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J.-L. Connat · D. Busseuil · S. Gambert · M. Ody
M. Tébaldini · S. Gamboni · B. Faivre
A.-L. Quiquerez · M. Millet · P. Michaut · L. Rochette

Modification of the rat aortic wall during ageing; possible relation with decrease of peptidergic innervation

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Abstract Structural changes of the male rat aorta were followed from birth to old age in male and female rats. In males, the vessel media width and area progressively increase concomitantly with a decrease of nuclei density during ageing, suggesting an hypertrophy of the smooth muscle cells. These correlations were however not evidenced in females. TUNEL-positive cells were found in media of 4 and 6 months in both sexes, mainly on the luminal side and in the adventitia. When biochemical markers were investigated with immunohistochemistry, media was uniformly stained by the anti-vimentin and anti-α-smooth actin at all stages investigated. On the contrary, the surface of media stained with anti-desmin decreased during ageing, especially on the luminal side. As observed with electron microscopy, with ageing the endothelium is replaced by small cells with pseudopodia adhering to the vestigial elastic lamina and infiltrating into the extracellular matrix left after the disappearance of smooth muscle cells. In addition, in the older rats (25–29 months) the elastic laminae are completely disorganised. Hypertrophy of the smooth muscle cells was confirmed by this approach. In parallel to this study, perivascular peptidergic innervation was stained with antibodies against calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), and vasoactive intestinal polypeptide (VIP) at different ages during the whole life of rats. These peptides are present in stages younger than 6 months, then gradually disappear. In one year animals and older, the peptidergic innervation has totally disappeared. We discuss the possible role of peptidergic innervation in the control of the vessel wall cellular stability during ageing.

Keywords Smooth muscle cell · Desmin · CGRP · SP · NPY · VIP · Apoptosis · Hypertrophy · Gender · Endothelium

J.-L. Connat (✉) · D. Busseuil · S. Gambert · M. Tébaldini
P. Michaut · L. Rochette
Université de Bourgogne, IFR 100,
Laboratoire de Physiopathologie Cardiovasculaire Expérimentales,
Biologie Animale Cellulaire et Moléculaire,
UFR Sciences de la Vie, 6 Blvd Gabriel, 21000 Dijon, France
e-mail: Jean-Louis.Connat@u-bourgogne.fr
Tel.: +33-80-396216, Fax: +33-80-395066

B. Faivre
Université de Bourgogne, UMR CNRS 5561,
Equipe Ecologie Evolutive, 6 Bvd Gabriel, 21000 Dijon, France

M. Ody · S. Gamboni · A.-L. Quiquerez · M. Millet
Université de Genève, Dpt de Biologie Animale, Sciences III,
30 Quai E. Ansermet, 1211 Geneve 4, Switzerland

Introduction

Morphological changes have been documented in the aorta during development and ageing in man (Bouissou et al. 1987; Lévy 1992). An increase in calibre of the arterial lumen has been found in all studies. Concomitantly, collagen, elastin and smooth muscle contents of the media are modified. Causes of these structural and functional changes are not understood. We used the rat model to better understand the evolution of the aortic wall during ageing. Indeed, embryonic development of rat aorta has been extensively studied with electron microscopy by Nakamura (1988) and changes within the tunica media associated with the growth of the thoracic aorta in the 3 months following birth were monitored by Cliff (1967) but no work until now is available on the modification of the structure during ageing. Similarly, many neuropeptides have been found in perivascular nerves of a range of mammals (Dhital and Burnstock 1987; Edvinson et al. 1989; Owman 1990) but most often the innervation was studied at an undefined adult stage. Only a few data are available about developmental changes of the innervation and none about densities of innervation in the elderly. A correlation between the occurence of the peptidergic innervation and differentiation of the vessel wall has been established (Woolgar and Scott 1989; Ody et al. 1993). Furthermore, CGRP modulates proliferation and differentiation of rat aorta smooth muscle cells in culture (Connat et al. 1993, 2001; Li et al. 1997). In guinea-pig, vasoactive intestinal polypep-

tide (VIP) and calcitonin gene-related peptide (CGRP) nerve plexuses of mesenteric and carotid arteries reached a peak at birth and then declined to about half maximum density in old age (Dhall et al. 1986). The same authors indicated that in renal and femoral arteries, these two peptides together with substance P (SP) peaked at 4 weeks after birth before declining in density in old age. The fact that abundance and nature of peptidergic innervation vary during development suggests that these peptides could act as trophic factors for the differentiation of the vessel wall, as already proposed by Burnstock (1982). In fact, the structure of the vessel wall also varies during ageing (Bouissou et al. 1987). In many animals, including man, smooth muscle cells (SMC) become more secretory and form collagen and glycosaminoglycans (GAG), which provoke an intimal thickening. In some cases, SMC can completely dedifferentiate, migrate into the intima and induce atheromatosis. In the rat aorta, the desmin content of media decreases with ageing (Nikkari et al. 1990). It is tempting to associate these morphological and biochemical changes with some alteration/modification of the peptidergic innervation of the vessels. In the rat aorta, a previous study reported that CGRP innervation occurred very early during development to reach its highest density at birth (Thiévent and Connat 1993). Although very scarce compared to other vessels, these densities remained constant for at least 5 weeks, then values found in 6-month-old adult rats decreased and became very low. No information is available on aortas in older rats. We decided to investigate the presence of CGRP and of other peptides, such as SP, NPY or VIP, that are commonly described around the blood vessels, during the whole rat life. In parallel, we studied the structural changes occurring in the aortic wall of the rat during ageing to find if they could be associated with modifications of the pattern of peptidergic innervation.

Materials and methods

Animals

Wistar rats from birth to 29-month-old were raised in our animal house and fed with food for laboratory rodents. Five males or five females were kept together in the cages. Lighting was 12 h day and 12 h night. Females never brought forth young. Half life span is approximately 36 months for males and females. However, in this study, females demonstrated an accelerated senescence compared to males.

Histology/cytology

The structure of the aorta was investigated on sections from the first 5 mm above the diaphragm. The aorta ring was dissected out of the rat following anesthesia (chloroform) and cervical dislocation.

Classical histology was performed on tissues fixed in Zamboni, embedded in paraffin, cross-sectioned at 7 µm and stained with hematoxylin-eosin. The histomorphological features were measured with a computer-based digitilizing image system (Biocom Inc) using a light microscope (Nikon Elipse 600) connected to a high-sensitive colour Coolsnap camera. The width and area of the media was delimited manually at low magnification on the computer screen. For counting nuclei, three randomly selected fields per cross section were digitalized at higher magnification (objective ×40) and nuclei manually selected. Results were given as the number of nuclei/mm^2.

For semi-thin and thin sections, tissues were rapidly immersed in fixative (2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer 0.1 M, pH 7.4, containing saccharose, 0.2 M). Postfixation was performed during 2 hours with 1% osmium tetroxide in the same buffer. Tissues were embedded in Epon 812 resin after dehydratation in ethanol. Semi-thin sections were observed after staining with toluidine blue (0.5% in distilled water containing borax 1%). Thin sections were stained with 5% aqueous uranyl acetate (20 min) and Reynolds' lead citrate solution (10 min), and examined with a Zeiss EM10 electron microscope. Electron microscopic study was conducted on two males (22 and 25 months) and two females (22 and 29 months) and compared to 2-month-old rats (one male, one female).

