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Examination of the Apoptotic Pathway and Proteolysis in the Pathogenesis of Popliteal Artery Aneurysms†

T. Jacob, A. Hingorani and E. Ascher*

Division of Vascular Surgery, Maimonides Medical Center, Brooklyn, New York, U.S.A.

Objectives: *to investigate the role of apoptosis, expression of death-promoting molecules and mediators of apoptosis in the development of popliteal artery aneurysms.*
Methods: *ten popliteal artery aneurysm (PAA) specimens were obtained from patients undergoing elective surgical repair. Normal controls were popliteal arteries obtained from patients without PAA undergoing infrainguinal bypass surgery (n = 8). Standard histochemistry techniques were used to assess elastic lamellae fragmentation and inflammatory infiltrate in PAA. Vascular smooth muscle cells (VSMC), macrophages, T lymphocytes, death-promoting molecules, CPP-32, Fas, p53, perforin, apoptosis-mediating Bcl-2 family proteins and apoptotic death substrate, poly(ADP-ribose) polymerase (PARP) were detected immunohistochemically. Detection of apoptosis was by TUNEL assay. Proteolytic activity was determined by 10% gelatin gel zymography.*
Results: *there is a conspicuous disruption and fragmentation of elastic lamellae in PAA as compared to normal arteries. Increased gelatinolytic activity was observed at 92, 84, 72 and 67 kDa in PAA tissues. There is a significant decrease of VSMCs in the PAA walls ($p = 0.02$). The control arteries had fewer CD68+ macrophages and CD3+ T cells in their media ($p < 0.01$). There was a significant increase in the number of cells undergoing apoptosis in aneurysmal tissue than in the normal vessels, ($p < 0.02$) as well as an increased expression of Bax, CPP-32, Fas, p53 and perforin.*
Conclusions: *the data confirm the architectural disruption of the PAA wall and illustrate an apparent biological response involving inflammatory infiltrate, apoptosis and signalling molecules capable of initiating cell death. In addition to compromising the mechanical integrity of the vessel wall, VSMC loss may contribute to imbalance in the protein profile, accelerating extracellular matrix degradation that could favour PAA development.*

Key Words: Popliteal artery aneurysms; Apoptosis; Proteolysis; Molecular mechanisms.

Introduction

Previous studies on aneurysms have primarily focused on the aetiology of the most common type of aneurysm, the abdominal aortic aneurysm (AAA). Changes in the extracellular matrix, proteolytic activity, cytokine expression, degrees of apoptosis,[1,2] and auto-immunoreactivity[3,4] between AAA tissue and atherosclerotic or normal controls have been well documented by multiple centers. These studies, in addition to epidemiologic data such as age, sex, MHC class distribution[5–7] suggest differences in the aetiology of AAA as compared to atherosclerosis. Based on these findings, some investigators have come to the conclusion that AAA may be caused by an auto-immune process. In contrast to the growing body of literature examining the aetiology of AAA, evidence of biochemical and molecular studies on the most common peripheral artery aneurysm, the popliteal artery aneurysm, is lacking. Since, there is a paucity of data investigating popliteal artery aneurysm (PAA) aetiology, we initiated this investigation into the basic changes taking place in PAA.

Deregulated apoptosis has been implicated as a fundamental pathogenetic mechanism in a variety of human diseases where faulty DNA repair mechanisms and cell cycle alteration are involved.[8–10] Apoptosis or programmed cell death is a normal process that counterbalances the cell proliferation by mitotic division. Programmed cell death plays a major role in determining the cellularity in tissues. Since loss of vascular smooth muscle cells, thinning of the tunica media and degradation of the extracellular matrix are the characteristics of aneurysmal arteries, we investigated the role of apoptosis in PAA development. The expression of death promoting proteins (CPP-32/YAMA/caspase3, Fas/APO-1/CD95, p53, perforin) by

* Please address all correspondence to: E. Ascher, Division of Vascular Surgery, Maimonides Medical Center, 4802 Tenth Avenue, Brooklyn, New York 11219, U.S.A.
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vascular smooth muscle cells, macrophages and T lymphocytes infiltrating the aneurysm wall, was examined. Presence of mediators of apoptosis (Bcl-2 family) and its downstream death substrate, poly (ADP-ribose) polymerase (PARP), was also investigated. The extracellular matrix and inflammatory infiltrate in PAA and normal popliteal arteries were compared.

