

Elastin variations implicating in vascular smooth muscle cells phenotype in human tortuous arteries

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Summary. The aim of the present work was to study the morphological implications between the elastin and the phenotypic expression of the vascular smooth muscle cells. For this purpose, sixty human tortuous arteries from different territories have been studied. We have measured the morphometric indexes **Intimal Thickening Index** and **Elastolyse Index** and they have been quantified with computer system analysis, image-colour corresponding to the orcein and Verhoeff reactions for detecting elastin and the a-actin in the smooth muscle cells. We compared both territorial arteries from the cranial and from abdominal origin. The elastin concentration was similar in both territories, but not its morphology according to its spatial distribution. We have observed a relationship between the elastin structural organisation from the media of arteries and of the internal elastic lamina in these territories and the variation of reactivity to the smooth muscle a-actin as a marker of the phenotypic state.

Our results confirm the hypothesis that elastin, besides intervening in the architecture of the arterial wall, is a factor implicated in the phenotypic variability of the smooth muscle cells and in the development and evolution of the intimal thickenings in human atherosclerosis.

Key words: Elastin, Vascular smooth muscle cells, Arterial thickening, Arterial tortuosity

Introduction

The study of the arterial wall is an important clue to increase our knowledge for the pathological events of the arterial wall, such as atherosclerosis or restenosis after angioplasty. In the wall can be found, as a dynamic constituent and of great influence, the cellular element, the vascular smooth muscle cells (SMC), and the

extracellular factor, the elastin. Both are main characters of first order in the development of the normal and modified arterial wall. Elastin is a protein that confers to the artery its distension capacity. In the foetal stage it determines the structure of the adult (Li et al., 1998). SMC are the predominant elements of the medial layer of a normal muscular artery. However in the medial parietal aorta elastin material represents an important structural constituent. There is strong evidence that the morphological structure of the extracellular matrix could influence the SMC functions, particularly the elastin (Atkinson, 1998).

The last investigations have made an effort to know the implications of all of the constituent elements of the arterial wall in a simultaneous way, studying the cell-extracellular matrix interactions. This fact has revealed a series of adherence proteins (laminin, fibronectin, vitronectin, tenascin and trombospondin) and other factors (integrins) that are of great importance. Their properties and characteristics generate a new hypothesis where both systems, cellular and extracellular, would be simultaneously decisive (Daniel Lamazière et al., 1997). Occasions, elastin could be the initial modulator and responsible for the phenotypic variations of the SMC.

In our study we have observed and measured the morphological and quantitative aspects of elastic material distribution of the arterial wall. We have correlated on serial sections the elastin organisation with the degree of SMC phenotypic modulation according to the immuno-detection of smooth muscle a-actin quantitation. We propose to associate the degree of elastin organisation and the level of SMC differentiation. This can be seen depending on the area of the arterial tree.

Materials and methods

Sixty human arteries of subjects ranging in age from 40 to 60 years taken from medical legal autopsies were tested. After background clinical investigation, they had not died of dyslipemias disorder, degenerative vascular processes or diagnosed high blood pressure. The areas studied and the samples taken from each of these

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subjects involved the following arteries: uterine (n=16), splenic (n=10), superficial temporal (n=12), lingual (n=11) and facial (n=11). After dissection, each piece was sectioned transversally in 1 cm-long segments. Only the medial and intimal layers were considered. The pieces were fixed following customary protocol for each case, cut using a Leitz microtome at 5 μ m and stained using Orceine and Verhoëff technique for elastic fibers and α -actin specific for SMC. For video analysis the sections were visualised by the operator through either an upright or an inverted Nikon microscope (Microphot-FXA/SA) and captured through a colour CCD camera (JAI 2070), the video signal being digitised with a Matrox "Meteore" device and saved onto a computer disk. In order to have reproducibility between image capture, we controlled the light intensity through the tissue specimen; each image was calibrated by its histogram values of the three colors (Red, Green, Blue). The morphometric study and colour intensity staining was carried out in INSERM Unit 441, Pessac-France using the QUANCOUL program (Daniel Lamazière et al., 1993) at power magnification of x400. The software Quancoul (Quant'Image-INSERM U441, France) defined true colours on the basis of three independent parameters (Hue, Intensity, Saturation). The parameters were calibrated against a background of control antigen which enabled extraction of the colour corresponding to the measured antigens. The thresholds were adjusted so that all but the faintest staining could be detected. Since the histologic reaction amount is expressed by both the number of the stained pixel (percentage of the labelled surface), and the intensity of colour. For our purpose, we described two types of staining as determined by measurement of the Hue Density (HD); strong or intense staining corresponding to HD>150 average, and a weaker or clearer reaction with HD<100. The strong staining corresponds to an important presence of α -actin,