Detection of apoptosis by the TUNEL method

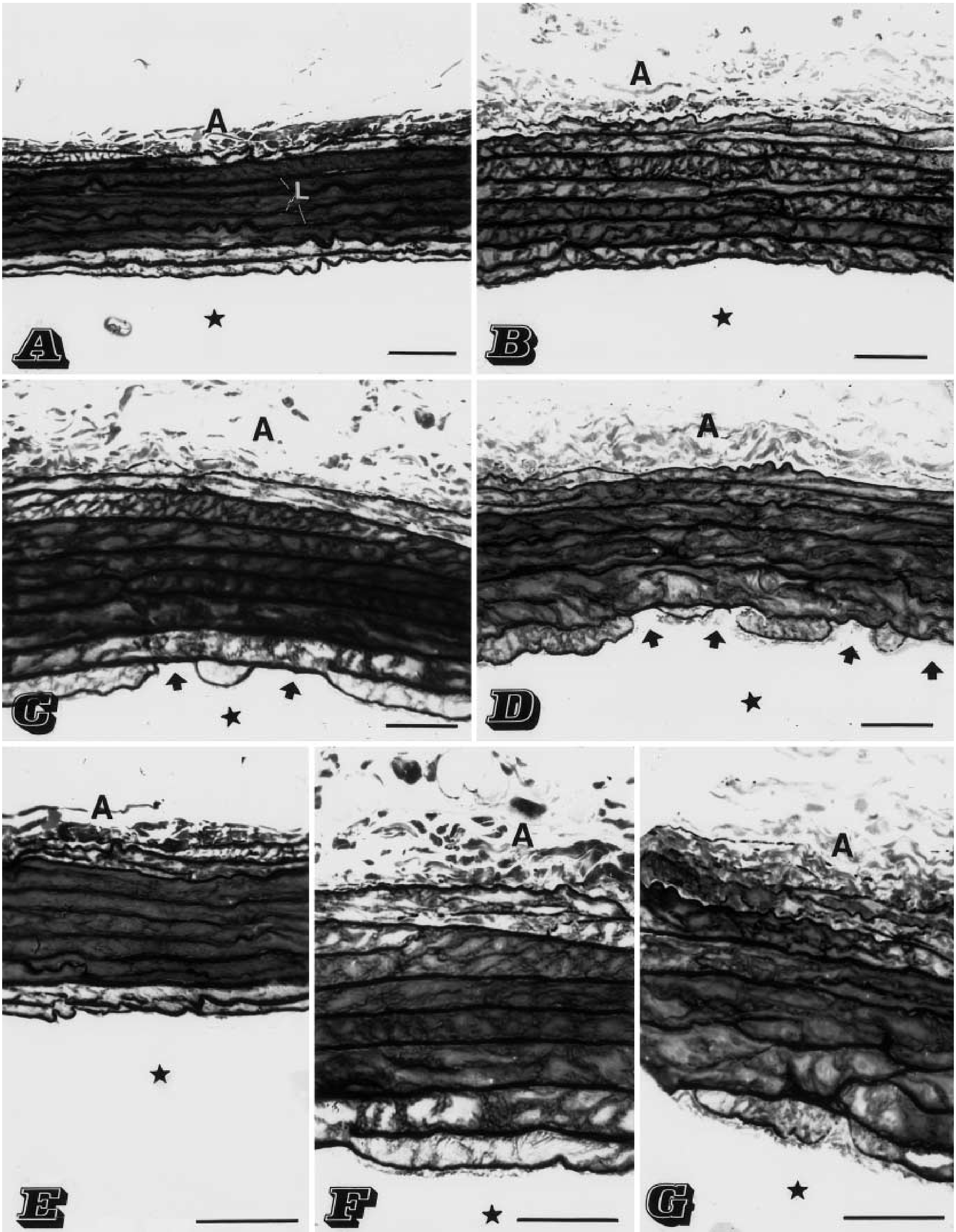
Tissues fixed with Zamboni were processed for paraffin embedding, cut at 7 µm with a microtome and collected on silanized slides (SuperFrost*/Plus, Menzer-Gläser, Germany). After rapid removal of the paraffin, sections were digested by incubation with 0.4% pepsin (Sigma) in 0.2 M HCl and washed in PBS (pH 7.4) The endogenous peroxidase activity was inactivated with 2% H2O2 in PBS. Sections were rinsed again and incubated in the mixture of terminal deoxynucleotidyl transferase and dUTP of ONCOR's kit (Apoptag, Gaitherburgh, USA). Protocol (stop wash and incubation with the peroxidase antibody) was then followed according to the manufacturer's instruction. Tissue-bound peroxidase was visualised by 3,3',5,5'-diaminobenzidine (DAB; 0.05%), 0.8% H2O2 30% in phosphate buffer (pH 7.4, 0.1 M).

Transverse sections of mouse retina graciously provided by Dr Dubois-Dauphin (University of Geneva, Switzerland) in which apoptosis was naturally occurring, or sections treated with DNAse I, were used as positive control. Negative controls were obtained from preparations in which terminal transferase was omitted.

Immunocytochemistry for cytoskeletal proteins

This study was performed on males. Three rats 5-days-old, 11 rats 5–8-weeks-old, 3 rats 6-months-old and 5 rats 1-year-old were used. Aorta was dissected out, rinsed in phosphate buffered saline (PBS, 0.01 M, pH 7.4) and frozen in isopentane at –20°C. Pieces of vessel were embedded in cryosectioning medium, and cut with a Reichert-Jung cryostat. Ten-µm sections deposited on gelatine coated slides were stored at 4°C and stained with the antibodies within 24 h. Slides were delipidized and fixed with a 5 min acetone bath at –20°C, dried for 1 h. at room temperature, then permeabilized and blocked for non-specific binding with 0.3% Triton X100, 0.1% BSA in PBS during 15 min. Tissues were incubated for 1 hour at 37°C with anti-desmin, anti-vimentin (diluted 1:10 in PBS 0.01 M) and/or monoclonal anti-α-smooth actin [anti-αsm-1 (Skalli et al. 1986) diluted 1:10 in PBS 0.01 M]. Three different anti-desmin antibodies were used and gave similar results: Rabbit antisera against desmin and affinity purified according to Kocher et al. 1984 (diluted 1:3 in PBS), polyclonal anti-desmin from Sigma (diluted 1:10 in PBS) and monoclonal anti-desmin from

Fig. 1A–G Evolution of the aortic wall of the rat during ageing (*A* adventitia, * lumen of the vessel, *L* elastic lamina). Bars 50 µm. **A** Aortic wall at 2 months, **B** at 22 months, **C** at 25 months, **D** at 29 months. Note the disaggregation of the internal muscular sheets during ageing (*arrows*). **E–G** Illustration of the increase in thickness of the aortic wall and desintegration of the elastic laminae during ageing (2 months, 25 months and 29 months respectively) ▶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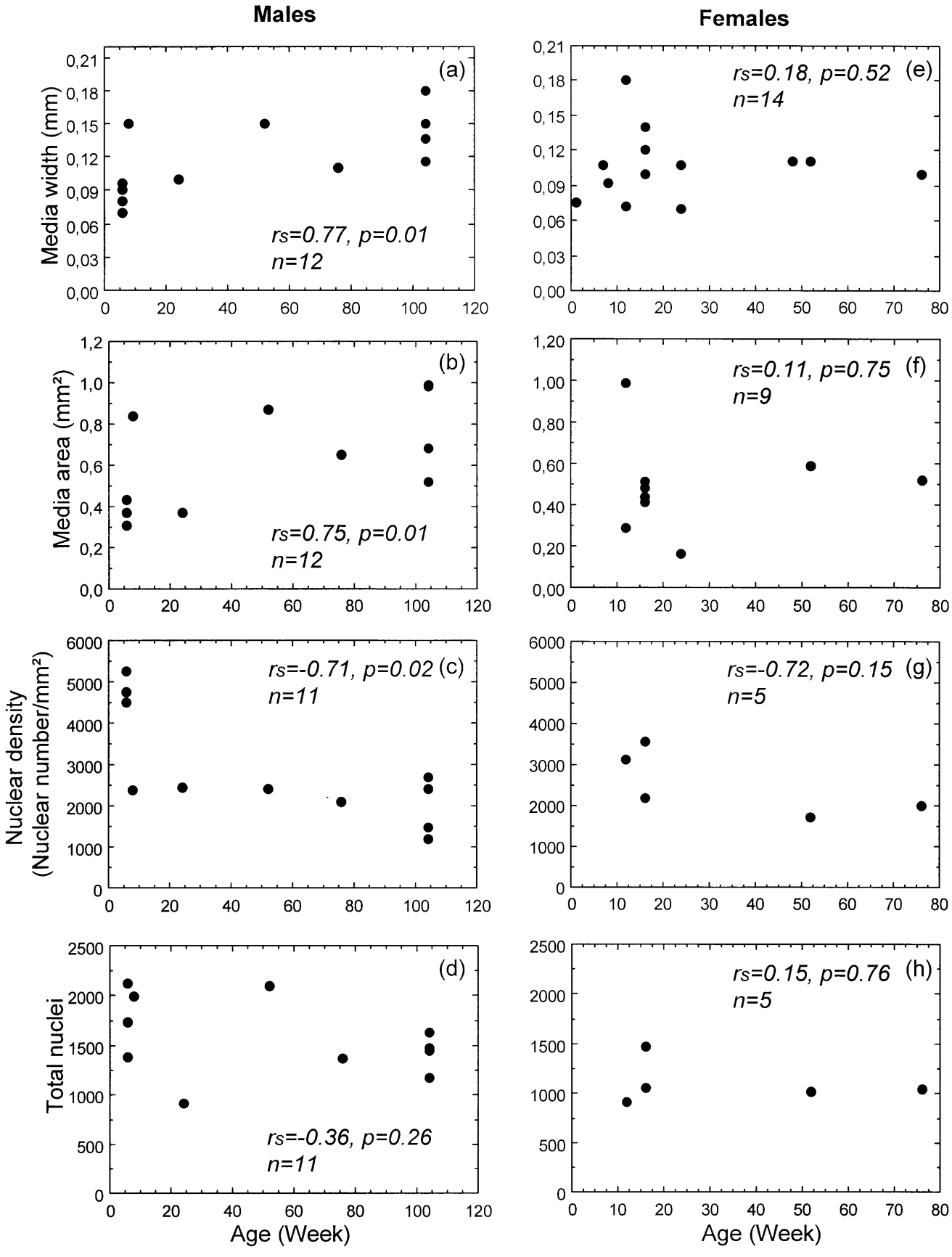


