Time Course of Apoptosis and Proliferation in vascular Smooth Muscle Cells after Balloon Angioplasty

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Abstract

Aim of our study was to evaluate SMCs changes in the media and neointima of the vessel wall at different time intervals after balloon injury in a rabbit animal model of intimal hyperplasia, with special reference to the relation between apoptotic cell death and SMC hyperplasia.

Forty male New Zealand White rabbits were used for this study. Wall injury was induced on the rabbit iliac artery with a Fogarty catheter (3F). The animals were sacrificed at 30 minutes, at 24 , 48 and 72 h, 7 , 14 , 21 and 30 days. Serial sections were assessed for light microscopy (histological, immunohistochemical, molecular and morphometric analysis) and for Transmission Electron Microscopy.

An high apoptotic index of SMCs was observed in the injured iliac arteries at 30 min, 24 h and 48h; though decreasing, this value remained high at 72 h when a high SMC proliferation index (MIB 1 positive cells) in the media was observed (64.3%). At 7 days intimal hyperplasia was detectable (13.24%), proliferation index appeared decreased (15.2%) and apoptosis was greatly reduced (0.60%). At 21 days SMCs showed an intermediate phenotype shifting from the contractile to the synthetic type, interstitial collagen was produced and the proliferation activity significantly reduced. At 30 days very few apoptotic cells were detected in the vessel wall.

Conclusions

Apoptosis could be viewed as the main cause of early SMCs loss after balloon injury. Apoptotic cells are detected in all the animals suggesting that apoptosis has a role both in the early and late response to vascular injury. Cell proliferation activity was maximal in the early period (between 48 and 72 hours) suggesting that this may be the optimal window for pharmacologic intervention aimed to modulate the remodelling process. Apoptosis is still present with fairly high figures at 72 hours suggesting that the phenomenon is a major determinant of vessel «remodelling». Later on when signalling for growth stops, minimal levels of apoptosis still persist, suggesting that this mechanism could also modulate the cellularity of the lesion.

Key words: apoptosis, intimal hyperplasia, proliferation kinetics, restenosis, smooth muscle cells.

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Restenosis continues to be a vexing and expensive complication of percutaneous transluminal angioplasty accounting for late failure within six month from the procedure in 40-50% of patients. There is an open debate on the mechanism of restenosis; intimal hyperplasia (IH), has been advocated as the predominant phenomenon [7, 27, 30, 31, 36-38] although remodelling and recoiling have been hypothesized to play a role in the process as well [19, 35]. The understanding of these processes is of paramount importance in the treatment of restenosis.

Multiple strategies have been employed clinically and or experimentally to inhibit recurrent intimal hyperplasia (IH), the predominant component of restenosis, either blocking proliferation or enhancing apoptosis, a genetically determined mechanism of cell death. Pharmacological and new interventional strategies have been targetted to stop IH and restenosis [16, 23, 28, 41, 44].

The proliferation activity starts immediately after balloon injury and persists for few weeks accounting for media repopulation and accumulation of SMCs in the intima. At the same time apoptosis has been shown to be present. There are many studies dealing with vessel wall proliferation [7, 8, 10, 18, 21, 30, 31, 38, 43], and apoptosis after balloon injury [4-6, 15, 17, 25, 34] but only few have been recently published on the combined role of the two phenomena over a prolonged period of time in a porcine animal model of balloon injury [20, 29, 40].

In this study we investigated the time course of apoptosis and of proliferation after hyperacute lesion, in a different experimental animal model of balloon angioplasty. The aim was to shed further light on the dynamic of wall disruption, cell death, and proliferation leading to intimal hyperplasia, including early and late events.

Materials and methods

Animal model of arterial injury

Forty New Zealand White male rabbits (weight 3.3-3.5 kg), kept on a normal diet were studied. All the animals were pre-anaesthetized with intramuscular injection of ketalar (25 mg/kg) and a mixture of atropine (40 μg/kg) and chlorpromazine (15 mg/kg). All the animals were anaesthetized with ketalar (15 mg/kg) and diazepam (5 mg/kg) ev. The area was prepared aseptically and the superficial femoral artery was surgically exposed by blunt dissection, and arteriotomy was performed by introducing a 3F Fogarty arterial embolectomy catheter (Baxter Health Care Corp., Edward's Div., Irvine, Calif.) into external iliac artery. Before inducing the intra-arterial lesion all animals received intravenous heparin in a bolus of 100 U/kg. After the procedure, no anticoagulants or aspirin have been given. Arterial injury was obtained with the inflation of the balloon by introducing 70 μl of distilled water into the catheter, and pulling back the balloon against resistance by continuous inflation and deflation. This procedure was repeated 4 times, rotating the balloon 90 degrees every time in order to get a well distributed lesion along the circumference of the artery. After the procedure, the arteriotomy was tied off with a 2.0 silk suture and the subcutis was closed with a 3.0 silk suture. Antibiotics were given immediately after surgery. All animals recovered from the procedure without problems.