Table 1. Summary of morphometric data in the different arteries studied.

	GROUP 1		GROUP 2		
	Uterine	Splenic	Temporal	Facial	Lingual
Elastin Media	15,06 \pm 4,1		13,70 \pm 3,8		
Elastin Intima	45,95 \pm 9,9		53,33 \pm 8,4		
ITI	27,38 \pm 5,8*	20,02 \pm 4,9*	11,71 \pm 3,3	7,14 \pm 2,8	14,86 \pm 3,1
EI	8,21 \pm 1,0**	9,45 \pm 2,9**	3,2 \pm 1,2	2,8 \pm 0,1	5,6 \pm 1,9*

ITI: Intimal Thickening Index (%); EI: Elastolysis Index (%); *: p<0.05; **: p<0.01

since the antigenic presence is at very high density.

Each case was analysed on five different slides, each slide containing five different sections. The medial and intimal layers were measured for morphometric evaluation and the elastin and α -actin for the quantitation in these areas.

In the statistical analysis, we have analysed the mean, standard deviation, variance and Newman Keuls q study for p<0.05 and p<0.01.

We define the Intimal Thickness Index (ITI) as the ratio for each section of the intimal surface (x100) divided by the medial surface. The Elastolysis Index (EI) is defined as the ratio between the values of elastolysis (mean distance between two fragments of the Internal Elastic Laminae, IEL) and the mean contour of the IEL. This can be proposed as an index of elastin organisation.