Fig. 2 Association between age and media characteristics in males (**a–d**) or females (**e–h**). Spearman rank correlation coefficient (*rs*), *P* values and number of individuals are indicated in each case

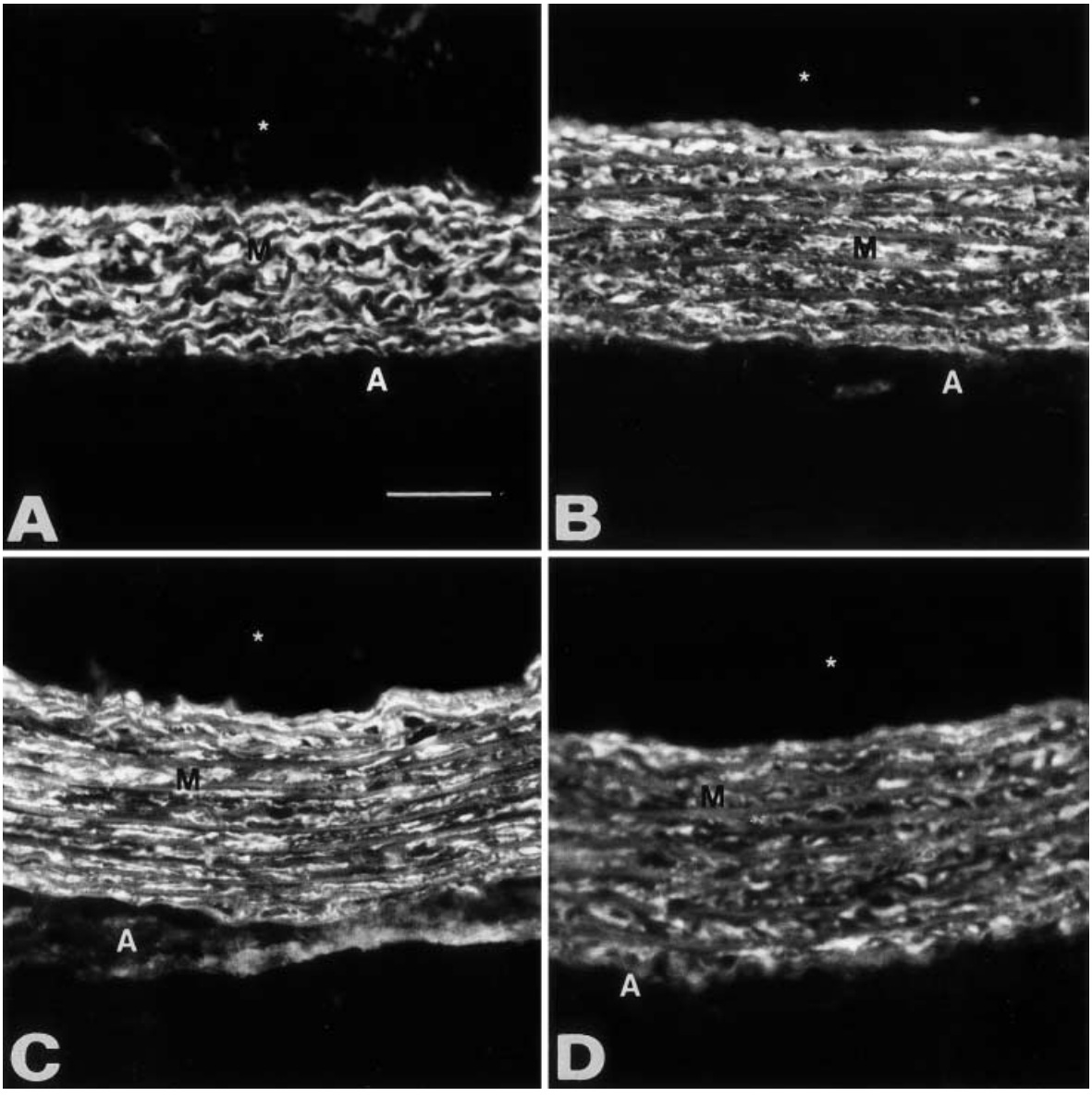


Fig. 3A–D Repartition of cells containing α-smooth actin in the rat aortic wall evidenced by immunofluorescence. Pattern found in aortas of 5-day-old rats (**A**), 5 to 8-week-old rats (**B**), 6-month (**C**) and 1-year (**D**) -old rats. The whole media is uniformly stained (* lumen of the vessel, elastic lamina appear as *grey lines*, *M* muscular sheet, *A* adventitia) *Bar* 50 µm; same enlargement for all figures

DAKO (diluted 1:10 in PBS). In all cases, staining was revealed with a second incubation (30 min. at 37°C) with fluorescein and/or rhodamin conjugated antibodies (Nordic) diluted 1:50 in PBS. Nuclei of the cells were stained with DAPI, 300 ng/ml (Sigma) for 10 min (data not shown). In parallel, controls were run without the primary antibodies to assess the specificity of the labelling.

Immunocytochemistry of the peptidergic innervation

After dissection, the whole aortae were immediately immersed for fixation in a fresh solution of 0.4% p-benzoquinone (Fluka) in phosphate-buffered saline (PBS) at room temperature in the dark, for 1 h 30 min to 1 h 45 min. Fixation was followed by 3×1 h rinse in PBS, during which the vessel was carefully freed of extravascular material. Vessels were then incubated in the presence of different antibodies (anti-CGRP, anti-NPY, anti-VIP, and anti-SP) purchased from Amersham and diluted 1:200 in PBS pH 7.2 containing 0.3% BSA, 0.01% NaN3 for 14 h at 4°C. After three 15 min washes in PBS, tissues were incubated for 1 h 30 min at room temperature in the dark in FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1:50 in the same solution as for primary antibody. After 3×15 min baths of PBS, the vessels were finally opened longitudinally and whole-mounted, adventitial side up, in a polyvinyl alcohol antifading medium. The preparations were observed using a Leitz Orthoplan microscope fitted for epiillumination with excitation filter from 450 to 490 nm and extinction at 515 nm.Young vessels (<5 weeks) innervation was observed on their whole surface. On bigger vessels, observations were realised along 6 lines drawn perpendicular to the vessel axis, taking care to include all nerves at different depths by adjusting the focus.

