Immuno-inflammatory Athero-arteriosclerosis Induced by Elastin Peptides: The Effect of Age

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Abstract

The emergence of cellular immunology in the second half of the twentieth century triggered the interest of scientists and clinicians to explore the potential role of -immune-mechanisms in degenerative chronic diseases, among others in athero-arteriosclerosis. These experiments were preceeded and encouraged by the important work of Klemperer, who coined the term collagenosis implying autoantibodies to collagen in such chronic diseases as disseminated lupus erythematosis and related chronic affections of connective tissues (Gardner 1965 for review). Several authors obtained reproducible vascular lesions similar to those observed in humans by

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immunizing rabbits with arterial wall homogenates. Using a fractional extraction procedure, we could show that the major antigen responsible for this experimental immune-atherosclerosis was elastin, considered previously as nonantigenic. The more hydrosoluble macromolecular fractions of the vascular wall, although strongly antigenic, as judged from the production of precipitating antibodies, did not produce the same lesions with the same regularity and severity. Immunization of rabbits with highly purified elastin induced only a modest increase of circulating antibodies but did produce arteriosclerotic plaques without any increase of dietary lipid administration. These results were completed and reinterpreted after the identification of the elastin-laminin receptor, activated by circulating elastin peptides by triggering a release of proteolytic enzymes and free radicals. The functional profile as well as the transmission pathway of this receptor, present on vascular cells and also on circulating white blood cells (WBC) was shown to change with age, losing its physiologically relevant regulatory functions and preserving only its harmful effects. Circulating elastin peptides acting on the elastin receptor (ER) can induce vascular damage by upregulation of proteolytic (elastolytic) activity and reactive oxygen species (ROS) production. These reactions form a vicious circle with autoamplifying feedback mechanisms and age-dependent increase of the harmful effects on the vascular wall. A large number of human blood samples were tested for antielastin antibodies and also for elastin peptides. All blood samples contained both of these markers of the (auto)immune atherogenetic process involving the activation of the elastin-receptor, its uncoupling which results in the progressive increase of the degradative processes leading to the age-dependent amplification of the athero-arteriosclerosis. Elastin peptides were also shown to induce oxidation of LDL. This experimental model is an example of the delicate interplay of immune-triggered reactions with cell-signaling events during the development of athero-arteriosclerosis.

Keywords

Elastin · Aorta · Vascular wall · Antielastin antibodies · Elastases · Immune-atherosclerosis

List of Abbreviations

CFA	Complete Freund's adjuvant
ECM	Extracellular matrix
ER	Elastin receptor
ROS	Reactive oxygen species
SMC	Smooth muscle cell

Introduction

Since the publication of this treatise on immunosenescence and its pathological consequences, the underlying basic science on immunopathology, vascular pathology continued to progress with several important results but also with some fallacious conclusions. For example, inflammation induced by a single pathogenic

agent was shown to induce vascular lesions without increasing the concentration of circulating LDL: this is not really new, already Metchnikoff in the nineteenth century showed in his monography on inflammation all the symptoms this process can present. Hyperlipidemia which occurs in the organism triggers multiple mechanisms to fight the ensuing pathological process, this is an evolutionary acquisition. Therefore, the determination of LDL cholesterol as a marker of hyperlipidemia can certainly not be replaced by the determination of inflammatory markers. LDL cholesterol with its receptor identified by Brown and Goldstein, who received the Nobel Prize, remains an important event in the long road to fight vascular pathology. As a result of long evolution, the human organism reacts with multiple defense mechanisms, one of them, probably the oldest is the immunological process. Multiple studies on immunobiology showed that immune pathological processes regularly result in inflammatory reactions, but this does not mean that all the other symptoms of a specific pathology such as hyperlipidemia could be neglected. These reflections underline the importance of a second volume of this book on immunosenescence.