Results

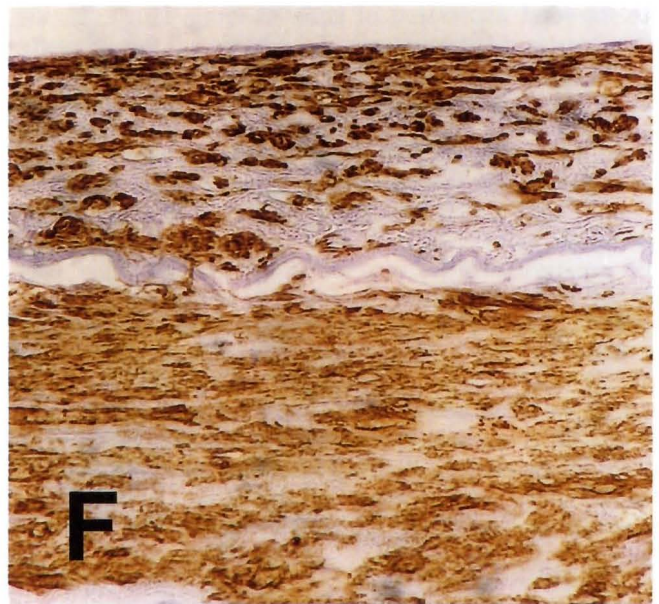
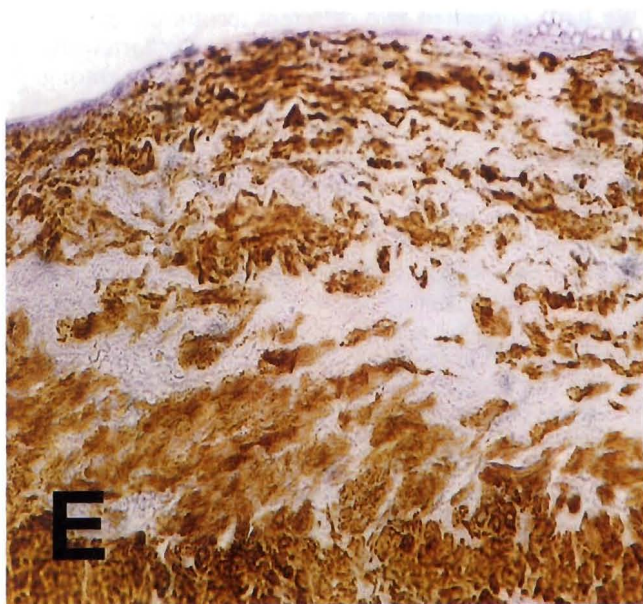
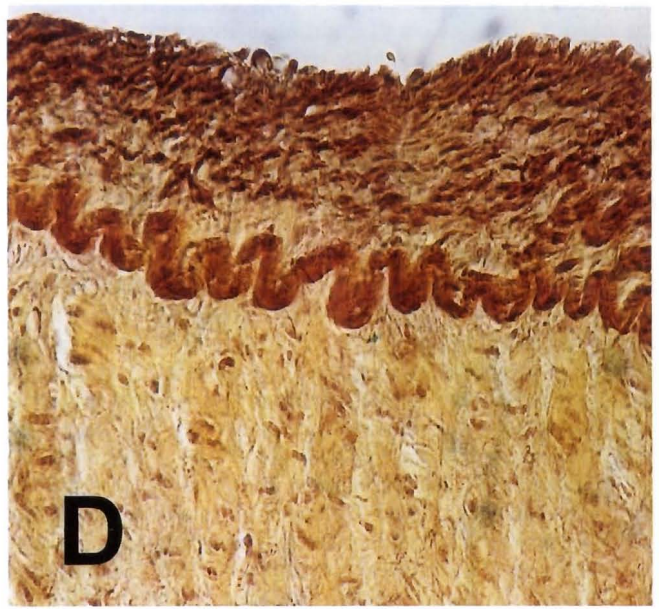
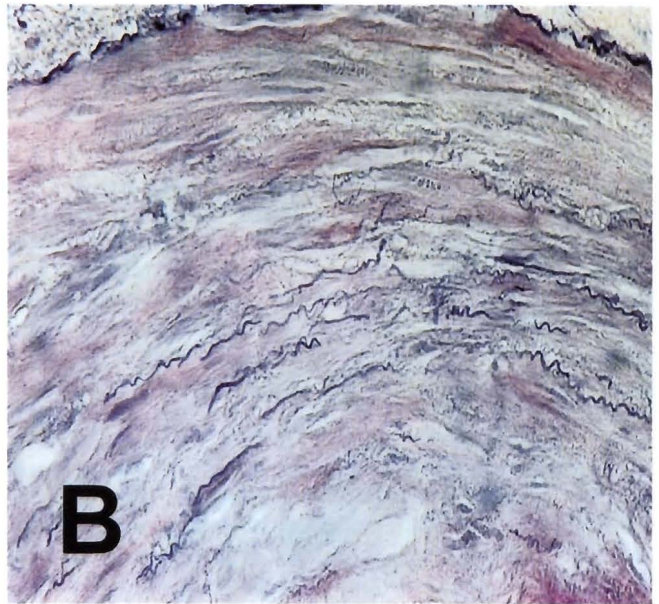
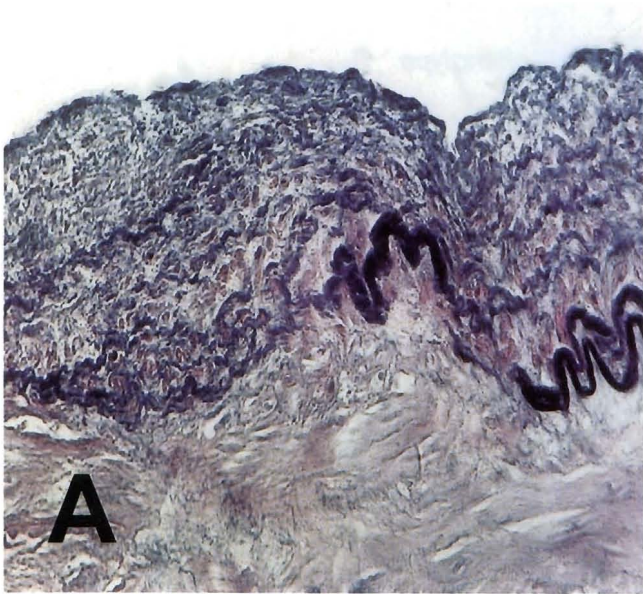
The gross morphometrical measurements obtained are shown in Tables 1 and 2. In relation to morphometric parameters (Table 1), the statistical analysis confirmed the existence of significant differences. We separated arterial specimens into 2 groups depending of anatomical origin. Group one corresponded to abdominal area and in group two concerned cranial territory. We did not find any statistical differences between the 2 groups concerning elastin concentration, both groups presented a higher concentration of elastin inside the intimal space than the media (Fig 1. A-D).