Preabsorption of primary antisera with excess of peptide was realised with CGRP (1.5 µM), NPY (1 µM) and SP (1 µM). In all cases, the primary antiserum was also replaced by a non-immune rabbit serum. In both cases, no immunostained nerve fibres were observed on preparations that are normally labelled. Positive controls (pieces of gut or young portal vein) were included in the different experiments when necessary.

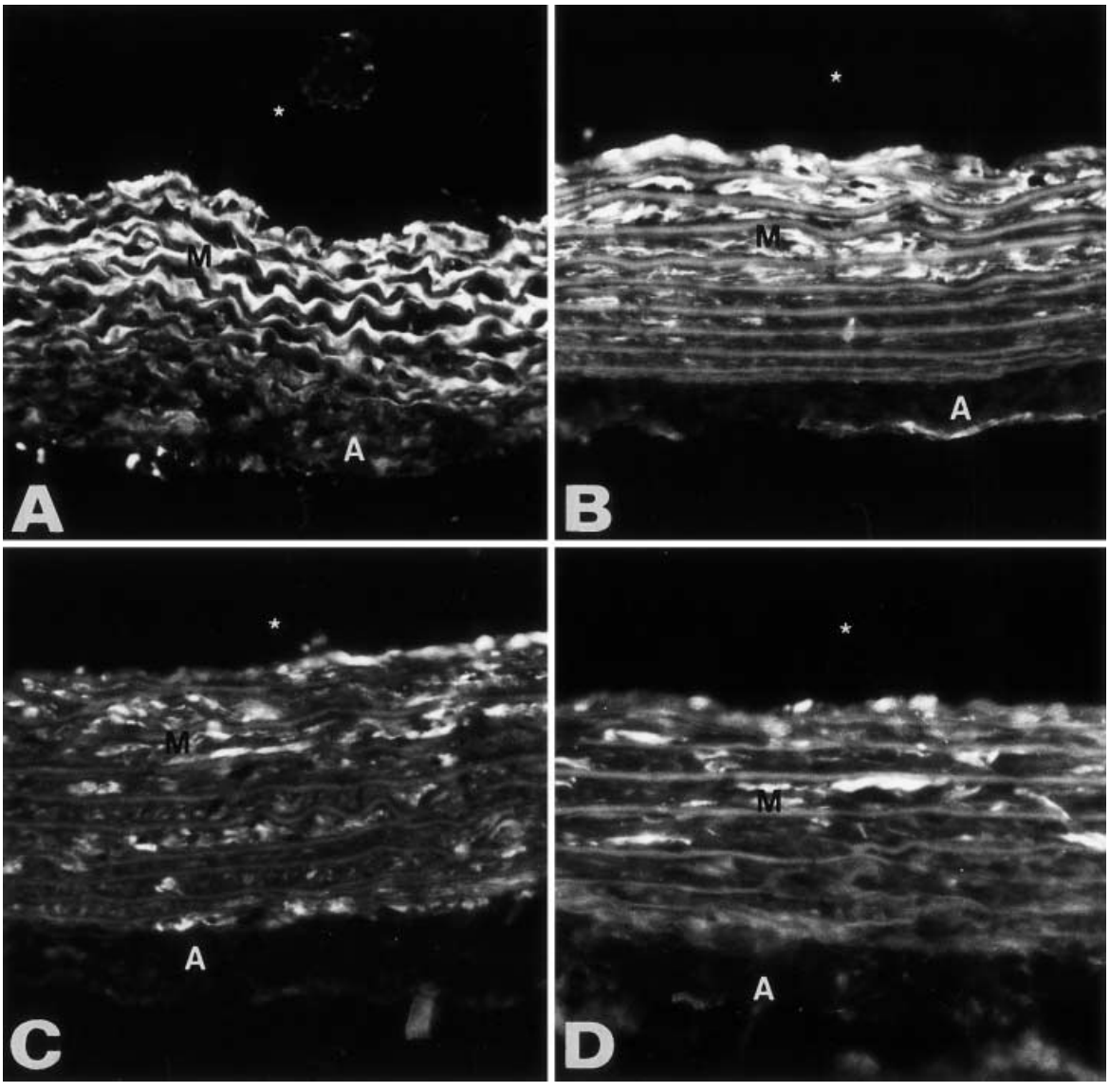


Fig. 4A–D Repartition of cells containing the intermediate filament desmin in the rat aortic wall evidenced by immunofluorescence. Pattern found in aortas of 5-day-old rats (**A**), 5 to 8-week-old rats (**B**), 6-month (**C**) and 1-year (**D**) -old rats (* lumen of the vessel, elastic lamina appear as *grey lines*) Magnification as Fig. 3

Statistical analysis

The statistics presented were performed using Statview 5.0. Because of the small size of the samples, normality of the population cannot be assumed, and therefore the Spearman rank correlation test was used to test associations between age and structural variables.

Results

Evolution of aortic wall thickness, elastic laminae and number of nuclei during ageing

The evolution of the media width and area of male and females rat aortae were examined during ageing on transverse sections with light microscopy combined with image analysis (Fig. 1). Nuclear density (number of nuclei/mm2) was also determined (see Fig. 6D). The total number of nuclei per section was estimated from the previous data. The different results obtained were plotted against the real age for each gender and a Spearman rank correlation test was computed (Fig. 2). In males, thickness of the media and area increase gradually significantly with age. All p values are smaller than 0.05. Thickness in animals older than 2 years reached approximately 1.5 times the width found in young adults (Fig. 1, 2a). The correlation rank study also demonstrated a decrease of the nuclear density, which became at 2 years approximately half of that found in 6–10-week-old rats. Although a general tendency to a reduction of the total number of nuclei was observed, no significant correlation was noted for this parameter (Fig. 2d, rs=–0.36, P=0.26).

However, it was impossible to demonstrate any significant correlation between the latter parameters and age in females (Fig 2e–h). Even if male values were computed in the same age range (<80 weeks), the correlation remains significative (P >0.05).

Table 1 Detection of apoptotic nuclei by the TUNEL method on transverse sections of male rat aorta of different ages. For each animals studied, at least three different sections were observed and the test was repeated twice (– no visible nucleus, + less than 5 nuclei positive on the whole section, ++ 10 to 50 nuclei, +++ 50 to 100 nuclei). A similar pattern was obtained for two female rats 6 months old

Age of the rats	Number of animals studied	TUNNEL score		
		Rat no. 1	Rat no. 2	Rat no. 3
2 months	3	+	–	+
4 months	3	–	++	++
6 months	3	+++	+++	+++
1 year	3	–	+	–
2 years and older	2	–	–	0

In both sexes, between 22 months and 25 months, when the thickness of the aortic wall was increased (Fig. 1E, F) the lumen side was irregular, as if some parts of the muscular sheet has been destroyed (Fig. 1C). This is even more evident at 29 months. In these vessels, the elastic laminae lost their typical parallel arrangement (Fig. 1D, G).