The animals were sacrificed (by an overdose of pentobarbital) at 30 minutes, 24, 48 and 72h, 7, 14, 21 and 30 days. Harvesting of normal and injured iliac arteries was done after flushing with saline and perfusion fixation in situ at 100±10 mmHg for 30 minutes with 4% solution of paraformaldehyde. The noninjured controlateral artery was used as control. Serial sections of each arterial segment were assessed for light and electron microscopy.

The animal protocol used in this study was approved by the institutional animal care of the Department of Biology, University of Padua.

Histology and immunohistochemistry

The arterial sections, after fixation, were progressively dehydrated in alcohol, and embedded in paraffin. Thin sections from uninjured and injured arterial tissue (~4μm) were stained with haematoxylin and eosin and Van Gieson.

Immunohistochemistry for actin (DAKO 1:100), desmin (DAKO 1:50), vimentin (DAKO1:100), Factor VIII (DAKO 1:100) and MIB 1, an antibody specific for Ki 67 antigen in paraffin-embedded tissue [24], (DAKO 1:50) was performed.

The specimens were analysed morphometrically using a computerised image analysis system. The internal elastic lamina (IEL) and the lumen (L) were delineated manually and the areas were automatically measured. The intimal hyperplasia was defined as the ratio of IEL-L/IEL [11].

In situ nick-end labelling

Terminal deoxinucleotidyl transferase-mediated d-UTP nick-end labelling (TUNEL) was used to detect DNA fragmentation [12]. Proteins were stripped from the nuclei of arterial sections by incubation in proteinase K (20 μg/ml; Boehringer Mannheim) for 10 min at room temperature. TdT (0.3e.u./ μl) and biotin-16-UTP (Boehringer Mannheim) in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) were added to sections before incubation in a humid atmosphere at 37°C for 60 min. Prior to diaminobenzidine staining, the sections were incubated with avidin and biotin-horseradish peroxides complex.

All solutions were prepared using DNAse-free distilled water and molecular biology grade reagents to avoid false positive results due to DNAse contamination. Sections pre-treated for 10 min with 0.7 mg/ml DNAse I (Boehringer Mannheim) in sodium cacodylate buffer (pH 7.2) served as a positive controls. After thorough washing in distilled water, the slides were processed according to the above protocol. TdT was omitted for the negative controls.

The apoptotic index (AI) was calculated on each sample by counting the number of TUNEL positively stained nuclei and dividing it by the total number of cells (%). The data were expressed as mean ± standard deviation. Apoptotic bodies (AB) were not included in the count of AI.

Transmission electron microscopy

Arterial sections of uninjured and injured vessels were fixed in Karnowsky's solution (2% PF, 2% GTA in Millonig, pH 7.3) for 24 h, post fixed with 1% osmium tetraoxide (Millonig, pH 6.8) for 1h, and then progressively dehydrated in alcohol and embedded in epon. Semithin sections were stained with 0.1% toluidine blue for light microscopic examination. Ultrathin sections were stained with uranyl acetate and lead citrate for