Methods

Patients

Ten popliteal artery aneurysm (PAA) specimens were obtained from patients undergoing elective surgical repair. All were males with ages ranging from 48 to 81 (mean 67 years). Three patients had bilateral PAA and one had a history of AAA repair. The mean diameter of the PAA was 2.14 cm $\pm$ 0.6 cm (S.D.). Normal controls were popliteal arteries obtained from patients without PAA undergoing infrainguinal bypass surgery ($n=8$). The biopsies for normal controls were taken from areas of the artery that was grossly and histologically free of atherosclerosis as they were sites for anastamosis. These were matched for age, sex and major risk factors to aneurysm specimens. No patient had signs of local or systemic infection. No evidence of arteritis, mycosis or osteochondromas was noted on clinical or histological exam. None of the specimens were ruptured or underwent any other treatment besides open repair. Preoperative arterial mapping by duplex ultrasound assessment was performed on all patients. This investigation was undertaken with the approval of institutional review board of Maimonides Medical Center and the patients' informed written consent.

Tissue specimens

The entire PAA was removed and utilised for analysis. All artery specimens were divided into two portions of which one half was cryopreserved in liquid nitrogen immediately. The remaining portion was fixed in 10% neutral buffered formalin solution containing about 3.7% formaldehyde (w:v) and paraffin-embedded. Paraffin blocks contained five different segments of each specimen. Transversal tissue sections of 5 μm thickness were mounted on 3-aminopropyltriethoxysilane coated slides.

Histology

Routine haematoxylin and eosin staining was performed for histological evaluation of the specimens. Gomori's one-step trichrome staining was used to identify changes in collagenous connective fibres and to differentiate between collagen and smooth muscle fibers in both normal and PAA specimens. Verhoeff's elastic tissue stain (with van Gieson's stain to counterstain) was performed to assess the pathological changes in the elastin network in all the artery specimens included in the study.

Detection of apoptosis

Detection of apoptosis in the arterial tissue was performed by terminal deoxynucleotidyl transferase (Tdt)-mediated digoxigenin-deoxyuridinetriphosphate (dUTP) nick end-labeling of free 3′ OH DNA termini of fragmented DNA present in the apoptotic cells (TUNEL) using the ApopTag kit (Intergen, Purchase, NY, U.S.A.) with minor modifications. The ApopTag method is based on the specific binding of TdT to 3′ OH ends of DNA and the ensuing synthesis of polydeoxynucleotidyl polymer. The sections were deparaffinised and rehydrated by transferring the slides through the following solutions: xylene four times for 5, 5, 5 and 2 min, 100% ethanol two times for 7 min each, 95% and 70% ethanol for 4 min each and finally PBS for 5 min. Tissue sections were incubated in 3% citric acid to remove all small calcium containing vesicles that can be responsible for non-specific binding of the nucleotides.[11] Nuclei were stripped of proteins by incubation with 20 μg/mL proteinase K (Oncor, Gaithersburg, MD, U.S.A.) for 15 min at 45 °C. Slides were then washed in four changes of distilled water for 2 min each and air dried. The tissue sections were equilibrated in ApopTag equilibration buffer for 5 min after which TdT enzyme and digoxigenin-dUTP in the reaction buffer were added to cover the sections. The slides were incubated in humidifying chambers for 1 h at 37 °C. One negative control slide (per batch) was incubated in the absence of TdT enzyme. Positive control used was tonsil tissue. After end-labeling, the slides were immersed in stop wash buffer for 20 min at 37 °C. Blocking solution containing anti-digoxigenin antibody (sheep polyclonal) conjugated to fluorescein was applied on tissues and incubated for a further 30 min at 37 °C in humidifying chambers. The antibody solution was washed away with three changes of PBS for 5 min each. End-labelling was visualised after counterstaining with propidium iodide and observing

Table 1. Antibodies used for immunohistochemistry in PAA and normal arterial tissues.

Antibody	Clone/origin	Manufacturer	Titre	Antigen retrieval
Alpha actin	IA4	DAKO	1:50	Pressure cooker, 0.25 M Tris base buffer, pH 9.0
Bax	Rabbit polyclonal	Oncogene	1:20	Pressure cooker, 0.57 M urea
Bcl-2	124	Santa Cruz	1:200	Pressure cooker, 0.25 M Tris base buffer, pH 9.0
CD3	Rabbit polyclonal	DAKO	1:150	Same as above
CD8	C8/144B	DAKO	1:50	Same as above
CD20	L 26	DAKO	1:75	Same as above
CD30	Ber H8	Pharmingen	1:10	Same as above
CD68	KP-1	DAKO	1:6000	Pressure cooker, 10 mM citrate buffer, pH 6.0
CD95/Fas	DX3	DAKO	1:20	Pepsin, 10 min at 60 °C
CPP32/YAMA	Rabbit polyclonal	DAKO	1:200	Pressure cooker, 10 mM citrate buffer, pH 6.0
p53	DO-7	DAKO	1:600	Pressure cooker, 0.25 M Tris base buffer, pH 9.0
PARP	F-2	Santa Cruz	1:10	None
Perforin	Delta G9	Endogen	1:50	Pressure cooker, 10 mM citrate buffer, pH 6.0

the fluorescence under Zeiss Axiophot fluorescence microscope (Carl Zeiss Inc, Thornwood, NY, U.S.A.).