Historical Remarks

Although inflammation was recognized by Hippocrates and his school and defined by its cardinal symptoms (tumefaction, redness, heat, pain, and tissue dysfunction), its description in cellular terms had to await the birth of histochemistry. The invention of specific stains by Paul Ehrlich and others during the last decades of the nineteenth century helped to designate the leucocytes as the essential cellular elements of the inflammatory process. With the development of clinical chemistry and biochemistry, more and more molecular markers of the inflammatory process became available. Besides the increase of leucocytes in the blood and in tissues, the rate of red-cell sedimentation, followed by that of the so-called acute phase glycoproteins (haptoglobine, α1 acid glycoprotein or orosomucoid) and of C-reactive protein were routinely determined and considered as the hallmarks of the inflammatory process. This same period was dominated by the birth and expansion of humoral immunity, dominated by the French and German schools (Pasteur, Roux, Behring and others). Refinement of methodology, with the routine practice of passive hemagglutination and the ELISA-methodology, an important progress could be achieved, enabling the detection and quantification of low concentrations of non-precipitating antibodies, cytokines, and other molecular players of the inflammatory process. Based also on refined methods of immunohistochemistry, it became difficult to maintain the strict distinction between degenerative and inflammatory processes, exemplified by the joint diseases. Arthrosis considered as a degenerative disease of articular tissue was more adequately designated osteoarthritis because of the inevitable development of the inflammatory process (Trentham 1984 for review). The possibility to create experimentally such articular pathology by immunizing with the major collagen component, collagen type II, of articular cartilage further blurred the frontiers between degenerative, inflammatory, or (auto)immune-mediated diseases. These conceptual and methodological advances prepared the

way for the birth and expansion of cellular immunity, which dominated the field over the second half of the twentieth century. This slowly emerging and finally dominating methodological and conceptual advances reached also cardiovascular pathology. During the last decades of the twentieth century, several teams entered the field of cardiovascular pathology and introduced the above summarized methodology. These studies which will be described in this review revealed progressively the immuno-inflammatory nature of the athero-arteriosclerotic process also. Further progress came from the rapidly advancing field of cellular signaling. The study of receptors, agonists, antagonists, and message transmission pathways considerably improved the understanding of the details of cellular-molecular immuno-inflammatory processes underlying such chronic, degenerative diseases as cardiovascular pathology. As however the lipid-based concepts continued to dominate the field of atherogenesis, the final junction between these different avenues of approach for the understanding of this family of diseases was difficult to achieve. Of great help for open-minded scientists and physicians was the relatively rapid increase of the senior population over the last decades. Thanks to progress made in preventive and curative cardiovascular medicine, the fatal outcomes of cardiovascular pathologies were progressively postponed to later years of life, still remaining, however, the dominant cause of fatalities. This fact is certainly the motivation for experimental gerontologists to reassess the diverse contributing factors playing crucial roles in the development and progression of cardiovascular pathologies. Inflammation in particular was more recently recognized as an important factor in most age-related pathologies. A number of studies over the last decades clearly showed the importance of the inflammatory process as an omnipresent player in geriatric pathology. Cardiovascular pathology is no exception. Our laboratory actively participated in this progressive evolution of our concepts elaborated for the understanding of vascular diseases as relates to aging. We shall briefly review the successive stages of the abovementioned shifts of emphasis in the description and conceptualization of vascular pathology, essentially its importance as an age-related disease.

Arteriosclerosis and Atheromatosis

Progressive hardening, rigidification of the vascular wall, termed later arteriosclerosis, was recognized by early pathologists (Robert 1996, 1999a for review). These observations were based on autopsies and could not be easily generalized. The importance of nutritional factors in general and especially of cholesterol started to gain acceptance with the demonstration by Anitchkoff in the early decades of the twentieth century of the induction of lipid infiltration and plaque formation in rabbits kept for several weeks on a cholesterol-enriched diet (Olsson 1987 for review). This model became the most widespread in laboratories of experimental medicine and formed the basis of the lipid-hypothesis of atherogenesis. The development of atherosclerotic plaques, as observed in human blood vessels at autopsy, could therefore be attributed to nutritional factors, cholesterol, and saturated fat in particular. As calcified plaques and lipid infiltration were regularly associated in human blood vessels, the term proposed by the German physician Marchand

athero-arteriosclerosis was progressively adopted as more adequate to describe the human disease. The vast majority of scientists engaged in this field adopted the lipid hypothesis further comforted by the characterization of lipoprotein-classes and during the second half of the twentieth century by the description of the LDL-recognizing receptor by Brown and Goldstein (Olsson 1987 for review). Some laboratories did, however, continue to explore avenues related to the immuno-inflammatory hypothesis.