Immunocytochemistry of cytoskeletal proteins in the aortic wall during ageing

Presence of the three different cytoskeletal proteins was investigated by immunohistochemistry on transverse sections of aortic wall at 5 days, 5, 7 and 8 weeks, 6 months and 1 year. The staining obtained with the anti-vimentin antibody was not different at any age investigated. The three tunicae of the aortic wall, which are of mesenchymal origin, were uniformly stained (data not shown). On the contrary, the anti-α-smooth actin antibody stained only the media (Fig. 3). Already at 5 days, although the aortic wall had not finished its maturation, a clear limit exists between adventitia (unstained) and media (stained; Fig. 3A). When the aortic wall was stained with the anti-desmin antibody (Fig. 4) the pattern obtained is different at the different stages investigated. At 5 days, when the aorta is not totally matured, the elastic laminae still being folded, and their number being lower than at the adult stage, the vessel wall presents a continuous gradient of staining, the intensity of which decreases from the lumen to the periphery (Fig. 4A) without a precise limit between media and adventitia. The latter becomes visible at 7 weeks (Fig. 4B) the adventitia and the outer part of the media being totally negative for desmin. At this stage, the adult media is composed of two cell types: desmin positive cells, preferentially located in the inner part of the vessel, in contact with the endothelium and desmin negative cells, located at the outer part of the vessel, in continuity with the adventitia. At 6 months and one year, the surface of media stained by anti-desmin antibodies decreases. Only some desmin positive cells were found in the media of 1-year-old rats. The positive luminal region has completely disappeared.

Ultrastucture of the aortic wall during ageing

Observation with electron microscope confirms previous paragraphs. At 2 months the aortic wall presents the typical structure of elastic arteries. The endothelium lies on a first thick elastic lamina (Fig. 5A). Smooth muscle cells (SMC) present the typical structure of contractile cells with myofilament and dense bodies, particularly visible in longitudinal section (Fig. 5B). On the contrary, the luminal side of old animals is considerably different in males and females. The first elastic lamina is very thin or disaggregating; instead of being covered by a typical endothelium, it is covered by connective tissue/ extracellular matrix, infiltrated with pseudopodia of small cells that seem to come from the lumen (Fig. 5C), which are probably leukocytes. The first row of smooth muscle cells is less compact than before, a lot of connective tissue being intercalated between the smooth muscle cells. SMC remain in their contractile phenotype and present a fragmentation of their cytoplasm (Fig. 5C) Necrotic cells are found at this age in the ECM material on the luminal side of the vessel (5D). In the more internal layers, according to the previous quantitative studies, SMC presented a large volume of cytoplasm compared to SMC from young rats and the quantity of extracellular material is increased (Fig. 5E, F).

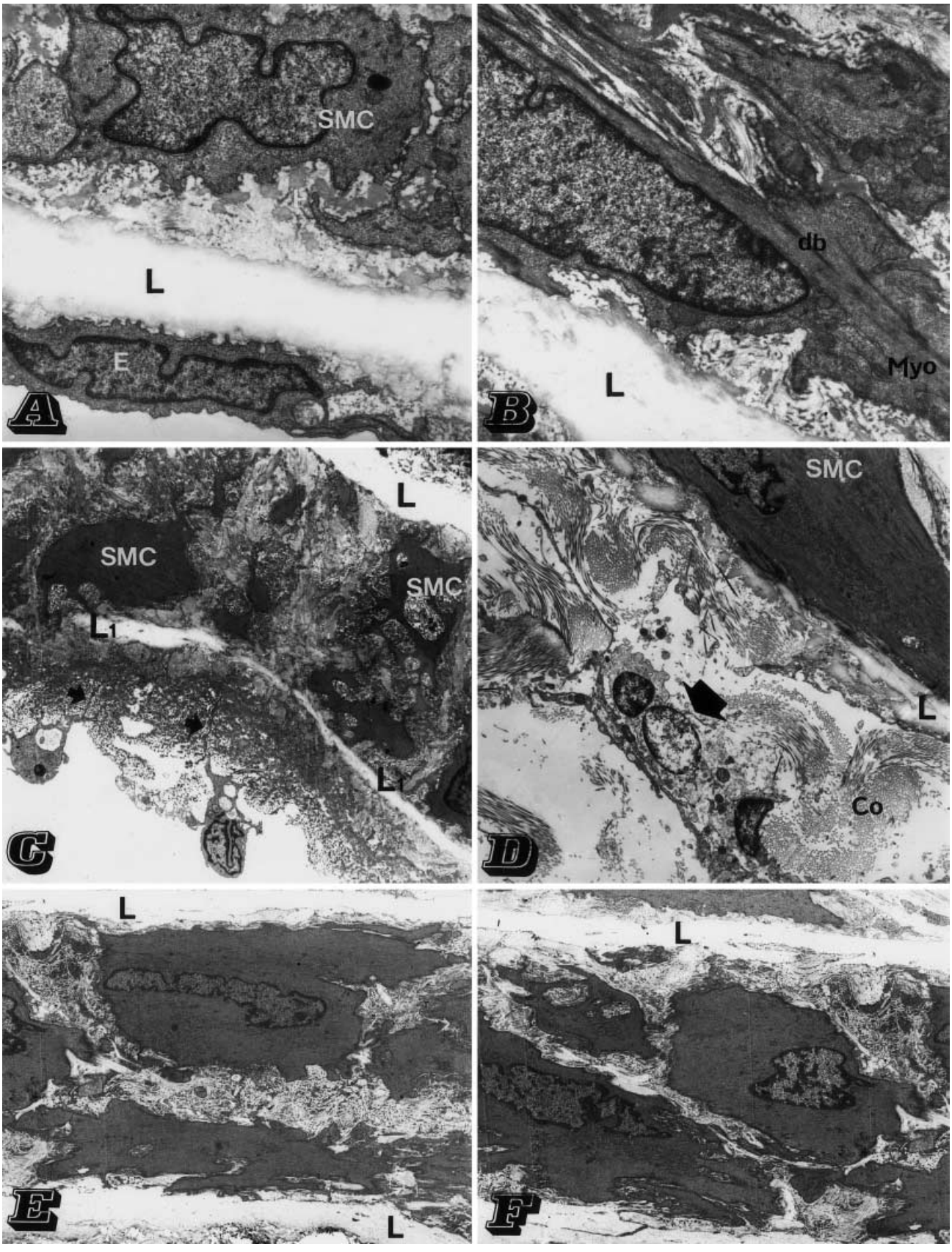
Detection of apoptosis in the aortic wall during ageing

Transverse sections of aortas from male rats of different ages were processed for visualisation of apoptotic nuclei (Table 1). A large amount of TUNEL-labelled nuclei were observed in smooth muscle cells of the media from 4- and 6-month-old rats. They were principally distributed along the luminal side of the vessel and at the limit between media and adventitia (Fig. 6). Sometimes, labelled nuclei were observed in the adventitia. On the contrary, only few or none positive nuclei were observed in 2-month or 1-year and more -old rats. Observation of hematoxylin-eosin stained sections confirmed the results obtained with TUNEL. Fragmented nuclei were observed in corresponding sections at the same places as TUNEL positive nuclei. Two 6-month-old female rats were also processed to compare with males. A similar pattern was observed in both females.