Zymography

Proteolytic activity was determined by 10% gelatin gel zymography under non-reducing conditions. Cryopreserved specimens were homogenised and dialysed overnight with saline and detergent as previously described.[12,13] Total protein in the samples was determined by Bradford's technique using a protein assay kit as per the manufacturer's instructions (Pierce Chemical Co, Rockford, Ill, U.S.A.).[14] Equalised samples were used for zymography under non-reducing conditions by 10% gelatin gel SDS-PAGE (Biorad, Hercules, CA, U.S.A.) as per the manufacturer's instructions.[15]

Immunohistochemistry

Serial formalin-fixed paraffin-embedded tissue sections were deparaffinised and rehydrated by sequential immersions in four changes of xylene followed by baths of 100%, 95%, 70%, 50% ethanol and phosphate-buffered saline (PBS). After protease digestion, the antigens in the arterial tissues were unmasked by heat treatment in the presence of suitable buffers/enzymes as shown in Table 1. After cooling, the endogenous biotin activity in the tissue sections was blocked by immersing the slides in dilute egg white solution, for 15 min at room temperature (RT). Following several rinses with tap water, the slides were placed in skim milk for 15 min at RT, rinsed with tap water and PBS containing 0.0001% Tween 20 (PBS/Tween). Tissue sections were incubated with primary antibodies at appropriate dilutions (Table 1) for 1 h at RT. Biotinylated secondary link antibodies (DAKO, Carpinteria, CA, Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) were added to the specimens and incubated for 30 min at RT. The endogenous peroxidase activity was quenched by immersing the slides in 0.3% H_2O_2 in PBS for 10 min. The bound primary antibodies were amplified by LSAB2-horseradhish peroxidase-labelled streptavidin complex and detected with 3, 3′-diaminobenzidene (DAB) substrate (DAKO). The reaction products were developed in DAB solution, and stain development was stopped by immersion of slides in tap water and then in PBS/Tween. Chromogenic enhancement was accomplished by incubating the specimens in 0.5% copper sulphate in PBS/Tween for 5–8 min at RT. Subsequently, the slides were counterstained with Mayers' haematoxylin for 1.5 min. Tissue samples were dehydrated and coverslipped with mounting medium. A section of a multitumour sandwich block (containing up to 79 different tumours in one block), placed on one end of each slide, served as positive control. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody and substituting it with an unrelated antibody at the same dilution. For tissue authentication, serial sections of each specimen were also stained with haematoxylin and eosin.

Quantification

Cells only positively stained by TUNEL and containing apoptotic bodies were referred to as "apoptotic cells". Thus, multiple criteria were used to identify apoptotic cells, TUNEL staining and morphological markings including chromatin condensation. Cells with these features have been confirmed to be apoptotic by electron microscopic analysis in previous studies by us[16] and others.[17] Ten random fields per specimen were

examined under a fluorescent microscope at high magnification ($\times 1000$) and apoptotic cells were counted manually. A total of 1000 cells were counted in each specimen. The expression of proteins was evaluated according to staining intensity and immunocytochemical distribution–cytoplasmic and nuclear staining, based on the results of two independent blinded investigators. The entire section for each specimen was scanned using the $20\times$ magnification to estimate the subjective level of staining. Immunoreactive cells were counted manually at $400\times$ magnification and quantified in six random fields per section. Five sections per specimen were analysed.

Statistical analysis

The data obtained were analysed by Student *t*-test and Chi-square. Fisher's Exact test was used to compare the results obtained in the different groups. *P* value of <0.05 was considered statistically significant. Statistical analyses were performed using Winks 4.21 program (Texasoft, Cedar Hill, CA, U.S.A.) and StatView software (SAS Institute, Cary, NC, U.S.A.).

Results

Histology

Histochemical studies revealed conspicuous disruption and fragmentation of elastic lamellae in PAA as compared to normal arteries. Consistent with our previous report and those of other investigators, PAA specimens exhibited a more disorganised architecture as compared to normal arteries. Gomori's trichrome staining showed visibly reduced collagen fibers in PAA as compared to normal arteries. Verhoeff's elastin staining demonstrated increased degradation of elastin network. Disruption in the elastic lamellae and increased proteolysis in PAA was evident (Fig. 1). PAA demonstrated significant increase in inflammatory cells as compared to the control arteries ($p<0.01$). A conspicuous decrease in smooth muscle cells was noted between PAA and normal tissue. Whether this may be due to a non-differentiated cell status is unclear.