Role of (Auto)Immune Factors

As mentioned in the introductory, historical section, immune-inflammatory factors were discovered as of crucial importance in some chronic diseases and especially in rheumatoid arthritis and osteoarthritis. Rapid progress in the field of extracellular matrix biology, in the characterization of collagen(s), major components of connective tissues (more correctly of extracellular matrix, ECM) led to the identification of collagen type II as a major component of articular cartilage (Comper 1996 for review). Immunization with purified collagen type II was shown to induce osteoarthritic pathology. Autoantibodies to collagen type II were demonstrated in patient's sera (Trentham 1984 for review). Simultaneously, several teams showed that immunization with arterial extracts could induce in rabbits athero-arteriosclerotic lesions.

Induction of Athero-Arteriosclerotic Lesions by Immunization with Arterial Homogenates

Apparently, the first description of the production of athero-arteriosclerotic lesions in rabbits with homologous aorta-extracts was produced by a Hungarian team (Szigeti et al. 1960, 1968). These results were obtained without excess cholesterol administration. The severity of the lesions could be increased by prolonged protocols of immunization. The principally involved antigenic fraction was considered by these authors present in the saline-soluble fraction containing among other components a fraction with β-globulin mobility. Delayed-type tissue-allergic reactions could also be demonstrated. Similar lesions were created in rats by injection of rabbit sera immunized with aorta extracts. Total plasma lipids were shown to increase during immunization, similar to that found in cholesterol-fed animals (Szigeti et al. 1968 for review). Soon after these reports, the French team of Scebat and Renais reported the production of immuno-atherogenic reactions in rabbits by immunization first with heterologous (rat) aorta extracts and later with homologous aorta extracts (Renais et al. 1968; Scebat et al. 1966, 1967). In between White and Grollman (1964) produced periarteritis nodosa in rats also by immunization. The team of C.R. Minick at the New York Hospital (1966) also produced immuno-arteriosclerotic lesions by combining "allergic injury" and lipid-rich diet. Altogether these experiments illustrated the possibility of an immune-mechanism underlying the atherogenetic process. As however all the above cited experiments were carried out with aorta-homogenates, the principal antigen(s) involved in this immune-

atherogenic process remained to be determined. Some of the authors opted for the β-lipoproteins (LDL) of the blood-serum, present in the soluble aorta-extracts also as the principal atherogenic antigen. The Hungarian team of S. Gero et al. (1959, 1960, 1967) induced lesions with anti-β-lipoprotein antibodies and proposed a variant of the immuno-atherogenic process based on these observations. The symposium organized by the French Atherosclerosis Society in 1964 in Bordeaux enabled the confrontation of these different views on the immune-factors involved in the atherogenic process. Our experiments, detailed in the next section, were also first presented at this meeting, proposing elastin as the main culprit as sensitizing antigen and as the target of the immune-pathological process underlying athero-arteriosclerosis (Robert et al. 1967, 1968, 1970b, 1971a).