Evolution of the peptidergic innervation during ageing

CGRP innervation

A diffuse plexus of CGRP immunoreactive fibres, more abundant in the arch, was always visible over the whole surface of the vessel within the first month of life (15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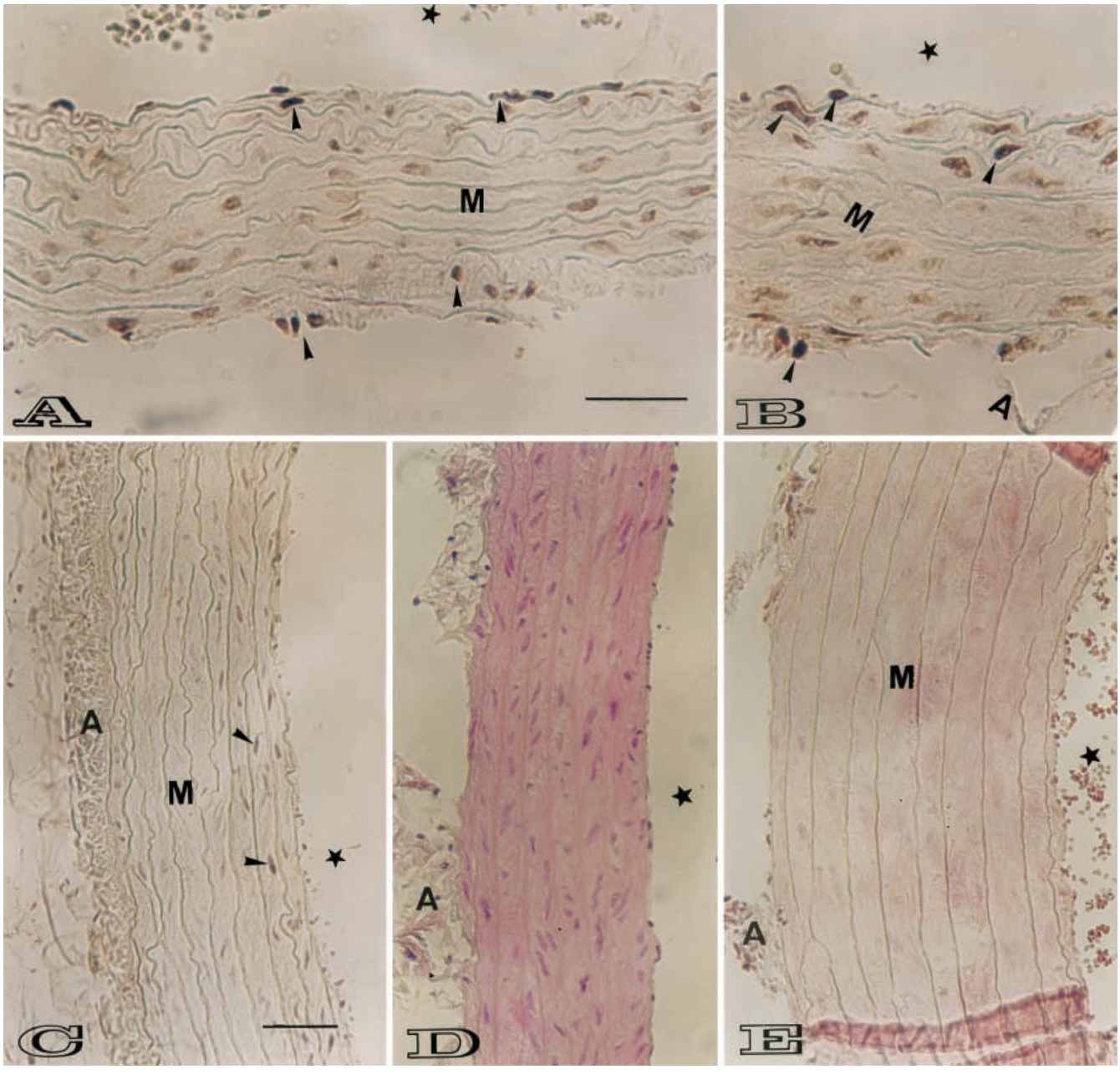

◀ **Fig. 5A–F** Ultrastructure of the rat aorta during ageing. **A** Endothelial side of a 2-month-old rat aorta (*SMC* transverse section of a smooth muscle cell, *L* elastic lamina, *E* endothelial cell). ×12,500. **B** Typical smooth muscle cell in contractile phenotype (longitudinal section) with myofilaments (*Myo*) and dense bodies (*db*). Media from aorta of 2-month-old rats. ×12,500. **C** Luminal side of an aorta of a 25-month-old rat. The first elastic lamina (*L1*) is thin compared to the second (*L*, right corner). On the internal side, smooth muscle cells (*SMC*) are sparsely arranged within an abundant connective tissue. On the luminal side, only connective tissue, infiltrated with cells presenting pseudopodia (->), is visible. On the right a SMC is fragmentized. Neutrophils involved in phagocytosis and often associated to the apoptosis process are present on the luminal side. ×3,150. **D** Connective tissue and fragments of cells containing vesicularized nuclei (*arrow*) on the luminal side of an aorta of another 25-month-old rat (*Co* collagen, *SMC* smooth muscle cell, *L* elastic lamina). ×4,000. **E, F** Examples of hypertrophy of smooth muscle cell in aorta from two different 25-month-old rats. Note the increase of the cytoplasmic volume compared to nucleus and the large spaces filled with extracellular material between cells (*L* elastic lamina). ×3,150

Fig. 6A–E Visualisation of apoptotic nuclei in the media with the TUNEL method on transverse sections of aorta at different ages (* lumen of the vessel, *M* muscular sheet, *A* adventitia). **A** 4-month-old rat (lens ×40) *Bar* 50 µm. **B** 6-month-old rat (lens ×40) Same enlargement as **A**. **C** 1-year-old rat (lens ×20) *Bar* 50 µm. **D** same transverse section as **C** stained with hematoxylin eosin Same enlargement as **C**. **E** Rat older than 2 years (lens ×20) Same enlargement as **C**. *Arrowheads* mark some example of positive nuclei

rats examined). At 2 months, no innervation was detected in 4 of the 8 animals studied, while in the others, fibres were extremely scarce. In aortas from 2–6-month-old animals (16 rats), the density of innervation was clearly less abundant in the arch than at previous ages. Very few fibres were visible across the whole length of the aorta, most often along the vasa vasorum (Fig. 7a). No immunoreactive fibres were detected on vessels from

