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The Evolution of Experimental Endarteritis in the Rabbit Abdominal Aorta

Light and Transmission Electron Microscopy

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Summary. Experimental aortic intimal thickening has been induced in rabbits by sheathing the vessel with a polyethylene cuff. The alterations have been examined by light and transmission electron microscopy, during 12 months. An irregular intimal thickening develops as soon as the 15th day and includes numerous myofibroblasts with some other cells of monocytic or endothelial type. Microfibrils, elastic aggregates and collagen fibers are found in the intercellular space. Simultaneously, the media undergoes a fragmentation of the elastic laminae and the adventitia shows a capillary angiectasis and a granuloma. After 3 months there is, between the intimal smooth muscle cells, a progressive increase of elastic and collagenous material. In the media, elastic break up becomes more frequent after the 4th month and myocytes appear increasingly atrophic, which facilitates the extension of fibrosis. This is accompagnied at times by a thinning of the arterial wall with or without localized disappearance of the media. All these modifications are discussed and compared to what we had previously found in the femoral artery [12].

Key words: Endarteritis – Smooth muscle – Elastogenesis – Elastolysis – Fibrosis – Medial atrophy

We recently [12] described the morphological characteristics of endarteritis after the temporary placement of a polyethylene cuff around the femoral artery. The study demonstrated that an early intimal thickening developed as soon as the 7th day. Moreover, these lesions were invariably associated with modifications of the media and the adventitia. The present study was undertaken to examine the reactivity of the aorta in the same experimental model in order to compare the behaviour of two different types of artery and see if the same elements are involved in the evolution of the phenomenon. A brief report of this work has appeared elsewhere [11].

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Materials and Methods

Material and Operative Protocol

The present study included 42 male rabbits "Fauve de Bourgogne", 2.5–3 months old (about 2,500 g body weight); the animals were fed by a standard food (type U.A.R.), and divided into 2 groups:

a) Group I comprised 30 rabbits. The animals were operated on under Urethane-Nembutal anesthesia. After a laparatomy, the subrenal segment of the aorta was mobilised over a length of 15–22 mm, and surrounded by a polyethylene cuff that did not constrict the vessel. It was not possible to remove the cuff as was previously described for the femoral artery [12], because of early fibrous organization, which lead to adhesions to vena cava. Post-operative complications forced us to replace 4 animals.

b) Group II comprised 12 untreated control rabbits which were maintained under the same conditions.

II. Samples and Preparatations of Tissues

a) Sacrifices. For group I, 2 rabbits were sacrificed every two weeks over a 3 months period, and then 2 every month between the 4th and the 12th months.

For group II, 4 rabbits were sacrificed on the 15th day of the experiment and the remaining 8 at intervals of 2 rabbits every 3 months.

b) Fixation, Samples, Embedding and Staining. Fixation was by perfusion with glutaraldehyde as previously descirbed [12].

For group I, after removing the cuff, the whole abdominal aortic segment was resected and divided into 5 fragments: The central part was kept for electron microscopy. For light microscopy, the 2 adjacent parts were transversly-sectioned and the 2 distal parts were longitudinally sectioned in order to study transitional zones.

Two other aortic samples were examined by light microscopy to verify the vascular condition away from the cuffed aortic segment: the aortic arch and the coeliac trunk.

For group II, samples of the aortic arch, coeliac trunk and abdominal aorta were also excised.

All electron microscopic samples were postfixed in osmium tetroxide, dehydrated in ethanol and embedded in Epon 812. Thick sections (1 μ) were stained with toluidine blue or methylene blue. Appropriate areas were selected and ultrathin sections (about 50 nm), were contrasted with uranyl acetate and lead citrate prior to being viewed in a Siemens I A electron microscope.

For light microscopy, the fragments were counterfixed in 10% formol-calcium, embedded in paraffin, sectioned and stained as follows: Masson trichome; Silver impregnation of reticular fibers; Orcein; Periodic-acid-Schiff (PAS); 0.1% Alcian blue in acetic acid (pH 2.7), before and after digestion by testicular hyaluronidase.

Results

I. Macroscopic Observations

The placement of the aortic cuff was followed by a more intense perivascular reaction than for the femoral artery. By the 8th day, the cuffs were encapsulated in a dense connective tissue which lead to adhesions to vena cava and rarely to abdominal organs. In one case, there was an adhesion to intestinal loops and this rabbit also showed a liver abscess and an encysted abscess around a cuff coating a partially necrosed aorta. Another rabbit also showed a pericuff abscess. These 2 animals were not considered in further investigations.