Immune-Atherosclerosis Obtained with Purified Aorta-Extracts

The most important step to follow up on the above summarized results showing that active and passive immunization with crude aorta extracts could induce vascular lesions similar to human athero-arteriosclerosis was the reproduction of such experiments with purified aorta extracts. These experiments were performed in our laboratory and will be summarized in this section. Human and porcine aorta extracts were prepared, using lesion-free portions by a fractional extraction procedure used previously for other ECM-rich tissues as cornea and skin (Robert and Parlebas 1965). The soluble macromolecules were extracted with a 1 M $CaCl_2$ solution buffered to pH 8.0 with Tris/citrate (termed CTC-extract). The insoluble stroma remaining after several extractions in ice-cold buffer with the Ultra-turrax was suspended in 2.7% TCA and heated to 90 °C for 30 min to hydrolyze selectively insoluble collagen. The remaining stroma contains essentially the elastic fibers. Adhering microfibrillar fraction (structural glycoproteins) was extracted by tour mixing the washed residue (0.9% NaCl) in 8 M urea in presence of 0.1% mercaptoethanol. After centrifugation and washing in sterile 0.9% NaCl, the final residue analyzed as pure, insoluble elastin. Elastin was shown to be selectively hydrolyzed to large peptides when suspended in 1 M KOH in 80% (v/v) aqueous ethanol at 37 °C for about 30 min (Robert and Poullain 1963). This large peptide solution (average MW about 70 kDa) was used for immunization. The other CTC, TCA, and urea extracts were dialyzed and lyophilized. All these operations were carried out in sterile conditions, in the cold, on tissues delivered in dry ice. Rabbits (New Zealand-white or Fauve de Bourgogne) kept on rabbit-chow and fresh vegetables were immunized with 1–5 mg proteins in complete Freund's adjuvant, two injections weekly as described (Robert et al. 1971a). After 4 weeks on this schedule the animals were left for 4 more weeks and received a final injection (i.v. or i.p. without Freund's adjuvant) with aluminum hydroxide as adjuvant. This was followed by testing animals for delayed hypersensitivity directly or using guinea pigs sensitized to the same antigens, as described (Robert et al. 1971a; Jacob et al. 1984). Control animals received saline injections with or without Freund's complete adjuvant. Some rabbits received a cholesterol-enriched diet (1 g cholesterol in 7.5 ml

peanut-oil homogenized with bran and barley). Titration of immune-sera was carried out with passive hemagglutination using glutaraldehyde-treated sheep erythrocytes. The antigens were fixed on treated erythrocytes using either a water-soluble carbodiimide or diazotated benzidine (Bing et al. 1967). The histological, histochemical, and electronmicroscopic procedures were described (Robert et al. 1971a). The CTC-extract contained a number of saline-soluble proteins and glycoproteins as shown by immune-electrophoresis and immune-diffusion according to Ouchterlony. The urea-extract contained several glycoproteins characterized by their size and glycan composition (Robert et al. 1967, 1971a). The κ-elastin solution had the typical amino-acid composition of purified elastin (Robert and Poullain 1963). Purified elastin before and after urea extraction was also examined by electron microscopy (Robert et al. 1971b) in order to demonstrate that urea-extraction largely eliminated the microfibrillar components. The amino-acid and glycan composition of the aorta fractions used as sensitizing antigens was described (Robert et al. 1971a). As shown by immune-diffusion, antisera to the CTC-extract showed strong precipitation lines to several soluble macromolecules, not further characterized. They also showed faint precipitation lines to the urea- and κ-elastin fractions. Antisera to the urea-extract showed one strong and several faint precipitation-lines to the urea extract and one to three faint precipitation lines to κ-elastin. Anti-κ-elastin antisera also gave faint precipitation lines to the κ-elastin solution as well as to 2–3 faint lines to the urea extract. This precipitation lines, observed with antiCTC and anti-urea extract antibodies to the κ-elastin solution can be considered as a first indication of the presence of elastin peptides in the soluble fractions of human and porcine aortas. Up to this time, elastin was considered as strictly insoluble. As shown by the passive hemagglutination tests, peak values of titrable antibodies were obtained at about 100 days after immunization, both to the urea extract and to κ-elastin. The titers fall than rapidly, suggesting absorption of antibodies by the proper tissue-antigens of the animals. Relatively high titers were obtained, about 10^{-5} of the urea extract and about 5×10^{-3} to κ-elastin (lowest hemagglutinating dilution of antisera). κ-elastin from human aorta gave higher titers than from porcine aorta. Cholesterol-fed animals showed low titers to all antigens tested (well below 10^{-2}). Delayed hypersensitivity reactions were regularly observed on the immunized rabbits as well as on guinea pigs sensitized to the same antigens. This can be taken as an indication that besides the humoral immune-reaction, immunizations with aorta-extracts did trigger also cellular immune-reactions. Total serum cholesterol did increase significantly 1-month after the onset of immunization (from 0.36 ± 0.07 to 0.88 ± 0.01 g/l in elastin immunized animals) and returned later to preimmunization values. β-lipoprotein (LDL) determined according to Burstein and Samaille (1959) did increase continuously with time in immunized as well as in control rabbit sera. This selective increase of circulating LDL might be attributed, besides immunization, to the diet and the aging of the animals. There were, however, significant differences between animals immunized with different aorta extracts. The strongest increase of LDL as compared to the starting levels (before immunization) were found in animals immunized with the urea-extracts (137–200% increase) followed by those immunized with elastin (without adjuvant!), 145% increase on the