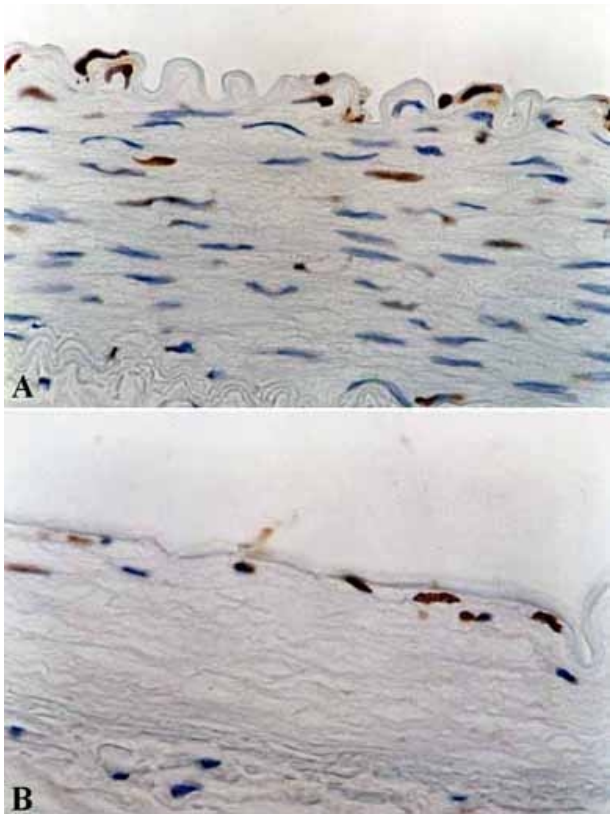

Figure 1. Histological sections of the iliac artery from an animal sacrificed early after injury at 30 min (a) and at 24 hours (b). a) The SMCs of the injured vessel showed a diffuse TUNEL positivity. TUNEL, Magnification 800X. b) Only few SMCs are present in the media and some of them are TUNEL positive. TUNEL, Magnification 800X.

transmission electron microscopy, performed using a Hitachi H-7000 (Hitachi Ltd., Tokyo, Japan).

Statistical analysis

All results were expressed as mean ± SD. Linear regression analysis was used when appropriate. A 5% difference was considered statistically significant.

Results

Occurrence of apoptosis

Light microscopy examination

The iliac arteries which underwent balloon injury showed at 30 minutes, 24 h, 48 h and 72 h clear signs of endothelial disruption together with fragmentation of the internal elastic lamina (IEL), and stretching of the medial SMC with loss of nuclei and condensation of chromatin in the few remaining ones (fig. 1). The phenomenon was sometimes circumferential, some others semicircumferential; cell disappearance was in some cases transmural, when it was diffuse to the whole wall thickness, or patchy when only scattered foci were involved.

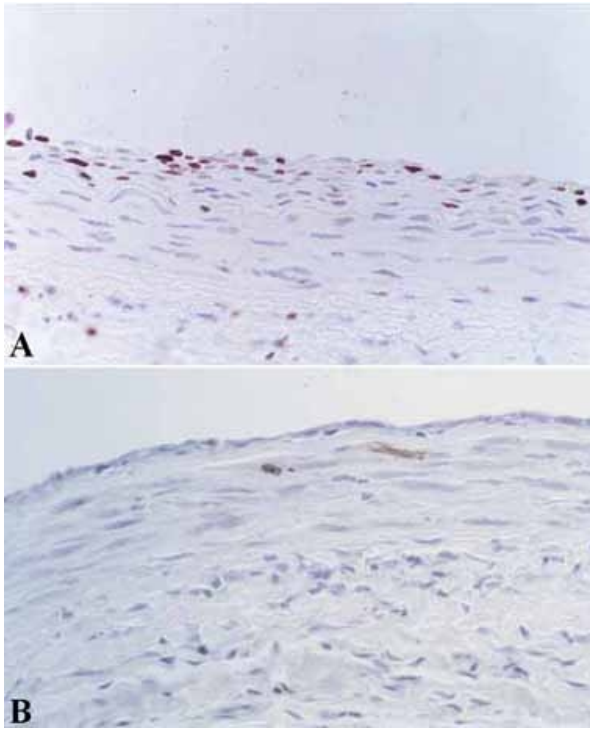

Figure 2. Histological sections of the iliac artery from an animal sacrificed at 7 days. a) The MIB 1 positivity of SMCs both in the intima and in the media indicate that active proliferation is occurring. MIB 1 stain, Magnification 400X. b) Few apoptotic cells are seen in the media underneath the internal elastic lamina. TUNEL, Magnification 400X.

Later on the vessel healing process took place with reendothelization and SMC proliferation, both in the media and in the intima (fig. 2). The media appears repopulated by an increasing number of SMCs which counterbalances the initial loss: at 14 days the site of the primitive lesions could be identified only because of the persistence IEL fragmentation or disruption. Sometimes the media thickness is decreased.

TUNEL (TABLE 1)

A large number of TUNEL-positive nuclei was detected among the residual SMCs of the vessel wall all along the wall circumference. There was no preferential site for TUNEL positivity (fig. 1b).

The percentage of TUNEL-positive SMCs was 55.2% ± 16.05 at 30 minutes, 55.5% ± 9.75 at 24 hours, 20.4% ± 14.9 at 48 hours and 24.43% ± 15.21 at 72 hours. At 7 days the positive response was sharply decreased to 0.60% ± 0.14 (fig. 2b), reaching 1.13% ± 0.89 at 14 days. At 21 and 30 days the number of TUNEL positive SMCs was minimal, (0.16% ± 0.03 and 0.32% ± 0.22 respectively).