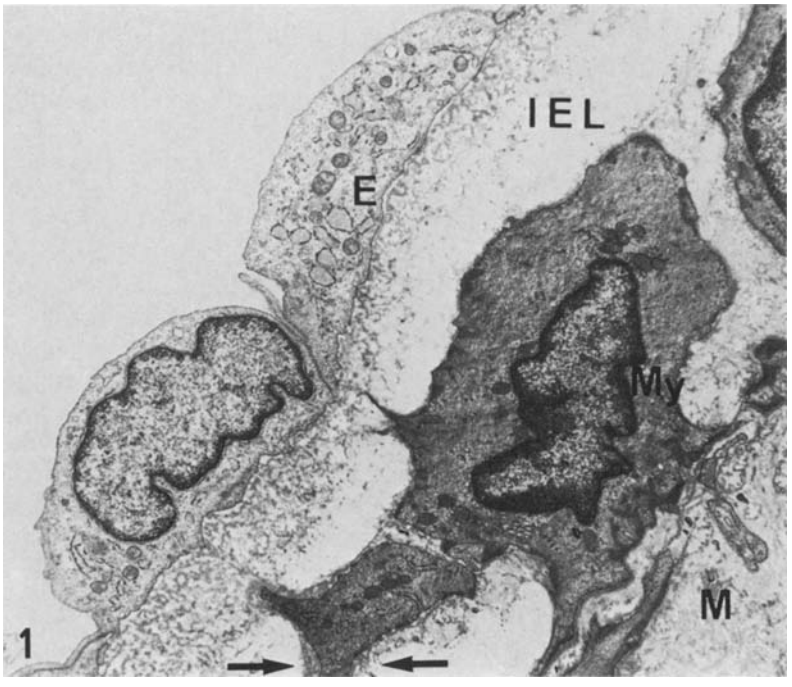


Fig. 1. Abdominal aorta of a 3 month old control rabbit. In this area, the sub-endothelial space is thin and shows elastic aggregates and numerous microfibrils. The internal elastic lamina (I.E.L.) is split (*arrows*) and myocytes (*My*) have cytoplasmic extensions going into the fenestrations of the I.E.L. Endothelium (*E*); Media (*M*). ×8,500

After perfusion and removal of the cuff, the sheathed aortic segments had a constantly greater diameter than the adjacent parts. This finding was at times accompanied by a thinner wall.

II. Remarks on the Normal Aortic Structure during Ageing

In the young animal (Fig. 1), the subendothelial space is discontinuous and includes microfibrils, some elastic and collagen fibers and a few myocytes perpendicularly oriented to the cells of the media.

After the 12th month, increasing cellular population and interstitial material leads to the formation of a constant and continuous subendothelial space. The interruptions in the internal elastic lamina (I.E.L.) became more frequent and the elastic laminae of the media grow thinner. Therefore, these regions of enlarged intercellular space show accumulation of cellular debris.

III. Observations during the Endarteritic Process

a) Light Microscopic Study. The aortic arch and the coeliac trunk do not show any lesion.

In the transitional zones, the longitudinal sections show that intimal proliferations and medial changes are restricted to the cuffed segment.

In the treated segments, the intimal thickening is sometimes circumferential but generally less extensive than in the femoral artery [12]. They are of 3 different anatomical types:

1. A more or less irregular band shape
2. In localised pads
3. In a form of a depression that appears to involve the media which sometimes completely disappears.

As of the 15th day, the irregular endarterial thickening shows 1 to 7 cellular strata (Fig. 2). The intercellular substance alcianophilic and PAS positive (similar to the internal media), includes collagen fibers, reticular fibers and numerous orceinophilic grains. The I.E.L. is discontinuous and the elastic laminae of the media are fragmented or collapsed. The adventitial reaction is characterized by capillary angiectasis, fibrous hypertrophy, fibroblast multiplication and histiocyte infiltration.

From the 1st to the 3rd month, elastic units merged to form lamellae which are better developed beneath the endothelium (Fig. 3), while the deep intima became enriched with collagen fibers. In the internal third of the media, fragmentation sometimes leads to the complete disappearance of elastic elements (Fig. 4).

Between the 4th and the 9th months, luminal widening and thinning of the arterial wall are observed. The extent and thickness of the intima are variable (1–15 cell layers). The well developed plaque can be either fibrous with a reduced cellular population and a few elastic elements, or very rich in elastic lamellae and fibers. In the latter case, when the I.E.L. is destroyed and the elastic laminae of the underlaying media are broken up, it is difficult to see clearly the intima – media boundary. One could ask whether the media is incorporated into the plaque, or if it is completely atrophied? Elsewhere in the media, the elastic laminae are condensed into

Fig. 2. Fifteen days after placing the cuff, the intimal thickening shows cells of variable density. In the media, the elastic laminae are thinned and discontinuous. Toluidine blue × 700