average. Immunization with elastin in complete adjuvant produced a less important increase (about 70%) compared to similar increase in nonimmunized control animals. The macroscopic and microscopic observations of the aorta of immunized rabbits revealed conspicuous modifications (Figs. 1, 2, and 3). The most important modifications were observed on the aortas of animals immunized with pure elastin: lipidic-sclerous infiltrations covering most of the intimal surface, from the origin of aorta to the renal arteries, most obviously on the cross and around the ostia. Calcified plaques imitating egg-shell appearance, sometimes with aneurismal dilations, were observed (Fig. 1). Migrating smooth muscle cells (SMCs) were observed in the intima, accompanied by fragmentation of elastic lamellae (Figs. 2, 3, and 4). Ultrastructural and histochemical studies confirmed the strong calcification of the fragmented elastic fibers. At some places necrotic modifications of the media were observed with SMCs undergoing lysis. In all cases of immune-lesions a striking difference was observed with lesions produced by the high cholesterol diet. The cholesterol-induced lesions were similar to those described by a number of authors in other cholesterol-fed rabbits (Table 1). Differences were observed between the two strains of rabbits used. White rabbits were more resistant to immune-induced lesions than

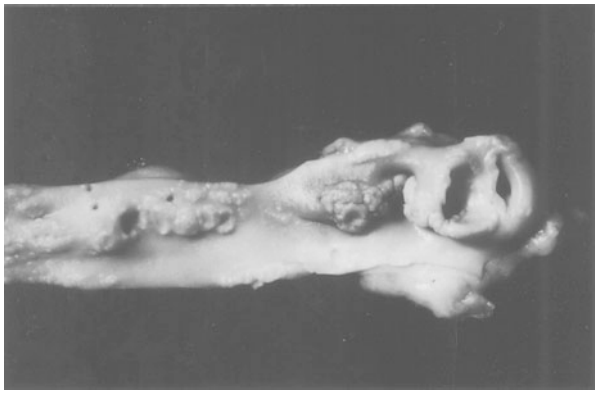

Fig. 1 Aorta of a rabbit immunized with κ-elastin from human aorta. Isolated or confluent fibrous calcified lesions covering most of the intimal surface (From Robert et al. 1971a)

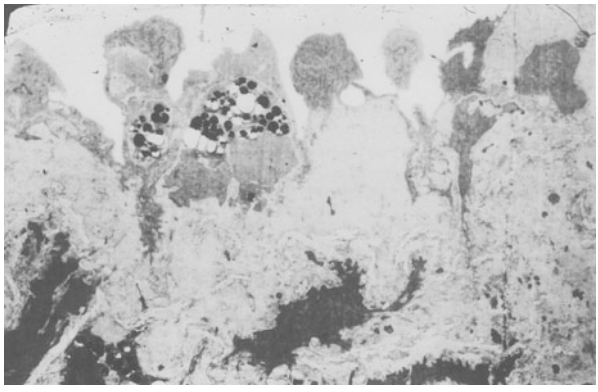

Fig. 2 Intimal portion of rabbit aorta immunized to urea extract of porcine aorta. Electron microscopy, 3,200x. Notice the granular infiltrate of strongly modified SMCs, fragmented, lyzed and calcified elastic lamellae and vacuolized and calcified endothelial cells (From Robert et al. 1971a)