Transmission electron microscopy

At 30 min and 72 h after balloon injury no intact endothelial cell lining could be detected in any specimen (fig. 3) SMCs were predominantly of elongated shape with large nuclei, narrow cytoplasmic borders and extended processes. The vast majority of SMCs showed different stages of typical apoptotic deterioration, including early chromatin condensation (fig. 3b, c), membrane budding, and apoptotic bodies, whereas the morphology of organelles membranes was not altered. Although a quantitation could not be performed, the number of apoptotic nuclei was extremely high confirming the TUNEL results.

Sections of uninjured rabbit arteries contained SMCs with normal-appearing nuclei and no detectable cytoplasmic shrinkage.

Arteries from rabbit sacrificed at 7 days frequently showed denudation of the luminal surface. Apoptotic cells were less frequently detected than in the previous group (fig. 4). At 20 and 30 days after injury, apoptotic cells were sporadically seen and apoptotic bodies showed various stages of degeneration inside cells of otherwise normal morphology (fig. 5).

Proliferation activity and vessel wall remodelling

Light microscopy

The pattern of intimal hyperplasia was characterized by SMC (actin positive cells) with different orientation, organized in two layers and restitution of medial cellularity. Closer to the IEL the SMC were oriented along the longitudinal axis of the vessels while in the outer part, closer to the lumen, they were orientated along the transverse axis. The same was found in the media where SMC closer to the IEL were oriented longitudinally in the attempt to migrate through the IEL. Although SMC proliferation, was the main feature of restenosis in few instances we also found mural thrombi attached to the vessel wall in different stages of organisation, which also contributed to worsen the degree of lumen patency.

In our series of observations the proliferative activity begins after 48 hours with initial reendothelization. At this time in the media MIB1, a marker of SMC proliferation [40], was found to be positive in 17.78% ± 10.20 of cells (TABLE 1). At 72 hours the proliferative response reached 64.3% ± 0.57. The proliferation index decreased rapidly to 6.3% ± 6.2 at 7 days stabilising round 10% at 14 days and persisting at 21 and 30 days round 2%.

Electron microscopy

At day 7 most of the SMCs present both in the media and the intima were found to be of the intermediate type between the contractile and synthetic phenotype, with moderate amount of rough endoplasmic reticulum (RER) and myofilaments mainly located in the periphery of the cytoplasm often enveloped by basement membrane-like material. At day 20, the luminal surface of the majority of arteries was covered by regenerated endothelial cells which were connected by a small number of junctions. At this point in time the prevalent pattern of SMCs was the synthetic one as demonstrated by the increased density of cytoplasm, dilatation of the endoplasmic reticulum, variable amount of mitochondria whereas microfilament bundles and invaginations of plasma membrane were located peripherally (fig. 5a, b); the intercellular space was oedematous, with occasional collagen fibres and ground substance. Complete reendothelization was observed 30 days after balloon injury. A large amount of collagen fibres between SMC, prevalently of synthetic type were

Table 1. Apoptosis, proliferation activity, and intimal hyperplasia (mean ± SD) in all the animals at each time point.

	30 min	24 h	48 h	72 h	7 days	14 days	21 days	30 days
AI*% media (mean ± SD)	55.23±16.05	55.5±9.75	20.44±14.92	24.43±15.21	0.8±0.91	1.14±1.03	0.26±0.25	0.18±0.24
AI*% intima (mean ± SD)	0.0	0.0	0.0	0.0	0.72±0.88	1.1±1.1	0.06±0.11	0.28±0.25
MIB†1% media (mean ± SD)	0.0	0.0	17.78±0.57	64.33±6.22	6.3±6.22	10.08±7.01	1.81±2.96	2.56±2.15
MIB†1%intima (mean ± SD)	0.0	0.0	0.0	0.0	24.82±15.27	8.42±7.34	5.44±2.21	6.76±3.93
AI* % TOT (mean ± SD)	55.2±16.05	55.5±9.7	20.44±14.9	24.43±15.21	0.60±0.14	1.13±0.89	0.16±0.03	0.32±0.22
MIB† 1% TOT (mean ± SD)	0.0	0.0	17.78±10.2	64.33±0.5	15.16±9.2	8.36±6.88	4.1±2.19	5.25±3.13
IH‡ % TOT (mean ± SD)	0.0	0.0	0.0	0.0	13.24±3.09	21±3.88	50±27.52	40±6.65