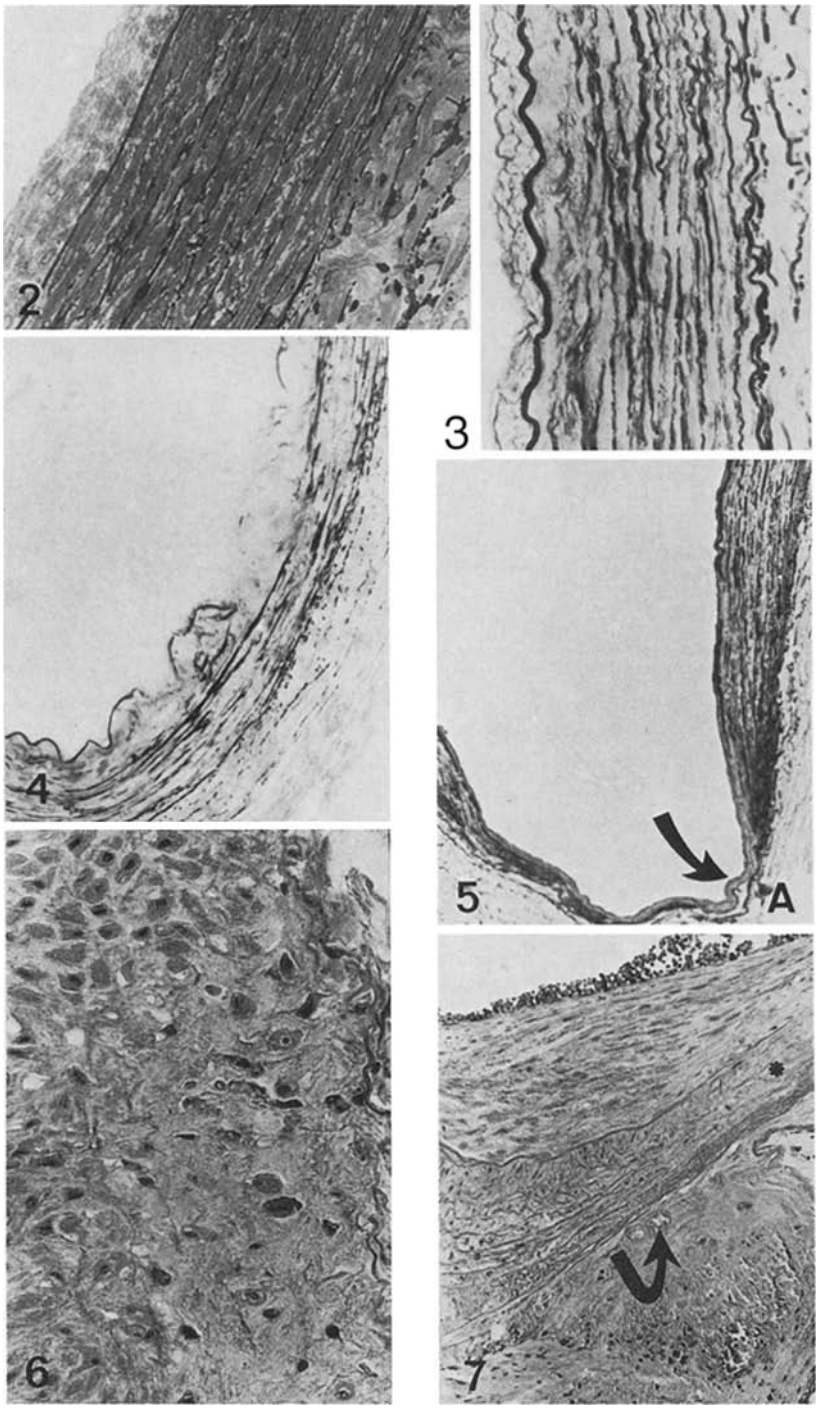
Fig. 3. After 3 months: elastic neogenesis prevails over the superficial layers. Orceine × 450

Fig. 4. Abdominal aorta $2^1/_2$ months after placing the cuff showing major fragmentation of the elastic laminae in the internal media. – Methylene blue × 400

Fig. 5. In this artery, 7 months after placing the cuff, the thickness of the media is variable. Sometimes (*arrow*) it is difficult to see if the media is completely atrophied or if it is incorporated to the intimal plaque, which rests on the adventitia (*A*). Orceine × 175

Fig. 6. Eight months after placing the cuff, extensive fibrosis is seen in the external media and the cells are going necrois. H.P.S. × 700

Fig. 7. After 8 months, in some areas fibrosis can spread to the whole thickness of the media (*) and to the deep overlying intimal thickening. Note a cartilaginous metaplasia in the adventitia (*arrow*). H.P.S. × 150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thick dark bundles. The thickness of the media is unequal and it frequently becomes atrophic (Fig. 5). The thinning and atrophy are either continuous over a certain area and localized, or discontinuous and recurrent giving, at a low magnification, a beaded-shape to the wall.

The myocytes of the external media have a pale cytoplasm. As early as the 4th month necrotic focus are seen in the external third-middle third of this coat (Fig. 6). In the vicinity of these degenerative lesions and within a fibrous tissue, we observed cartilaginous metaplasia in a sample at the 8th month. The same is true for the adventitia (Fig. 7). These appearances are also noted after an experimental trauma [50], or as spontaneous lesions [17].

The interstitial space is occasionally oedematous. The fibrosis which appears in the external part can spread to the whole media, thus the media-adventitia boundary become vague.

After the 10th month, the endarterial plaque irregularly but partially regresses. The cells maintain a variable orientation: circular or longitudinal and rarely radiating. The elastogenesis and the fibrosis vary according to the animals and within the same animal depending on the sections examined. The modifications noted above are more marked in the upper cuffed segment than in the lower segment.

With time, the number of the cells decreases in the adventitia, the capillary angiectasis persists and the fibrosis increases.

b) Ultrastructural Study. Fifteen days after placing the cuff, the endothelium shows numerous organelles. In the thickened intima there are different cellular types, but the myofibroblasts prevail. Sometimes the cytoplasmic membrane of superficial myofibroblasts is in close contact with endothelium. Some other cells showing Weibel and Palade bodies, are joined to the basal face of endothelial cells and seemed to be of the same nature. A few myocytes and monocytes are also noted (Figs. 8, 9).