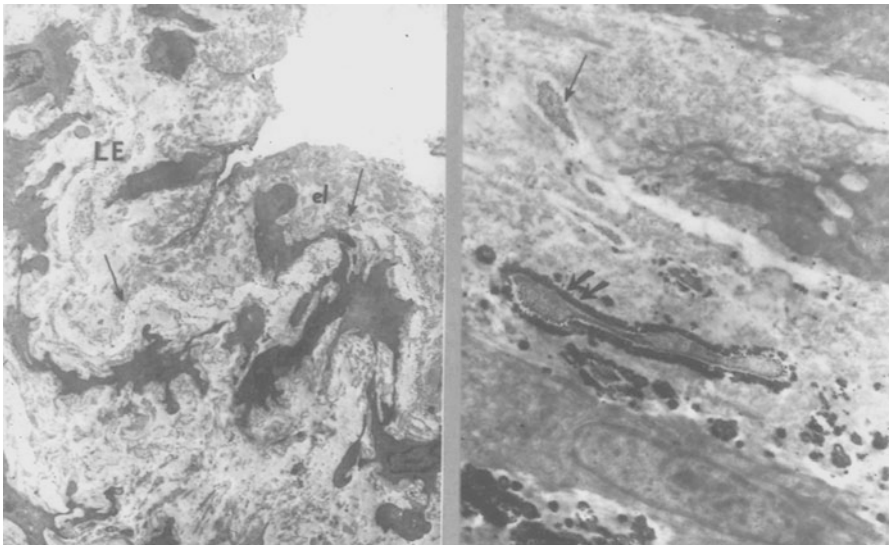

Fig. 3 Modified, fragmented, lyzed, and calcified elastic lamellae (LE) in the thoracic aorta of rabbits immunized with human κ-elastin. Fibrillar deposits (arrows) and strong perielastic calcification (double arrow; Electron microscopy by Grosgogeat Y. in collaboration with Robert et al. 1971a)

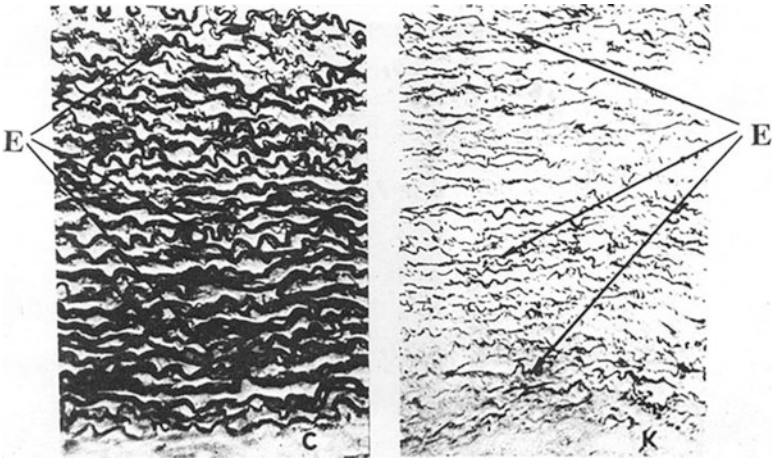

Fig. 4 Fragmentation of elastic lamellae in aortas of rabbits immunized with κ-elastin (right, K) as compared to a control aorta (left, C). *E* elastic fibers (From Jacob et al. 1984)

the fauve de Bourgogne strain as observed after several 1-month cycles of immunization followed by 1-month without. In our first experiments (Robert et al. 1971a), about 40% of all the immunized animals presented the above summarized lesions with 25% presenting wide-spread macroscopic and microscopic lesions as those shown on Figs. 1, 2, and 3. There were, however, important differences in the frequency and

Table 1 Macroscopic, microscopic and ultrastructural lesions of the aortas of rabbits immunized with aorta extracts

Antigen injected	n animals with visible lesions	Nature and intensity of lesions		
		Macroscopic	Microscopic	Ultrastructural
Controls NaCl 0.9% + adjuvant	0 out of 5	No lesions	No fibrosis Normal elastic lamellae	No lesions
CTC extract	1 out of 7	No calcified lesions Nor plaques	No fibrosis of intima or media Elastic lamellae intact	No necrotic SMCs No intimal hyperplasia
Urea extract	8 out of 12	Diffuse lesions covering part of the intimal surface	Necrotic plaques in media Fibrosis Calcified elastic lamellae	Fibrotic infiltration of the intima Elastic lamellae fragmented, fibrillar, calcified Degenerescence of cells in media
κ – elastin	5 out of 7	Heavy lesions covering the whole intimal surface Strong Calcification	Fibrosis of intima Elastic lamellae lysed, fibrillated, calcified Medial necrosis SMCs disoriented	Medial fibrosis Calcified deposits in elastic lamellae and collagen fibers
Cholesterol feeding	2 out of 4	Soft lipidic lesions at the cross No calcification	Lipidic infiltrate of intima Foam cells, fibrosis	No calcification Endothelial cells with lipid Infiltration Intercellular fibrosis Rupture of elastic lamellae

