. In the present study, we demonstrated that bcl-xs mRNA was selectively induced in the balloon-injured carotid artery (Figure 4), and positive staining for immunoreactive Bcl-x was co-localized with that of nuclei identified by in situ DNA labelling (Figure 5). We could not detect bcl-xl mRNA in the balloon-injured carotid artery. However, Pollman et al. [38] reported up-
regulation of Bcl-xl mRNA and protein expression in the intimal thickening of balloon-injured arteries using the high-cholesterol-fed rabbit model. Although we could not explain the reason for the difference in Bcl-xl expression, it may be due to experimental methods. Further studies are required to elucidate the role of the Bcl-2 family in VSMC apoptosis in cardiovascular diseases.

In conclusion, two opposite cellular responses, VSMC proliferation and apoptosis, existed together in the intimal thickening of the rat carotid artery after balloon injury, and Bcl-xs was a key player to switch VSMCs from proliferation to apoptotic cell death in this lesion.

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