In the intercellular space, elastic aggregates composed of amorphous elastin surrounded by microfibrils are seen in close relationship to subendothelial basal membrane or with glycocalyx sheathing the myofibroblasts. Most of the cells of the media show numerous organelles, and those of the internal layers are engaged in the widened fenestrations of the I.E.L. (Fig. 10). At the media-adventitia junction zone, occasional myocytes with well developed organelles are seen inserted in the fenestrations of external elastic lamina (Fig. 11).

After 6 weeks "superposed" endothelial cells are sometimes still seen. At about the 3rd month, the subendothelial intimal space includes cells particularly of smooth muscle type. However, at the 4th month, when the plaque rests directly on the adventitia, myofibroblasts continue to appear in the intima and occasional monocytes are close to endothelium in the vascular lumen (Fig. 12). In the very large interstitial space of the intima newly formed elastic lamellae containing abundant microfibrils developed at about the 3rd month (Fig. 13). The media rarely shows modified myocytes at this time, but myofilament disappearance and vacuole formation become

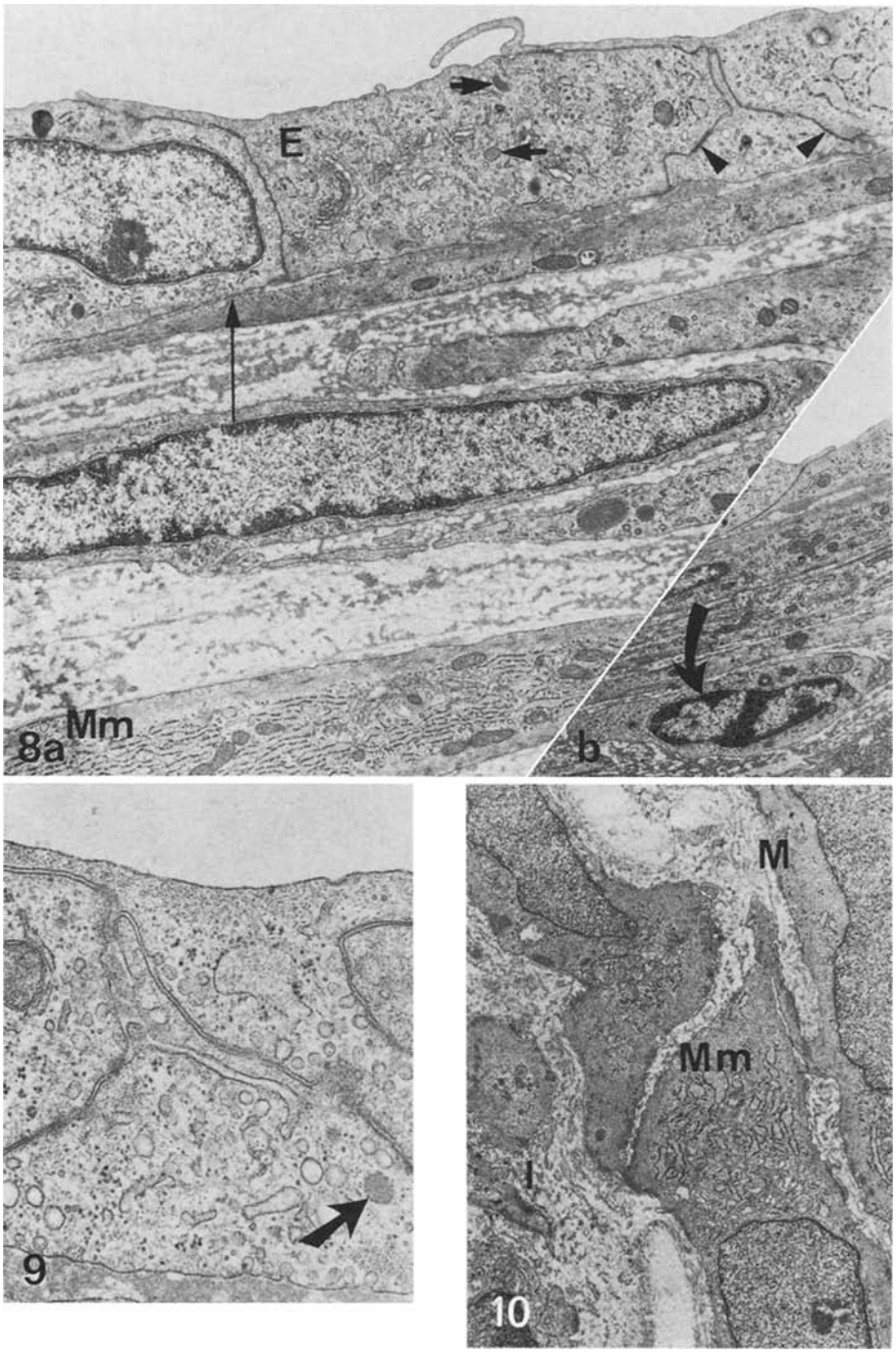

Fig. 8a, b. Intimal thickening 15 days after placing the cuff. **a** Note the prominent organelles in the endothelium (*E*) and the Weibel-Palade bodies (*horizontal arrows*). A subendothelial cell showing junctions (*arrow heads*) with the overlying endothelium. Another contact between this layer of cells and a superficial myofibroblast is seen (*vertical arrow*). Modified myocytes (*Mm*) × 10,400. **b** Monocyte (*arrow*) is occurring in this plaque. × 5,500