Zymography

Increased gelatinolytic activity was observed at 92, 84, 72 and 67 kDa in all of the PAA tissues (Fig. 2).

Apoptosis

Apoptotic cells were identified in all the PAA specimens (100%). A significant increase in the number of cells undergoing apoptosis in aneurysmal tissue than in the normal vessels was observed. There were $10.2\% \pm 1.4\%$ apoptotic cells in the PAA tissues as compared to that of $1.5\% \pm 0.4\%$ cells in the control arteries ($p<0.02$). Apoptosis was predominantly localised to the inflammatory infiltrate. There was significant amount of VSMC apoptosis. Figure 3 demonstrates apoptosis in inflammatory cells and VSMCs. Anti-actin immunostaining demonstrated a significant decrease of VSMCs in the PAA walls ($p = 0.02$, Fig. 1).

Molecular mediators of apoptosis

Immunohistochemical studies on PAA specimens revealed significantly increased expression of apoptosis mediators bax, CPP-32 and Fas as compared to normal popliteal arteries ($p<0.02$). There was no significant difference in the expression of bcl-2, a known negative regulator of apoptosis. P53 expression was also significantly upregulated in aneurysm specimens ($p<0.001$). PARP, a downstream substrate for apoptosis, was present in more cells of PAA as compared to normal arteries (Figs 4, 5). Perforin expression was observed only in the inflammatory infiltrate and was significantly upregulated in PAA tissues ($p<0.01$). Only aneurysmal arteries showed CD8+ T cells expressing death-promoting molecules. VSMCs of PAA show presence of markers of apoptosis and signalling molecules capable of initiating cell death. The control arteries had fewer CD68+ macrophages and CD3+ T cells in their media. CD3+, CD8+, CD20+, CD30+ and CD68+ immunoreactive cells were significantly more in the aneurysmal tissues than in the normal popliteal arteries ($p<0.01$, Fig. 6). The inter and intra observer variation for these data was noted to be <2%.

Discussion

The present investigation compares the presence of apoptosis-related proteins that affect cell turnover, extracellular matrix and inflammatory infiltrate in normal and PAA tissues. A higher amount of cells undergoing apoptosis was apparent in PAA tissues. In prior studies it has been noted that apoptosis occurs in a low percentage of cells in normal arteries.[2] We observed that