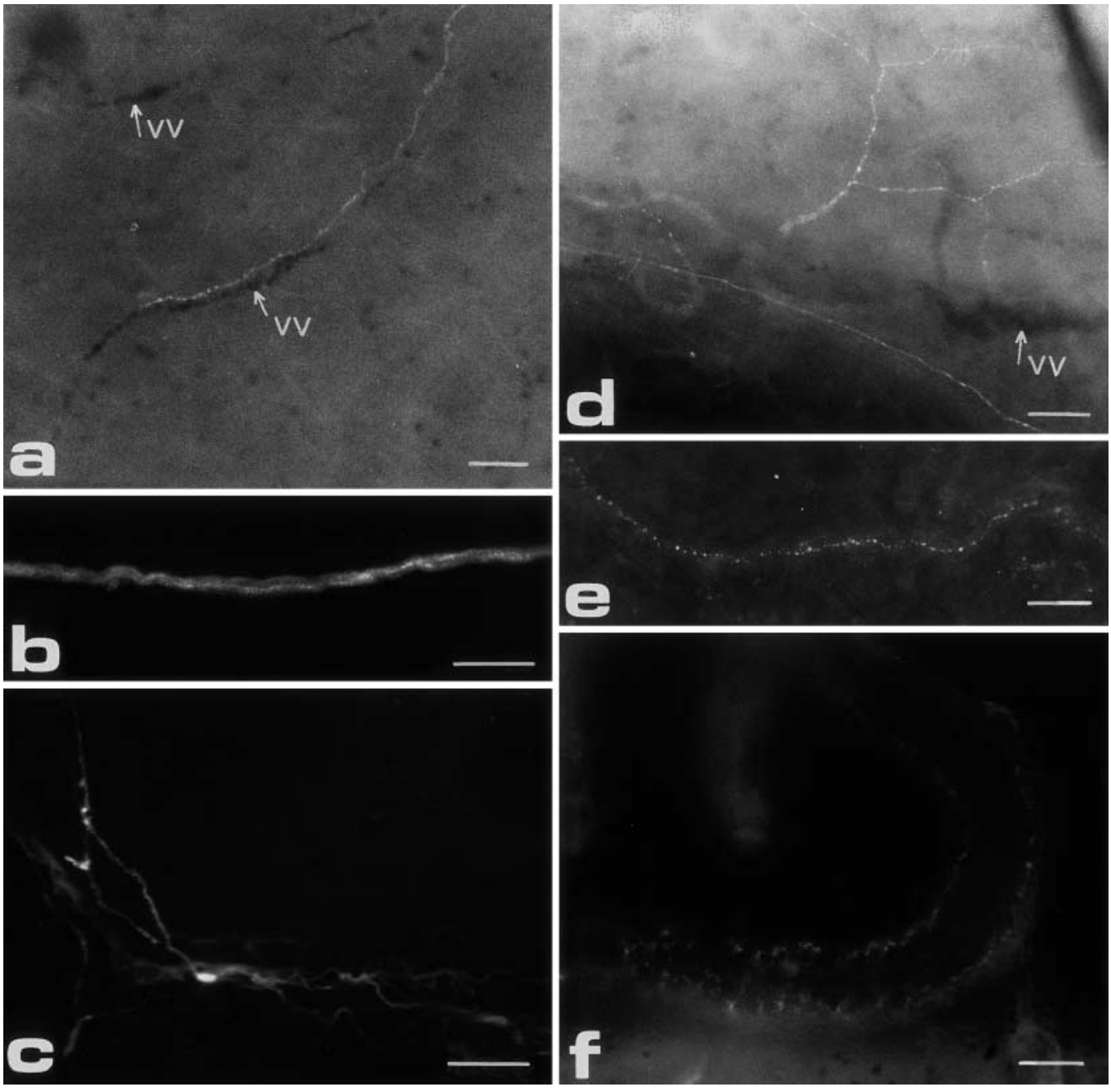

Fig. 7a–f Illustration of the peptidergic innervation found on the young rat aorta. *Bar* 50 µm. **a** CGRP-immunoreactive varicose fiber running along a vasa-vasorum (*vv*). Rat 3.5 months old. **b** VIP-immunoreactive nerve bundle at the surface of the aorta of a 2-month-old rat. **c** VIP-immunoreactive varicose fibers running in the outer adventitia of the arch of a 6-month-old rat. **d** SP-immunoreactive varicose fiber running on the arch of a 25-day-old rat. **e** Example of NPY-immunoreactive varicose fiber found in the rat duodenum (28 days) used as positive control. **f** NPY-immunoreactive network found around one of the lateral branches of the aorta. The network is not clearly visible on the picture because the focus corresponds to an optical section

1-year-old rats (8 animals) and older (9 animals) although positive controls (rat portal veins) were run simultaneously.

VIP innervation

Aortas from rats 1-day to 4-week-old (total of 20 animals examined) showed a slight antibody staining of the connective tissue, but no positive nerve fibres. At the same time, rat duodenum and stomach were used as positive tissue controls and gave clear staining of fibres. Furthermore, thin VIP-immunoreactive networks were present on the lateral branches of the vessel (data not shown). Between 1 and 6 months (32 animals exam-

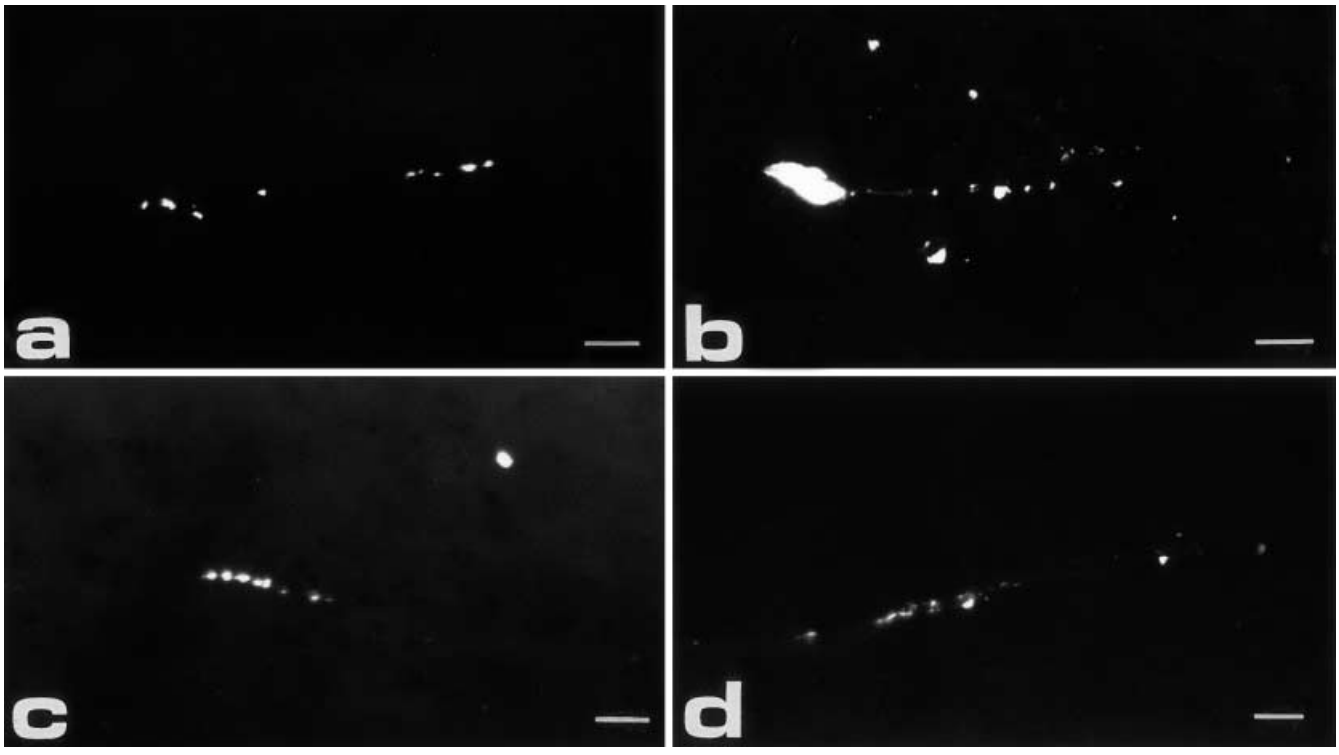

Fig. 8a–d Illustration of the peptidergic innervation found on the 6-month rat aorta. *Bar* 20 µm. **a** Staining obtained with the anti-NPY serum correspond to "on line" varicosities. In this case, the axon could be depleted of peptide and not stained. **b** Staining obtained with the anti-NPY serum corresponding to a labelled neuron and its neurite. **c, d** Staining obtained with the anti-SP serum, which corresponds to staining of varicosities. In all cases, continuous immunoreactive fibres were never detected

ined), some discrete fibres were detectable on the aorta of some animals (Fig 7 b,c). However, all these fibres were weakly fluorescent; more fluorescent fibres were found in the outer adventitia. In 1-year (6 rats) and older animals (4 rats), no fibres were seen.

SP innervation

Within the first month (9 rats studied), a few tiny fibres immunoreactive for SP were regularly seen on aorta, mainly on the arch, where they mainly formed a discrete but dense network (Fig. 7d). At the end of the 4th week, a long fibre was always observed, running along the upper part of the aorta. Between 5 and 7 weeks (14 animals examined) all these fibres were still present, but intensity of fluorescence was decreased. In 2-month aortas, innervation of very weak intensity was only noted in 2 from 4 animals and no innervation was detected in the 2 remaining animals. In 4- (7 animals) and 6-month-old (8 animals) aortas, innervation only remained in the arch. At the thoracic level, isolated varicosities were observed disposed along a virtual line that may correspond to the depleted axon on some preparations (Fig. 8 c,d). In rats of 1-year (6 rats) and older (8 rats), immunoreactive fibres at the surface of the aortas were never detected.

NPY innervation

No innervation was detected at the surface of 7 aortas taken from 3, 5 and 11-day-old animals although a clear staining was observed as a thin varicose chain (Fig. 7e) on rat duodenum that was used as a positive tissue control for this antibody. Very scarce short and thin varicose fibres were sometimes observed dispersed on the whole surface of the vessel at 22–25 days (8 animals). On one-month-old aortas (12 rats) some little fluorescent dots, visible at low magnification (×10 objective) regularly arranged on straight lines, and corresponding to varicosities of nerve fibres (Fig. 8 a,b) were visible. This scattered innervation was visible on the arch and the upper thoracic part of the aorta, but no innervation was seen on the lower part. However by 7 weeks (7 rats studied) the NPY innervation had almost completely disappeared. Only one vessel demonstrated a short fibre in the arch. At 2 months (7 rats), no more fibres were noted at the surface of the aorta itself ; although a local dense plexus of immunoreactive fibres was sometimes seen on small areas around the lateral trunks of the aorta (Fig. 7f). Later, in 4-(7 rats) and 6-month-old (9 rats) animals, clear immunoreactive fibres were no more visible. Only a very short distinct fibre was present on one vessel from the 6-month-old rats. In half of the other observed vessels, only lines of isolated varicosities (Fig. 8a) were visible. In 1-year-old animals (6 rats) and older (4 rats), no nerve fibres at the surface of the aorta were detected. However, large peripheral nerves running near the arch were positively stained. On half of the preparations, "lines" of varicosities were still detected.