Fig. 9. Weibel-Palade bodie (*arrow*) in a sub-endothelial cell, 15 days after placing the cuff. × 27,600

Fig. 10. Intima (*I*) – Media (*M*) after 15 days. Two modified myocytes (*Mm*) are seen in the enlarged fenestrations of the I.E.L. × 7,800

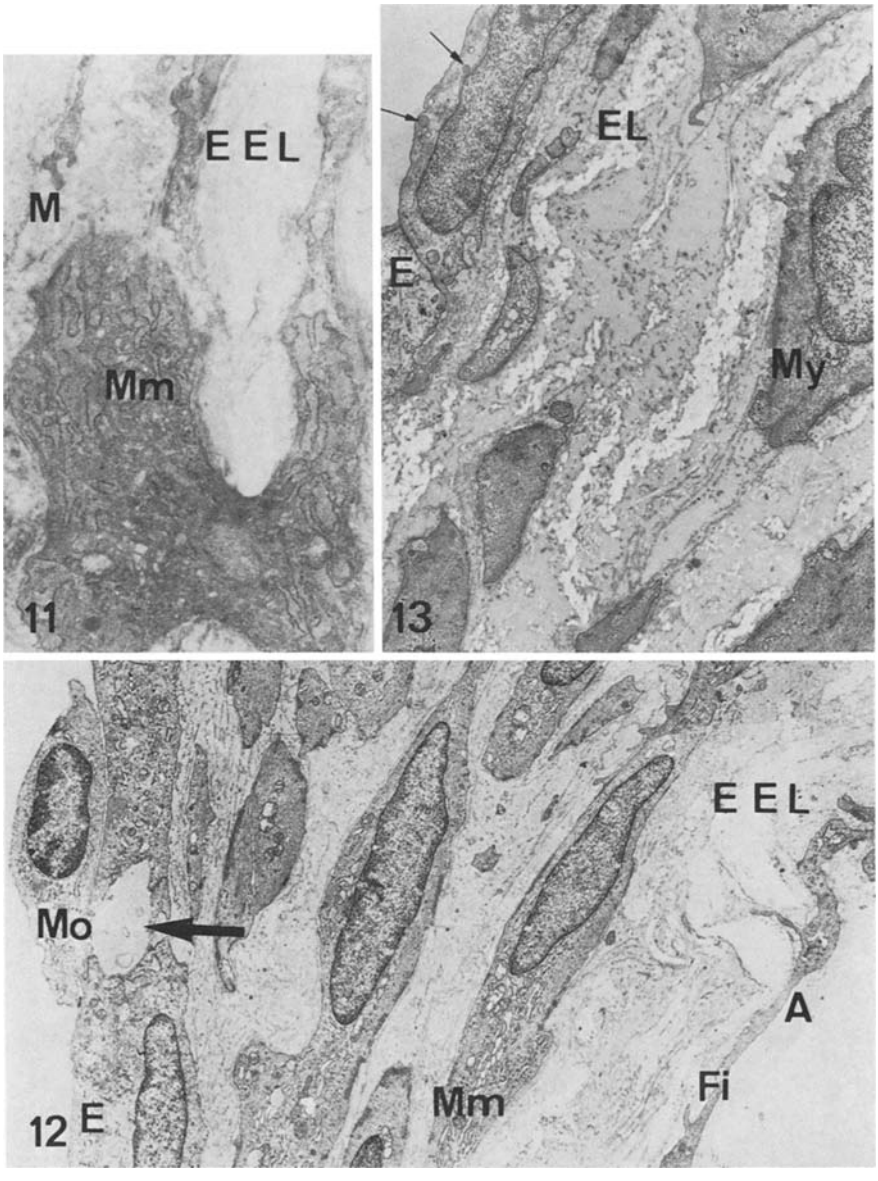

Fig. 11. Modified myocyte (*Mm*) is trapped in a fenestration of the external elastic lamina (EEL), 15 days after placing the cuff. Media (*M*). × 9,000

Fig. 12. Four months after placing the cuff: here, the media is absent and the intimal plaque lays directly on the adventitia (*A*) Note a monocyte (*Mo*) adhering to the endothelium (*E*), modified myocytes (*Mm*) and the poorly developed elastic fibers in the interstitial space. External elastic lamina (*EEL*). Fibroblast (*Fi*). The dilatation in the interendothelial junction (*arrow*) is probably artifactual. × 5,000

Fig. 13. Intimal thickening of an artery 3 months after placing the cuff, showing typical myocytes (*My*) separated by a more or less developed elastic lamellae (EL). Endothelium (*E*) with Weibel-Palade bodies (*arrows*) × 7,500