* AI % = apoptotic index, % of TUNEL positive cells;
† MIB 1 % = proliferation index, % of MIB 1 positive cells;
‡ IH % = % cross sectional area, IEL-L/IEL

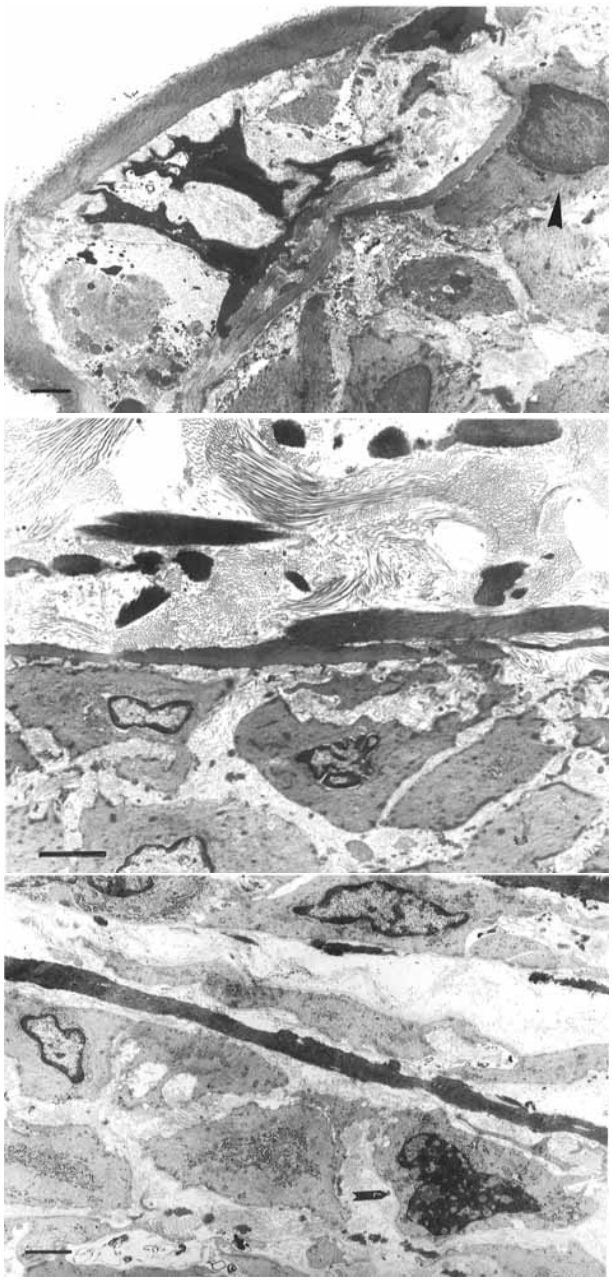

Figure 3. Transmission electron micrograph of arteries from animals sacrificed at 30 min. and 72 hours. a) Complete endothelial denudation and late ultrastructural changes of apoptosis of SMC; for comparison no such changes are evident in the adjacent cells (arrow). Note the duplication of the IEL due to the balloon injury. (9 mm = 2 μm). b and c) Two muscle cells in the lower left corner illustrate ultrastructural changes of apoptosis including compaction of nuclear chromatin and cytoplasmic condensation (arrows). In the lower right corner a typical «mazelike» features of apoptosis is viewed (arrow). (14 mm = 2 μm; 11 mm = 2 μm).

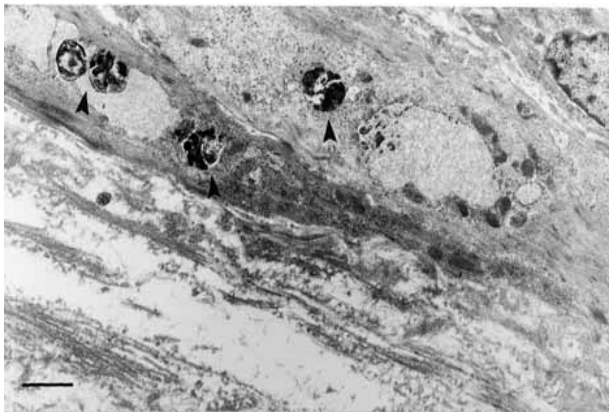

Figure 4. Transmission electron micrograph of an artery from an animal sacrificed at 7 days. Apoptotic bodies showed various stages of degeneration inside smooth muscle cells. (12 mm = 1μm).

observed in all the samples (fig. 5c).