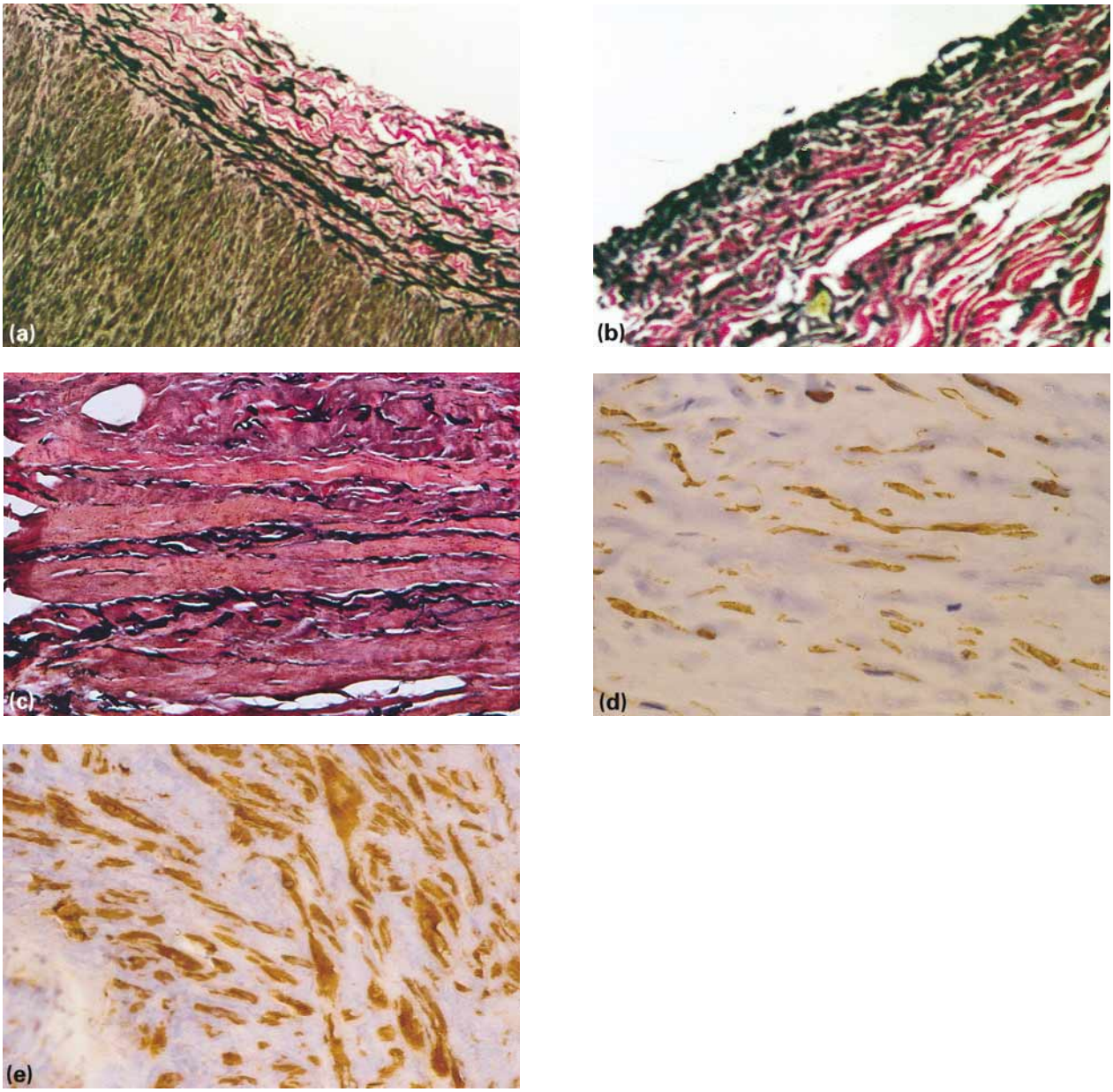

Fig. 1. Representative photomicrographs of histology and immunohistochemistry in arterial tissues. (a) Van Giesson staining for elastin in normal arteries (original magnification 100 ×). (b) and (c) Elastin staining in popliteal artery aneurysm tissues. Note the fragmentation of elastic lamellae (original magnification 400 ×) (d) Rarefaction of VSMCs identified by anti-alpha actin immunostaining. Brown colour (peroxidase staining) indicates cells positive for smooth muscle cell actin (original magnification 400 ×). (e) VSMCs in control arterial tissue (original magnification 400 ×).

immunoreactivity to pro-apoptotic mediators was significantly higher in the aneurysmal arteries. Our data reveal increased presence of inflammatory cells and matrix disruption in PAA, consistent with previous reports on the pathogenesis of AAA.

Other investigators have long recognised a link between generalised arteriomegaly and aneurysms.[18,19] In addition, patients with AAA have been noted to have aneurysms in a variety of other beds.[20] These would suggest that a generalised or multifocal process might be contributing to the development of these aneurysms at least in some patients. In the past, atherosclerosis was felt to be the main aetiologic factor perhaps due to the not infrequent finding of atherosclerotic changes within aneurysms. Recent data investigating the histology, biochemistry, and molecular biology of aneurysm disease and atherosclerotic disease have called this association into doubt and suggested an inflammatory or autoimmune aetiology involving proteolysis, matrix destruction and

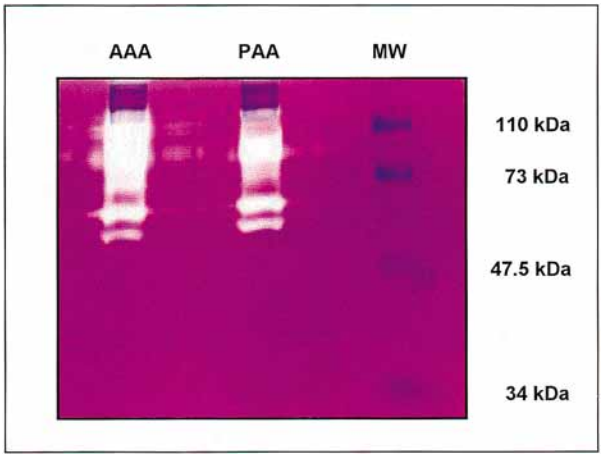

Fig. 2. Representative gelatin gel zymogram. 10% gelatin gel zymography was performed with arterial tissue extracts under non-reducing conditions as described in the methods section. Popliteal aneurysm tissue extracts demonstrated proteolytic activity comparable to the gelatinolytic observed in abdominal aortic aneurysms (AAA).

apoptosis.[2,21–23] Our preliminary data on PAA suggest that a similar phenomenon may be taking place in these aneurysms. Therefore, a unifying aetiology that may link aneurysms of possibly many types is suggested. The histological data noted in PAA in this study suggest that the unfavourable balance between destruction and repair of connective tissue proteins noted in AAA[24] may also play a role in PAA.