Discussion

In this study, we followed structural and biochemical changes in rat aorta wall during ageing. An important thickening of the media occurs in old age in males. Concomitantly there is disappearance of the more internal layers of smooth muscle cells as attested with electron microscopy or immuno-histochemistry for desmin positive cells, but also nuclear density measurements. The latter could be due, in view of the TUNEL results, to an apoptotic wave occurring between 2 and 6 months. The more internal desmin positive cells are then diluted in the surrounding hypertrophying cells. A previous study with gel electrophoresis also demonstrated that the proportion of desmin positive cells in the aortic wall decreased (Nikkari et al. 1990). This intermediate filament is characteristic of the muscle differentiation, suggesting that the rat aortic wall lost some of its muscular characters during ageing. With age, the proportion of beta-actin also increased at the expense of the alpha isoform (Nikkari et al. 1990). This is in agreement with the fact that cells become more secretory and that quantity of extracellular matrix increased. Hypertrophy of the media should thus be due principally to desmin-negative cells. These results also explain the results of Stemerman et al. (1982) who noticed a difference in response of SMC to injury with age. At that time, the authors suggested that "ageing produces a change in the vascular SMC that enhance proliferation". It should be suspected from our data that ageing operates a selection of desmin-negative cells, which have a greater proliferation potential than desmin-positive cells, as already suggested in Connat et al. (2001). It could be thus assumed that thickening of muscular sheets is due to a hypertrophy of the remaining SMC and to a hypersecretion of extracellular material, particularly collagen. Both were observed with the electron microscope. In parallel, a lysis of the elastic laminae and a disorganisation of their parallel structure occur. This agrees with the extreme fragility of the old aorta observed upon dissection.

In females, although a tendency was discerned, we did not detect any correlation between age and thickness/area/nuclei density using the same statistical test as used for males. This seems to indicate a gender difference in the evolution of the aortic wall, females being slightly less affected by ageing. In human this gender-difference was not observed for distensibility of the aorta, which decreased similarly with age in both sexes; but in the case of the muscular brachial artery, the diameter increase was more pronounced in women and, contrary to men, compliance increased with age(van der Heijden-Spek et al. 2000).

Results from the TUNEL study indicate that positive cells are principally located in the luminal side of the media. This technique allows detection of nuclei that present a fragmentation of DNA and thus are suspected of entering the apoptotic process. Our morphological study with light and electron microscopy showing nuclear fragmentation and degeneration of cells confirms the fact that these cells are dying. Immunocytochemistry for desmin also showed a disappearance of luminal muscular layers. The electron microscopy revealed that the lumen of the vessel in old rats is considerably modified. The typical endothelium made of flattened cells closely attached to the internal elastic lamina is replaced by cells with pseudopodia adhering to an abundant extracellular matrix mixed with necrotic smooth muscle cells. Morphology of these cells, containing a bilobed euchromatic nuclei and typical clusters of electron-dense inclusions in the cytoplasm suggest that they are leucocytes (neutrophils and/or eosinophils) attracted to the inflammation site to phagocytose the cell debris. Two papers reported that endothelium was modified during ageing. Yeh et al. (2000) also remarked these cells with pseudopodia and cytoplasmic inclusions and indicated that connexin expression was modified. Aliev et al. (1995) described a large number of leucocytes adhering to the endothelium and a shift of expression of the NOS towards the nNOS. This isoform is primarily expressed by brain but is also expressed by muscles and neutrophils. We thus hypothesize from these data and ours that during aging the primitive intima of the vessel is progressively lost, to be replaced by a pseudoendothelium made of adhering leucocytes. It should be thus easier to understand why with age, the vascular wall modifies its reactivity to pharmacological agents.

The purpose of this study was to find if structural changes in the vessel wall could be due to an alteration/modification of the peptidergic innervation. On one hand, several lines of evidence indicate that chronic absence of sympathetic innervation in vivo induces morphological and biochemical perturbations of the vessel wall. For example, it increases collagen synthesis and decreases activity of tricarboxylic acid cycle enzymes in the rabbit vascular wall. Also in pigeons, abdominal aorta of animals that present susceptibility to atherosclerosis (White Carneau strain) has less sympathetic innervation and declines faster with age than in the Show Racer strain (Fronek 1983). On the other hand, in vitro, Chamley and Campbell (1975) noticed that coculture of SMC with sympathetic ganglia delayed the dedifferentiation and the subsequent onset of proliferation of the cells. We investigated the presence of peptidergic fibres, which are in close contact with SMC of the media with antibodies directed against four different neuropeptides. Although rat aorta is scarcely innervated (as already described for large elastic arteries in general) we noted that innervation to CGRP and SP was regularly present in the young rats. As previously noted for the rat hepatic portal vein (Ody et al. 1993), CGRP innervation occurs around aorta before the innervation for the other peptides. It is already present at embryonic day 17 (ED 17) while SP is not yet present at ED 17 (n=2) and ED 20 (n=2; A. Thiévent, unpublished results). SP immunoreactivity occurs around the vessel only 11 days after birth. On the contrary, NPY is not yet present at birth and occurs at 22 days.Then NPY innervation develops within the 2nd month after birth. Innervation to VIP, already described

as little (Udmann et al. 1981) occurs only later and is only visible in the young adult. In all cases however, we showed that peptidergic innervation gradually decreased from around 3–4 months and was no longer detectable in the elderly. We already noticed a decrease for the CGRP innervation during the first 6 months of life around male hepatic portal vein (Carrier and Connat 1996) and aorta of both genders (Thiévent and Connat 1993). In renal, mesenteric and carotid arteries of the guinea-pig, SP and CGRP density also decrease during ageing (Dhall et al. 1986). On the contrary, in cerebrovascular arteries of rats, while the density of NPY- and SP-containing nerve fibres was not changed, the expression of VIP and CGRP (which are vasodilator for the vessels) was strikingly increased in the elderly (Mione et al. 1988). Thus, cerebral circulation and arteries in the body seem to present a different adaptation during ageing.

Large central conducting vessel such as aorta play only a minute role in the control of blood pressure. The physiological role of vasoactive neuropeptides of their perivascular nerves is thus not clear. We suggest that these peptides could play a dual role. On one hand, they could be released in response to dilatation of the vessel due to hypertension and regulate in turn by a vasodilatatory peripheral effect the pressure. On the other hand, their presence around blood vessels could maintain the muscular characteristic of SMC. It was demonstrated a long time ago that coculture of SMC with sympathetic ganglia delayed dedifferentiation of the SMC (Chamley and Campbell 1975). Similarly, denervation of vessels leads to phenotypic modulation of the SMC (Dimitriadou et al. 1988). We showed a general decrease of peptidergic innervation of rat thoracic aorta during ageing, which is correlated with drastic structural changes such as loss of luminal desmin-positive SMC, hypertrophy of the remaining SMC (mostly desmin-negative), hypersecretion of extracellular material, disorganisation of elastic laminae. This is an inverse process to what is observed during development. In view that the occurrence of peptidergic innervation is concomitant with the differentiation of the vessel wall, we can wonder if disappearance of the peptidergic innervation could be responsible for an alteration of the vessel wall structure. The alteration of this innervation during ageing could explain the gradual increase of blood pressure, due to a loss of peripheral regulation together with a loss of local trophic regulation that leads to hypertrophy, intimal thickening and rigidity of the vessel wall by hypersecretion of extracellular matrix.

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