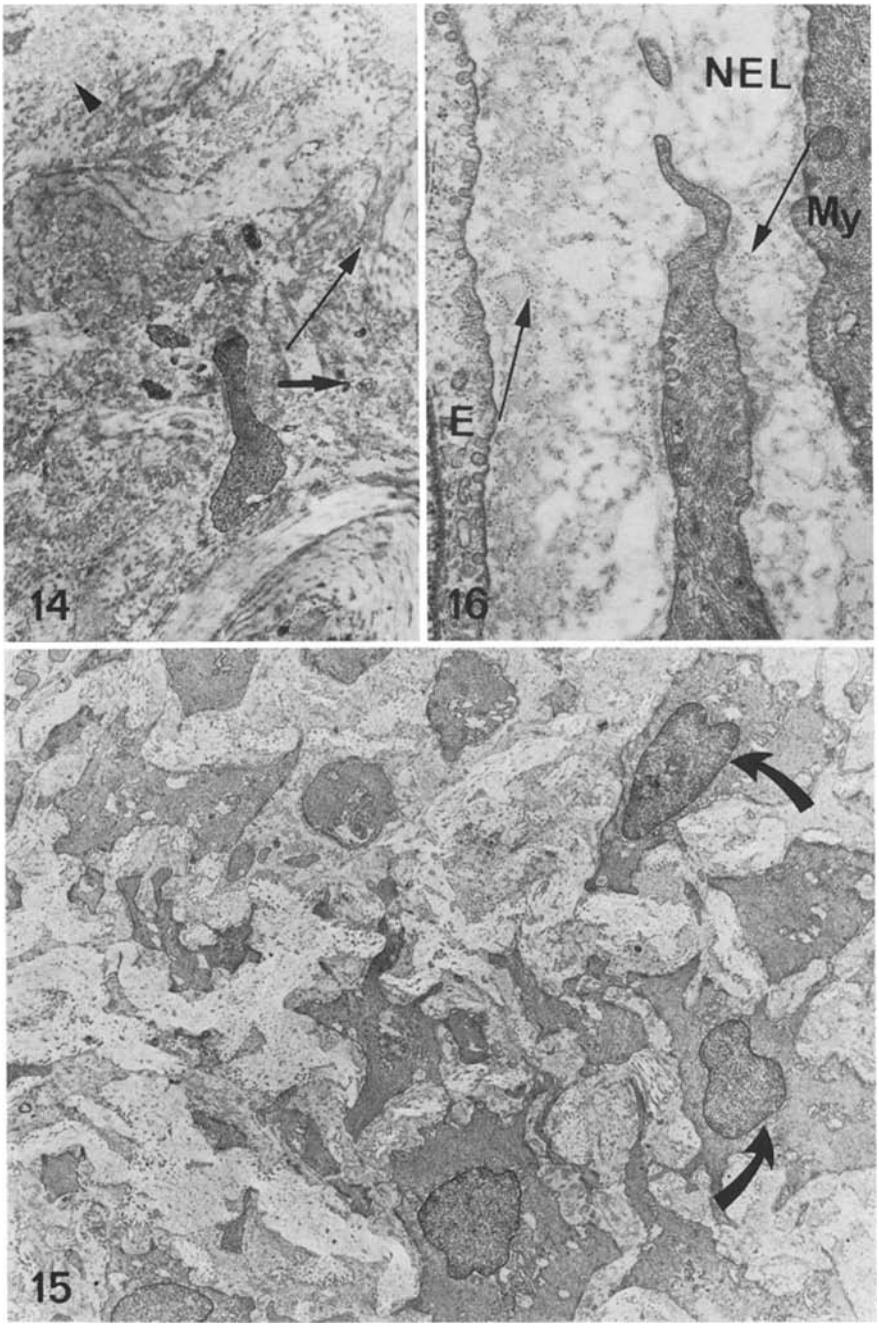

Fig. 14. Interstitial space of a media, 4 months after placing the cuff, showing major fragmentation of elastic laminae: the amorphous matrix is rarefied and the microfibrils very numerous (*thin arrow*). Cellular debris (*thick arrow*). Collagen fibers (*arrow head*) ×14,300

Fig. 15. In this media after 4 months, between the broken elastic laminae, the myocytes show numerous slim cytoplasmic extensions. The nuclei are often excentric (*arrows*) ×4,700

Fig. 16. Intimal thickening of an aorta 12 months after placing the cuff. Beneath the endothelium (*E*) or the basal membrane of myocytes (*My*) small elastic aggregates are still seen at this time, and surrounded by microfibrils of 11 nm (*arrows*). New elastic lamellae (NEL) ×27,600

more frequent. Within some specimens, elastic break up is very frequent as early as the 4th month: the amorphous matrix is rarefied and the medial interstitial space is crammed with microfibrils, collagen fibers and cellular debris (Fig. 14). In such areas, the cells of the media have a veiled aspect similar to fibroblasts: their ramified cytoplasm includes numerous organelles and a residuum of myofilaments and dense bodies (Fig. 15).