The half-life of apoptotic cells is only a few hours. Previous reports have emphasised that a small proportion of apoptotic cells visualised in tissue sections can represent a considerable magnitude of cell loss.[25] Hence, by quantifying apoptotic cells in the PAA tissue at a specific time, we may be underestimating the contribution of apoptosis to vascular remodelling and the disease process. The TUNEL assay detects apoptotic DNA cleavage that actually is a rather late stage of programmed cell death. Apoptosis occurs in at least two stages.[26] After a signal, which may be either intrinsic or extrinsic to the cell, the cell enters a committed phase. This is terminated in cell autonomous fashion by a transition to a final execution phase that includes DNA fragmentation and is brief and decisive.[27] We used the expression of CPP-32 and other mediators of apoptosis to detect the committed phase. Since apoptosis plays a major role in tissue homeostasis and cell number maintenance, it could influence PAA development. PAA wall layer cells may become dysfunctional due to the deregulation of cell cycle and apoptosis.

Apoptosis is a multifactorial phenomenon, and there exist multiple death pathways inducible by different stimuli. Expression of death promoting molecules investigated in this study correlate with the degree of apoptosis observed and with their spatial and topographical organisation. P53 detected in the PAA tissues studied, has been long identified as a promoter of programmed cell death and known for its "gatekeeper" role in cell cycle regulation.[28] Presence of the death-promoting perforin in the inflammatory infiltrate could have a bystander effect and promote apoptosis of the cells in its vicinity. Caspase 3 is a marker for early apoptotic phase. The significantly increased expression of CPP-32 indicates that several cells in the PAA are committed to programmed cell death. The presence of CPP-32 is predominant in the infiltrating cells of the vessel wall. A similar observation was also made in case of Fas which is a known death domain protein that is activated by FasL ligand in the apoptotic pathway. Thus, the presence of several death-promoting molecules of the apoptotic cascade identified in the PAA, implicate that apoptotic cell death may have a significant role in the development of popliteal aneurysms.

Programmed cell death may be positively or negatively regulated by the members of the expanding

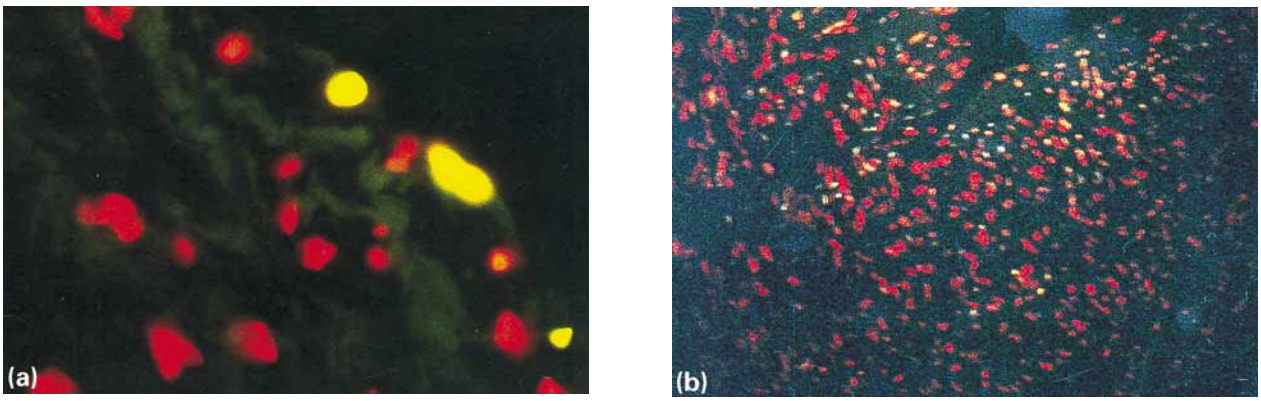

Fig. 3. Representative photomicrographs of TUNEL assay for detection of apoptosis. (a) TUNEL positive cells in PAA wall are the fluorescing cells (original magnification 1000 ×). (b) TUNEL positive cells in PAA tissues at low power (original magnification 100 ×).