Correlation between TUNEL positivity, MIB1 positivity and intimal hyperplasia (fig. 6)

The time course of TUNEL and MIB1 positivity in the media and intima and their relationship are summarized as follows:

Media (fig. 7)

We found TUNEL SMCs positivity in the media immediately after balloon injury at 30 min, and the phenomenon persisted with the same magnitude at 24 h to progressively decay to 50% of the initial value at 48 and 72 hours. At 7 days the number of TUNEL positive nuclei dropped dramatically to a value of 1.16% ± 0.85 to remain stable round similar values at days 14, 21 and 30. MIB1 positivity was 17.7% ± 10.2 at 48 h. A peak of positivity was reached at 72 h with 64.3% ± 0.5. At 7, 14 , 21 and 30 days proliferation activity decreased to 6.3% ± 6.2, 10.08% ± 7.0, 1.8% ± 2.9, 2.5% ± 2.1 respectively. This phenomenon could account for restoration of media cellularity.

Intima (fig. 8)

In the intima MIB1 positive cells could be detected starting at days 7 (24.8% ± 15.2) to persist at days14, 21 and 30 with a positivity of 8.42% ± 7.3, 5.44% ± 2.2, and 6.7% ± 3.9 respectively. *Intimal hyperplasia,* in terms of SMCs layer above the IEL was not observed at 72 hours. It appeared at 7 days leading to 13.24% ± 3.09 stenosis of the cross sectional area, that was the time of highest MIB1 positivity. It increased to 21% ± 3.88 at days 14 and reached 50% ± 27.52 at days 21. At 30 day it persisted with a degree of stenosis of 40% ± 6.65, when proliferation activity was minimal. Linear regression analysis between apoptotic index and intimal hyperplasia at 21 and 30 days did not show any statistically significant correlation (p = 0.63)

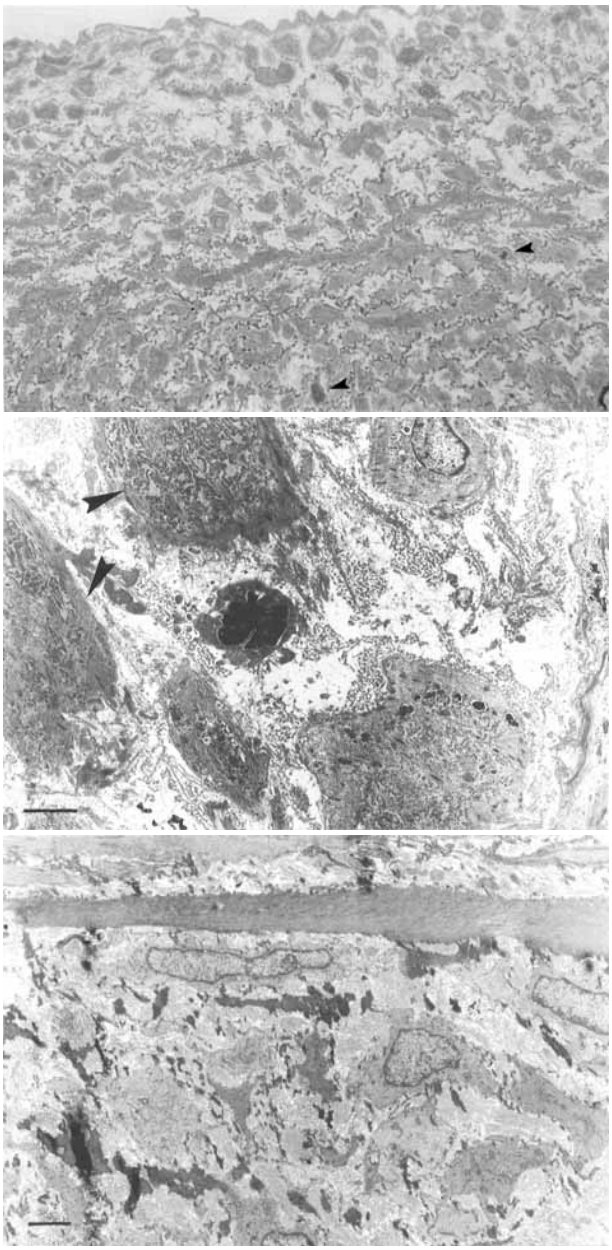

Figure 5. Semithin section and transmission electron micrographs of arteries from animals sacrificed at 20 and 30 days: a) Semithin section with intimal hyperplasia characterized by SMCs and extracellular matrix. Note two SMCs with apoptotic nuclei (arrows). Toluidine blue x800. b) TEM showing prevalent synthetic type of SMCs with well developed RER and myofilaments mainly in the periphery of the cytoplasm (arrow). Apoptotic cell is also seen. (12 mm = 2μm). c) TEM showing large amount of collagen fibers encircling SMCs of synthetic type. (10 mm = 2 μm).