Table 2. Smooth muscle α -actin quantification.

SM α -ACTIN	UTERINE AND ARTERIES	TEMPORAL, LINGUAL AND FACIAL ARTERIES
<i>Artery without thickening</i>		
Total Media	79.55 \pm 5.62	78.25 \pm 6.28
Media (weak reaction)	61.25 \pm 3.26	63.55 \pm 3.12
Media (strong reaction)	18.30 \pm 2.32	15.78 \pm 3.08
<i>Artery with thickening</i>		
Total Media	61.25 \pm 8.23	70.32 \pm 6.21
Media (weak reaction)	42.3 \pm 4.24*	52.09 \pm 4.38
Media (strong reaction)	19.95 \pm 3.78	17.23 \pm 2.21
Total intima	15.22 \pm 3.59	17.40 \pm 4.06
Intima (weak reaction)	11.40 \pm 2.23	10.18 \pm 3.11
Intima (strong reaction)	3.82 \pm 0.65	6.22 \pm 1.57*

Positive reaction anti α -actin smooth muscle cell in arterial wall (%). *: p<0.05.

Fig. 1. Human uterine artery. Intimal thickening with anarchical and desestructured elastin neoformation. IEL is fragmented and disorganised in lamellae with variable thickness. Verhoëff technique. **B.** Human uterine artery. Elastic fibers in the medial layer. Isolated and finely undulated lamellae corresponding arteries with high ITI. Verhoëff technique. **C.** Human temporal superficial artery. IEL compact corresponding with less EI. Elastic fibers in the medial layer are rolled, with irregular undulations and radial direction from the IEL according with less ITI. Orceine technique. **D.** Human lingual artery. Intimal thickening with elastic material organised in parallel fibers. IEL is much more compact than in the abdominal arteries. Orceine-Van Gienson technique. **E.** Abdominal arteries group. α -actin reaction in the intimal (weak reaction and randomly distribution) and medial (strong reaction and change in the orientation) layers. **F.** Cranial arteries group. α -actin reaction in the intimal (strong reaction and parallel distribution) and medial (weak reaction and concentric orientation) layers. x 40