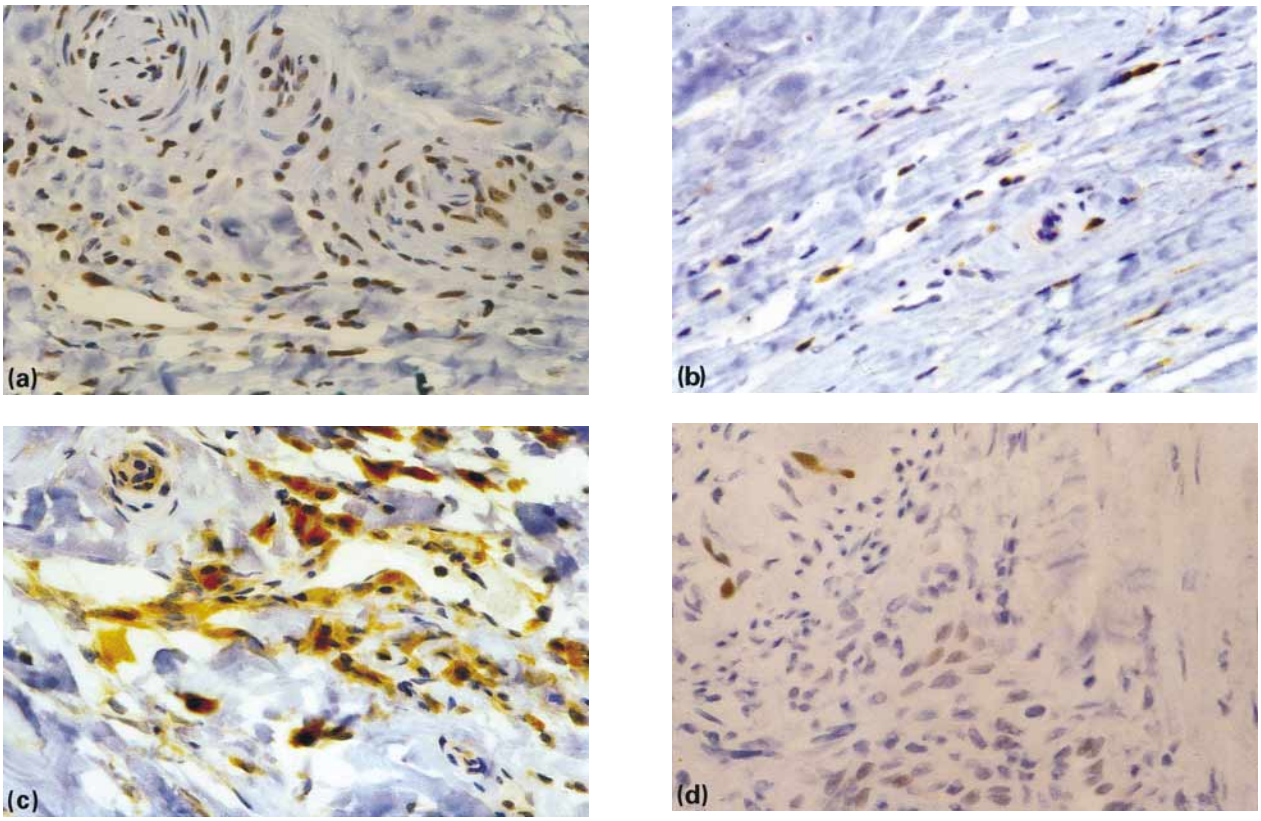

Fig. 4. Representative photomicrographs of immunohistochemical analysis for expression of apoptosis-related molecules. Formalin-fixed paraffin-embedded tissues were processed for localisation of apoptosis-related molecules as described under Methods. Brown staining (peroxidase) indicates immunopositivity. (a) Anti-bax staining in PAA tissue. (b) Anti-bcl2 staining in PAA tissue. (c) Anti-CPP 32 staining in PAA tissue. (d) Anti-p53 staining in PAA tissue (original magnification 400 ×).

bcl-2 family. While bcl-2 is a negative modulator, rescuing cells from undergoing programmed cell death, bax is an apoptosis inducer.[29] Bax is also a downstream effector of p53 known to promote growth arrest via the p53 pathway. In the PAA wall layers, we observed a high incidence of bax expression in VSMCs as well as infiltrating cells. This overexpression of bax is consistent with the apoptosis observed. The activity of bax is such that when overexpressed it is able to counter bcl-2 activity by promoting apoptosis. Bax expression can activate a common pathway of apoptosis either caspase-dependent or caspase-independent.[30] Moreover, although not examined in this report, other bcl-2 family members that interact to form heterodimers, such as recently described bag-1, bim, noxa and other proteins may be involved in the regulation of apoptosis in the vessel wall. Therefore, even if Bcl-2 overexpression protects many cell types against apoptosis, it also depends which other proteins of this family are expressed simultaneously. Also, we are not sure whether Bcl-2 rescues the vascular cells from programmed death as several investigators have failed to locate its expression in arterial tissues. Hence, the significance of bcl-2 expression in this study remains unclear.