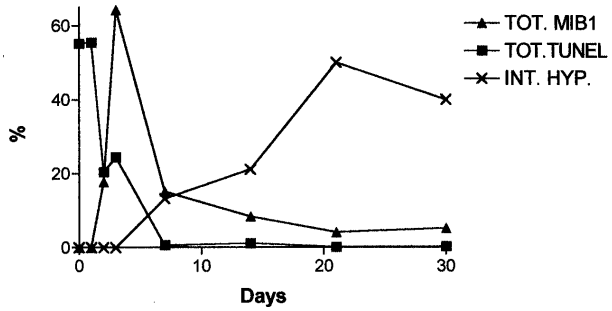

Figure 6. Diagram illustrating SMCs kinetics of injured iliac arteries for intimal hyperplasia, apoptosis and proliferation activity at each time point in all the animals.

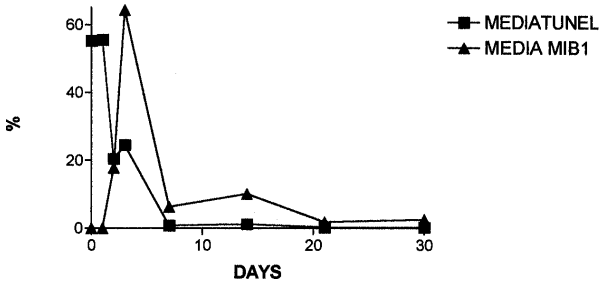

Figure 7. Diagram illustrating SMCs kinetics in the media of injured iliac arteries of all the animals at each time point for apoptosis and proliferation activity.

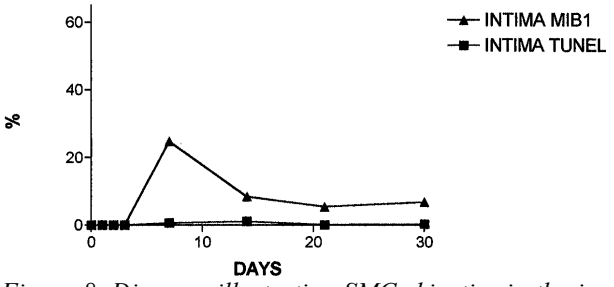

Figure 8. Diagram illustrating SMCs kinetics in the intima of injured iliac arteries of all the animals at each time point for apoptosis and proliferation activity.

Discussion

Apoptotic cell death has been suggested to influence the size and stability of atherosclerotic and restenotic lesions. The percentage of SMC apoptosis reported in previously published papers varied considerably between 2%, in native atherosclerotic plaques and in restenotic lesions in man [17], 8% in the media of a porcine model of coronary angioplasty at 18 hours [29], 14% in the rat carotid artery at 20 days [6] and 70% in the rabbit 30 min after balloon injury [34]. Other authors have reported that balloon injury produces a 25% loss of medial SMCs in the rat carotid arteries 24h after the injury and a 35 to 40% loss in DNA content of medial cells in rabbit iliac arteries 20h after injury [7, 30, 18]. It is known from a series of animal studies that intimal hyperplasia following balloon injury is a consequence of cellular proliferation [7, 8, 10, 18, 21, 30, 31, 43], while the contribution of cell death and apoptosis to this phenomenon is still under investigation [6, 15, 20, 29, 34, 40]. Malik et al., in a recently published study