From the 5th to the 8th month, beneath a metabolically hyperactive endothelium, the main cells of myocytic type are fitted with numerous extensions. Elastogenesis appears to be slower and more complex than in the femoral artery. Elastic elements are seen at different stages of maturation with the well developed lamellae close to the endothelium only if the I.E.L. is still present. When this is damaged, elastic scraps are numerous in the deep intima and seem to incorporate into the intimal plaque.

Between the 8th and the 12th months, ocasional myofibroblasts are found in the intimal regions where fibrosis is widespread. Elastolysis which has appeared in the media, also affects the newly formed structures after the 9th month, but elastogenesis continues in the last samples (Fig. 16). In the media, the myocytes are more or less altered, with vacuoles which are sometimes huge.

Discussion

Although the cuffs had not been removed before the sacrifice, the dilatation of the aorta is larger than that seen in the femoral artery [12]. The former contains more connective tissue (elastic fibers, collagen fibers and mucopolysaccharids) than the latter. Changes in those macromolecules responsible for the biomechanical properties of the aortic wall [15, 40] can result in the observed dilatation. Traumatic over-stretchings upon release of the aorta may be a cause of this, but the more dilated aorta has a damaged elastic framework.

Rupture of the elastic laminae in the media, while favoring disinsertion of the cells, could lead to intimal proliferation. The reaction is of variable magnitude. Similar individual and segmentae variations have been observed and discussed [12]. Although individual variations are described in the literature, segmentae variations have not, as far as we know. This is probably due to a practice of taking a smaller number of samples and sections from the same animal.

All the alterations can be roughly divided into 2 phases, with regard to their chronological development:
- an early phase characterized by a general activation of the arterial wall with a proliferation and fibrillogenesis;
- a late phase characterized by a certain "stabilization" of the appearent elements, however with degenerative aspects.

I. Early Modifications

On the 15th day, the main cellular component in the intimal proliferation is of modified smooth muscle nature.

Myocytes can proliferate in response to a mitogenetic factor released by platelets in vitro [38, 43, 44, 45, 46, 48]. The inhibition of platelet function also leads to the inhibition of intimal plaque formation [8]. But for a platelet agregation, the endothelium must be destroyed and the subendothelium (basal membrane and collagen) exposed to the circulating blood. In our experimental model, there is no platelet agregation, and even if there were any endothelial lesion, it would be discret, fast, and not very extensive. In addition, according to Malcsak [36] even damaged endothelial cells do not attract the platelets.

However, Haudenshild [23] has showed that endothelial regeneration precedes the proliferation of intimal myocytes. The intimal areas that are rapidly covered by endothelium are therefore protected from the development of a fibro-cellular intimal lesion. If there is an early endothelial necrosis, the distal parts of the cuffed segment should not show an intimal thickening; on the other hand, intimal thickenings in process of overlapping must be visible. But, these appearances are not found in our samples. Does platelet function play any role in our experimental model? Perhaps, when there is modified endothelial permeability (which could be demonstrated by radiolabeled substances) a mitogenetic substance would diffuse in vivo through the endothelium. Recent observations [9] suggest that endothelial cells create a growth factor for smooth muscle cell in vitro. One may speculate as to the importance of the endothelial growth factor in the origin of intimal thickening.

In any case, numerous modified smooth muscle cells appear in the intima. This phenomenon is a typical aspect of developing intimal plaques [6, 22, 28, 29, 42, 50]. The abundance of cytoplasmic organelles, the rarefaction of myofilaments and the histoenzymatic changes [1], all suggest an active fibrillogenesis. Indeed, the first microfibrils appear in close relation to glycocalyx. Later on elastic units merge to form lamellae far from the cells. This sequence of maturation has been studied during the different stages of human aortic growth [26] and the embryonic development of the arterial system [2].

It is generally agreed that the modified myocytes or myofibroblasts are responsible for the synthesis of extracellular material [6, 25, 26, 29, 41, 47].

However, as we had reported for the femoral artery [12], the endothelium also participates in the elaboration of this material.

The origin of the cells populating the endarterial plaque has been discussed for a long time using the femoral artery model [12]. The same neointimal cells are found in the 2 types of artery. However, the most important source is the media. Indeed, the myo-intimal cells and the mediacytes have a similar structure, modified myocytes are seen in the fenestrations of the I.E.L. at the 15th day and the cells of the media activated before their proliferation in the subendothelial space.

We should also mention monocytes, because recent data [13, 14] suggest that these cells are the major precursor involved in the atherosclerotic lesion. During the femoral and aortic endarteritis a few monocytes appear in the

first stages of the process. They do not seem to occupy an important role in our experimental model.

A characteristic modification of the aortic media affects its elastic network which, as of the 15th day undergoes enlarged alterations made up of thinning and fragmentations. These images are observed after sheathing the aorta by plastic cuff [28] or BAPN administration [3, 5, 33] whereas the elastic laminae are preserved after ligature or orthostatic collapse [6, 34].

In the internal third of the media, the lesions in the elastic laminae are described along with the ageing aspects of large arteries [10] as an "intimalisation of the media" [4].

The adventitia reacts to the cuff placing by a fibroblast multiplication and activation, and a subsequent fibrosis also seen after other methods of enclosure of the aorta [29, 52] or after allylamine administration [32]. Such a thickening of adventitia which increase over time can contribute to the maintaining of the mechanical properties of the arterial wall (considering that the media undergoes important atrophy) and prevent possible vascular rupture.