Our data indicate an increased presence of PARP in the aneurysmal tissues, as compared to the control group. Although we could not detect this death substrate in the aneurysmal tissues in a uniform manner, this is an important finding. This may be due to the potentially multi-focalised nature of PAA. PARP is a macromolecular downstream substrate of apoptosis. Proteolytic cleavage of key substrates is an important biochemical mechanism underlying the apoptotic process.[31] PARP, the first protein noted to be degraded into specific fragments in apoptotic cells, is an enzyme involved in genome surveillance and DNA repair.[32] Cleavage of PARP into its 89 kDa (85 kDa) form, considered a hallmark of apoptosis,[33] results in the separation of its important functional domains from the rest of the molecule.[34]

Signalling events in vascular remodeling and development of PAA has not been the focus of much investigation. We tried to elucidate the molecular mechanisms involved in the pathogenetic process of PAA formation by studying the expression of mod-

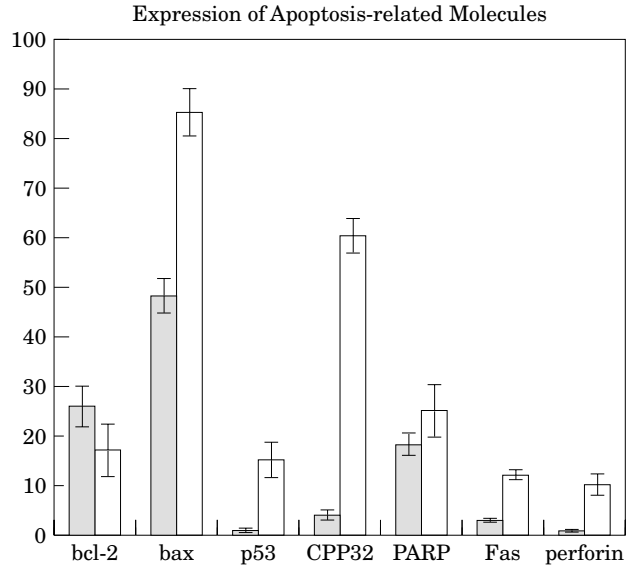

Fig. 5. Comparison of the percentage of cells expressing apoptosis-related molecules in the PAA and normal tissues. All values are expressed as mean ± SEM. (■) Normal; (□) PAA.

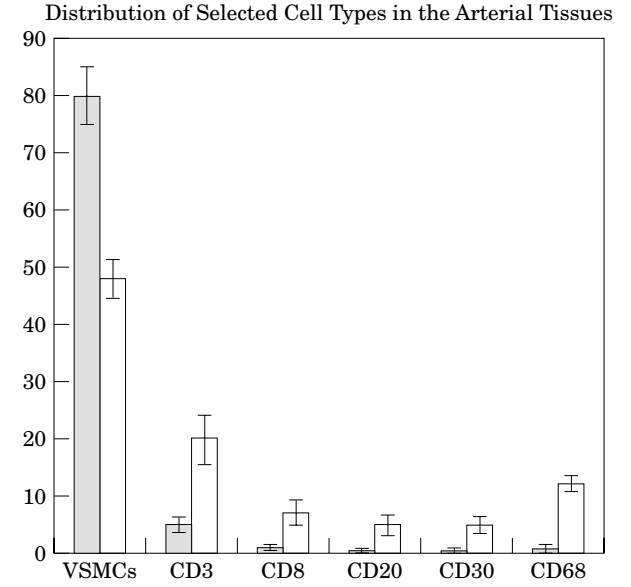

Fig. 6. Comparison of the percentage of selected types of cells identified in the PAA and normal tissues. All values are expressed as mean ± SEM. (■) Normal; (□) PAA.

ulators of apoptosis and death substrates in normal and popliteal aneurysmal arteries. We were convinced that localising the expression of apoptosis-related molecules, their spatial organisation and topographical relationship, by immunohistochemical techniques had more potential than quantifying their total content in the tissue. However, this study does not address the question whether apoptosis is the cause or effect of aneurysm formation, as we could not obtain any "potential" aneurysm specimens. Hence, further work is mandatory to better elucidate the molecular pathways that lead to the pathogenesis of PAA. Additional areas for future study include further identification of the proteolytic activity observed and mRNA studies for signalling molecules in the apoptotic cascade.

In conclusion, we have observed disruption in the elastic lamellae and increased proteolytic activity in PAA. VSMCs of PAA show presence of markers of apoptosis and signalling molecules capable of initiating cell death. Programmed cell death may contribute to the rarefaction of cells in the wall layers of PAA. This loss of VSMCs may contribute to imbalance in the protein profile, causing extracellular matrix degradation. This study indicates that the inflammatory infiltrate expressing death-promoting proteins in the aneurysm tissue may have an essential role in the pathogenesis of aneurysmal disease.

Acknowledgements

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