described the time course of apoptosis in a porcine model of coronary angioplasty [29], while Song [40] and Kamenz [20] focused their attention on the incidence of intimal proliferation and apoptosis after balloon angioplasty in an atherosclerotic rabbit model. Our findings, are based on a model of balloon injury and restenosis, that is devoided from inflammatory infiltrates and therefore cannot lead to speculation on the role of macrophages that is important in the human lesions [42]. Our results show that the remodelling of the vessel wall after balloon injury is accompanied by the early appearance of TUNEL positive nuclei and by the persistence of this phenomenon for an extended period of time [1-3]. This is confirmed by the early onset and the persistence, for the following 24h, of TUNEL positivity in the SMCs of the media and by the electron mycroscopy observation. We can speculate that TUNEL positivity is not a synonym of apoptosis; in fact acute media-intimal disruption can results in DNA-strand breaks, that produce TUNEL positivity, without necessarily resulting in activation of the apoptotic process. However electron microscopy clearly shows the occurrence of morphological changes consistent with apoptosis. Apoptosis was detected and quantitated by in situ end-labelling of fragmented DNA with TdT and biotin-dUTP, that is a commonly accepted method for the detection of the apoptotic process and the EM technique confirmed the reliability of our observation. Some authors have reported that the TUNEL assay can overestimate the number of truly apoptotic cells because it includes necrotic and autolyzed ones [13] or label preapoptotic cells, that do not have apoptotic morphology [26] and might not undergo apoptosis. Recent studies comparing TUNEL and laser scanning confocal microscopy have confirmed its utility and accuracy in identifying and quantifying apoptosis [22, 33]. The extremely high numbers of TUNEL positive cells could be explained by the balloon injury model which consists of a hyperacute lesion and, at least in part, by the DNA strand breaks induced by mechanical cell disruption. The magnitude of the process, though remaining significant, starts to decline at 72h. At day 7 apoptosis is still present with the same magnitude as at days 14 and 30. These results are in keeping with those previously reported in the same animal model both in terms of early onset and magnitude [34], but the persistence of the phenomenon is far more prolonged in terms of cell death that follows.

The kinetics of SMCs apoptosis is different from that reported in the rat carotid artery wall model of endothelial denudation where it appears later, follows proliferation and reaches the maximum 20 days after the injury [6]. We can speculate that, at variance with our balloon dilatation which causes intimal disruption and medial damage, the endothelial denudation in the rat does not result in massive loss of SMCs in the media. The different types of lesion could therefore explain differences in the magnitude and timing of the apoptotic loss and in proliferation kinetic.

In our model at 7 days when the proliferative activity of the intimal layer was higher and intimal hyperplasia could be clearly detected and quantitated, apoptosis was found within the intimal thickening. In the neo-intima at 7 and 14 days the magnitude of TUNEL positivity was greater in the layers closer to the lumen as previously described by Gabbiani et al. in the rat carotid artery [6]. No polarity in TUNEL positivity was however found in the media until 72 hours. We observed a sharp reduction in the apoptotic index between 72 h and 7 days in the media at the same time that the maximal proliferative response is ongoing and specific cytokines and growth factors are maximally secreted [4]. These may act as survival factors, stabilising the lesions and modulating its cellularity. Proliferation appears at 48th hours in the media, and reaches the peak value at 72 h: The proliferative activity clearly appears long after the peak of TUNEL positivity and may counterbalance the SMCs apoptotic loss, which was in our model still relevant between 72 h and 2 days. The proliferative activity in the intima appears later than in the media and persists at an even higher level until 30 days accounting for intimal hyperplasia. At 30 days the remodelling process of arterial wall is still ongoing.

Implications of the study

Our data confirm that apoptosis is present, though with different degrees of magnitude, at all the time points during vascular «remodelling» following balloon angioplasty.

a) Since we know that apoptosis is a fairly rapid occurring phenomenon which only last for 4 to 6 hours. We suggest that there are some other mechanism that can prolong the persistence of apoptosis: 1) prolonged clearance of apoptotic SMCs bodies. In fact the in vivo rate of clearance of TUNEL positivity cannot be assessed in tissue sections. 2) sublethal injury of SMCs at the time of angioplasty: the arterial injury can produce a type of injury that make SMCs enter later on the apoptotic cell death mechanism when eventually activated by some other triggers. 3) Levels of cytokines produced by proliferating SMCs or endothelial cells that are inadequate to complete the cell cycle of neighbouring cells. Mitotic arrest may follow and apoptosis consequently activated.

b) The presence of apoptosis at 30 days, which is in keeping with previous reports on restenosis [24, 25, 34], at the time of atherectomy, once again, suggests that apoptosis could counterbalance the proliferation activity of SMCs and could be responsible for lesion stabilisation and arterial wall remodelling. If that was true modulation of apoptosis could be one of the targets for future novel therapeutic strategies.

Conclusions

Our findings could raise future therapeutic perspectives for treatment of IH leading to restenosis through

modulation of early apoptosis occurring immediately after acute injury, and of "late" apoptosis persisting when cell proliferation and migration are at their higher magnitude [14, 32, 39, 41]. Whether this could be achieved by enhancing apoptosis at this two levels need to be confirmed.

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