The widening of vasa vasorum, also seen by Lusztig [35] and Huth et al. [28], and their multiplication, produce an adventitial hypervascularization. This phenomenon may compensate for the local metabolic deficiency.

II. Long Term Modifications

After the 4th month, beneath a normal endothelial layer, the myocytes may be numerous and separated by elastic lamellae, or rare and modified (myofibroblasts) in the deep fibrous intima. Therefore, the same gradient in the distribution of intercellular elements is seen as in the femoral artery [12]. It seems that there is an equilibrium between collagen and elastin biosynthesis; this was noted by Jones et al. [30] in a culture of fibroblasts of the ligamentum nuchae. Furthermore when in vivo in the very young fetal calf, the ligamentum is composed of a collagenous matrix and the microfibrils of elastic fibers are visible only after 100 days [31].

The fibrosis increases over time and the newly formed elastic structures show some damage after 9 months. This fragmentation is much more difficult to describe than in the femoral artery where elastogenesis was more distinct. However, the presence of elastic units beneath the endothelium at the 12th month proves that there is a continuous synthesis of the 2 macromolecules: glycoproteins and tropoelastin.

The long term evolution of the process in the media shows more degenerative images than what we have observed in the femoral artery [12]. As early as the 4th month, elastic laminae become dissociate in fragments, where the matrix become rarefied and bordered by numerous microfibrils. These morphologic data can express the so-called "contamination of human aortic elastin by glycoproteins" [27] and resemble the images observed by Haust [24] in the degeneration of elastic tissue in aortic explants. Also,

the signs of damage which appeared in the cells of the media can result in the necrosis of the middle third-external third of this tunic. Rarefaction of the myofilaments and myocytic necrosis are described by numerous authors [16, 17, 22, 32, 34, 52], as a primary reaction to various trauma whereas the images we obtain are secondary to the intimal thickening. The rabbit aortic media is not vascularized and this is in agreement with the works of Wolinsky and Glagov [51]. An hypoxia and a metabolic reduction develop in this critical zone (middle third -external third), where the cells are chronically underprivileged and in danger of necrosis. Hypoxia is expressed by a decrease of some oxydoreductases and ATPase activities [1].

Other factors will take place: the probable maintaining of the cuff in the course of the experiment and the fact that the abdominal aorta is metabolically less active than the femoral.

Over time, these focalised lesions evolue towards slow elimination of necrosed myocytes and their substitution by fibrosis. The long persistence of activated myocytes with a fibroblast-like shape, explains the increasing production of collagen fibers.

However, we have never seen total necrosis of the cells of the media replaced by fibrosis of the whole cuffed segment as noted by Huth et al. [28] (after 4–6 months). This is probably due to the adventitial compression by the cuff.

The irregular thinning of the media frequently leads to a total but localised atrophy which sometimes enhances intimal development. This decrease in the medial thickening can be the result of both the passage of internal mediacytes into the intima and of myocytic necrosis.

Repopulation of the media was proposed by Webster et al. [50] to occur from the advential pericytes. They took into account their capacity for transformation into myocytes [7, 37]. Indeed Webster et al. have noted an early incorporation of tritiated thymidine within the pericytes, and their presence in the interruptions of the external elastic lamina. In our experiment only one such image has been seen, at the 15th day. This weak and early occurence of such a cell does not explain the disappearance of the cells of the media, and probably contribute to the thinning observed.

Conclusion

The experimental data underline the importance of cellular intraparietal exchanges. As in the femoral artery the aortic wall showed a great plasticity. However, comparison of the femoral and aortic reactions introduces some differences, after the cuff placing. In the femoral artery, intimal proliferation seemed isolated and directly responsible for the thickening, apparently showing a "lesion by addition" [39], whereas for the aorta, the intimal thickening which appeared later, is of a more complex aetiology. Occasionally, the important destruction of elastic laminae of the internal third of the media, erase the intima-media boundary giving an image comparable to what Bouissou et al. [4] described as "intimalization of the media". Frequently, the intimal thickening sits on top of the media. Indeed, neoelas-

togenesis is always more developed beneath endothelium and obvious when the first elastic lamina is still present. Furthermore, the mitotic figures seen early in the femoral artery suggest that the neointimal cells come from a proliferation rather than from the reorientaion of smooth muscle cells of the preexistant media.

The development of intimal plaque preceeded the damage and necrosis of aortic mediacytes. These alterations of media in relation with a decreasing nutrition, may result in a more or less significant atrophy of this coat.

If we admit the reality of an incorporation of the internal layers of the media into the intima, this phenomenon can only be accessory to a much more important process of cell proliferation. It is accompanied by medial alterations more accentuated than in the case of the femoral artery.

Most likely such parietal injuries, at least in the large arteries [18, 20, 21, 49] constitute a preferential site for further lipid deposition if metabolic changes lend themselves to this and if endothelial permeability is altered. Moreover, rapidly proliferating cells in the neointima of injured aortas may synthetize increased quantities of cholesterol [19].

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