However, for the ITI values, we found two different arterial groups, one made up of the lingual, facial and superficial temporal arteries which presented values for intimal extension to a lesser extent than that observed in the uterine and splenic arteries ($p < 0.05$). Moreover, the integrity of the IEL, expressed by the EI, enabled us to reinforce the difference between the two groups ($p < 0.01$). However, the lingual artery presented an intermediate EI between the groups which concerned some elastin disorganisation ($p < 0.05$). In uterine and splenic arteries, the IEL was separated in two or more concentric lamellae with variable thickness (Fig. 1A). In the tunica media, the elastic fibres were distributed as thin, isolated and finely undulated lamellae (Fig. 1B). In superficial temporal, lingual and facial arteries, the IEL was generally a thicker lamina, usually single and compact. In the medial layer it was common to observe the elastic fibres as small undulated lines but also with a random distribution, with irregular undulations and rolled forming elastic knots and sometimes with a radial direction from the IEL (Fig. 1C). Underlying the intimal thickenings, the IEL stayed much more compact than in the other group of arteries, with an aspect of undulated parallel fibres that reduced the arterial light (Fig. 1D).

Table 2 quantitatively compares smooth muscle α -actin distribution in the above defined arterial territories. After numeric video analysis quantification we compared the content of smooth muscle α -actin. In order to express the variations in the immunostaining intensity we calibrated our numerical device in two groups according to color intensity. For strong staining we measured the mean Hue density as above 150, however for weak staining the average of Hue density was lower than 100. In both groups the positive reaction to the α -actin in the medial layer, without intimal thickening, did not show any significant difference even for the staining intensity. In the two groups of arteries with intimal thickening, we did not observe any significant difference for the total expression of actin staining. However, we found some differences when we screened the intensity of staining. Concerning the arteries from abdominal origin (Fig. 1E), the actin in the medial layer was 61.25% of the total, where 42.3% corresponded to weak reaction and 19.95% to strong reaction; in the intimal thickening, the values were of 15.22%, 11.40% and 3.82% respectively. In the cranial artery group (Fig. 1F), in presence of intimal thickening, the positivity to the α -actin in the medial layer was 70.32% corresponding to 52.09% for weak reaction and 17.23% for strong reaction. Inside the intimal thickening, the global values were of 17.40% with 10.18% and 6.22% for the weak and strong reactions respectively. We found statistical differences in presence of intimal thickening between weak staining of group 2 in the media layer ($p < 0.05$). Moreover, inside the intimal thickening we found statistical differences concerning the strong staining between the 2 groups ($p < 0.05$).

Discussion

Extracellular matrix plays an important role in controlling vascular integrity and vascular tone through the regulation of SMC differentiation (Daniel Lamazière et al., 1997). A recent feature increases the role played by the elastin organisation through the arterial wall (Curran et al., 1993; Li et al., 1998a). The production of elastin occurs mainly in the foetal period during the first stages of development (Pierce et al., 1995). After the fifth month of the gestation, the accumulation of elastin is accelerated in all the arteries but most rapidly in the thoracic aorta (Bendeck et al., 1994). In the arterial wall of arteries, elastic material is mainly concentrated in concentric elastic laminae. The elastin content is decreased along with the degree of muscle cell amount. We chose to analyse the degree of elastin organisation in different tortuous arteries from different origins, cranial and abdominal, so we proposed to compare the degree of elastin organisation and the level of muscularity according to the variations of smooth muscle α -actin.

Vascular SMC are able to modulate their phenotype and their function which allows a balance between cell proliferation or secretion of extracellular matrix and a decrease in the positive reaction to the α -actin (Bergweff et al., 1996; Thyberg, 1996, 1998). This is much more frequent in the tortuous arteries, where we observed important vascular diameter variations which induced laminar sanguine turbulence (Robert et al., 1986; Borley et al., 1995). Both elastin distribution and SMC phenotype had variations along the arterial tree. This paper compares the elastin distribution as well as SMC α -actin expression in different tortuous arteries.

Our results concerned elastin organisation as evaluated by morphometric indexes such as ITI and EI. It has been suggested that the qualitative aspects concerning elastin organisation could play a major role in vascular response (Whyte et al., 1995; Alconchel et al., 1997; Ortiz et al., 1997, 1998, 1999). In this paper we have compared two different territories, cranial and abdominal arteries, which present similar tortuosity. We have not found any significant difference among the concentration of elastin of the medial and intimal layer in either group. However, we describe a finely undulated elastin organisation of the medial layer with a concentric organisation for the abdominal group, contrary to the cranial group where we found several concentric elastin fibers as well as radial organisation. We have observed that the size and morphology of the intimal thickenings in these two territories are strongly different according to elastin organisation. The IEL organisation is very different between the two groups; in the abdominal group the organisation is more anarchical than in the cranial group, where the elastin disposition is predominately in strata (Fig. 1A-D).

We have linked this study of elastin morphological organisation to the phenotypic expression of SMC according to smooth muscle α -actin content. The quantification by other authors (Dufourcq et al., 1997) of

the content of α -actin in the normal arterial medial layer (76% in rabbit aorta), it is lightly inferior obtained in our study (79.55%). This can be explained by the fact that human arteries are typically muscular and not elastic. We found a decrease in α -actin amount in the medial layer of the arteries underlying generalised intimal thickening (61.25% in the uterine/splenic group arteries and 70.32% in the facial/lingual/temporal artery group) regarding to the control. This fact confirmed the results of Blank et al. (1988), Kocher et al. (1991) and Dufourcq et al. (1997) it has been attributed to SMC dedifferentiation or proliferation. The weak-reaction α -actin smooth muscle is significantly lower in the abdominal artery group than in the other group (42.3% to 52.09%). We postulate that in the medial layer of the first group, a stronger tendency exists to the dedifferentiation of the SMC, associated with an important secretion and cell migration toward the neointimal space.

In the intimal thickenings we have detected more reactivity to the α -actin in the cranial group arteries in comparison to the abdominal group. We can say that this result indicates that SMC of the cranial group presents a more differentiated phenotype. The phase of dedifferentiation implicated in the migration and proliferation to produce the intimal thickening from the medial layer would be easier in the abdominal group arteries, since the SMC are already less differentiated. We postulate that this situation is favoured by a less organised elastin. Our results confirm the works of Whyte et al. (1997) in the uterine artery and those of Sartore et al. (1997) in other territories, where it is observed that in the vascular processes characterised by the migration and proliferation of the SMC, different degrees of cellular differentiation and an heterogeneous phenotypic state presentation coexist (Thyberg, 1998).

We can deduce the existence of some important morphological implications between the elastin and the differentiation of the SMC. We have described a link between elastin organisation and SMC differentiation state. When we found a polyform of organisation of elastin fibers inside the media (cranial group) it was associated to a more differentiated phenotype. Moreover, the morphology of the intimal thickening depends on the underlying medial organisation. This situation could be important in the development of the atherosclerosis. The presence of differentiated phenotype of SMC can limit the plaque extension and prevent the fibrous plaque from rupture, the differentiated state is a factor of stability (Kockx et al., 1996; Weissberg et al., 1996; Carr et al., 1997).

When a deficit of elastin exists (Curran et al., 1993; Li et al., 1998b) or its structural properties are not the appropriate ones (Borg et al., 1995; Mari et al., 1995) a high incidence of pathological arterial wall thickenings is observed, with abundant proliferating SMC (Ito et al., 1998). Moreover, most of SMC are oriented in a radial position leading to vascular occlusion. Between a gene lack and a defectus, several possibilities exist to influence elastin conformational structure; local factors

like the haemodynamic changes (Levy et al., 1994; Safar et al., 1996) the collaterally degree, the metallo-proteinase presence (Patel et al., 1996; Marshman, 1998), and the activity of the lissyl-oxidase system (Hungerford et al., 1996; Peyrol et al., 1997).

The relation with this cell proliferating ability and the differentiated state is not yet clearly established. We have tried to associate arterial elastin of different territories and the state of differentiation. Compared to abdominal area, cranial artery elastin had a high degree of organisation linked to a more differentiated state and a lesser extent of ITI.

In conclusion, we have observed that the elastin of the media, besides its structural function, is an element that modulates the phenotypic expression of the SMC and determine the development and the evolution of the intimal thickenings, according to the territory of the arterial tree studied.

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