Modified from Robert et al. (1971a)

severity of lesions according to the antigen used (Table 1). Immunization with the soluble extract (CTC-extract) gave only infrequent lesions (1 out of 7 animals immunized) and only at the ultrastructural level. Immunization with elastin produced lesions both at the microscopic and macroscopic levels in more than 70% of the immunized animals. Immunization with the urea extract gave macroscopically detectable lesions, only in about 15% of animals, and microscopic lesions in about half of the immunized animals. These results suggested the presence of the most pathogenic antigen, supposed to be elastin, in all extracts, but in highly variable proportions: lowest concentrations in the CTC extract, somewhat higher concentration in the urea extract, and the highest in the purified elastin fraction. Further studies largely confirmed these observations (Jacob et al. 1984). The most severe

microscopic and macroscopic lesions were seen in elastin immunized animals, followed by the urea extract. Such lesions included vacuolized, calcified endothelial cells (Fig. 2) above strongly dislocated, lyzed, and calcified elastin lamellae. Table 1 shows a comparison of most frequent lesions seen in the aorta of animals immunized with the different aorta extracts. One of the crucial observations was the inverse relationship between the three aorta extracts used for immunization as far as the induction of precipitating antibodies and lesion-induction is concerned. The soluble (CTC) extract induced high titers of precipitating antibodies and only infrequent and relatively mild lesions. Purified elastin, whether used in its insoluble, fibrous form or as soluble, κ-elastin, did induce regularly the most severe lesions but gave only low titer hemagglutinating or precipitating antibodies, the urea extract occupying an intermediary position. The low titer of circulating antibodies could best be explained by their adsorption on elastin fibers and was confirmed by the fixation of immunofluorescent anti-rabbit-IgG antibodies on elastic fibers (Jacob et al. 1984; Robert et al. 1967, 1968, 1970b). The predominant localization of the lesions at the level of elastic fibers was certainly the most conspicuous observation: fragmentation and calcification. Degenerescent modifications of endothelial cells and SMCs were also a constant observation. Extracellular calcium-containing crystals could also be observed occasionally. The macroscopic and microscopic ultrastructural lesions were quite similar to those observed in human athero-arteriosclerosis but quite distinct from those produced by the cholesterol-rich diet. Although a more or less important and transitory increase in β- lipoproteins (LDL) was observed in immunized animals, we also did observe unexpectedly an increase of antielastin antibody titer in the sera of cholesterol-fed animals, although the titers remained relatively low ($\leq 10^{-2}$). Further experiments on the elastin receptor (ER) of vascular cells confirmed the role of elastin peptides in the lipid-induced lesions also (*See* later). Some years later, these experiments were repeated in our laboratory, using only κ-elastin as the sensitizing antigen (Jacob et al. 1984). These experiments will now be described.

Extension of Elastin-Immune-Pathology to Pulmonary Vessels, Metabolic Effects

The above described experiments were repeated using this time only κ-elastin of high molecular weight purified by gel filtration ($\geq$ 70 kDa, designated κ_1 – elastin) from bovine ligamentum nuchae-elastin as the immunizing antigen with New Zealand white rabbits, carried out essentially as in the first experiments (for methodological details, *See* the original publication, Jacob et al. 1984). This time however, besides the aorta, detailed investigations were carried out on the small arteries in the lung parenchyma also. The macroscopic, microscopic and ultrastructural investigations were completed by experiments on the biosynthetic capacity of aorta-wall explant cultures using 14C-lysine and 14C-glucosamine incorporation. Another innovation as described in more detail in the next section was the determination of elastase-type endopeptidase activity in aorta extracts from control and immunized animals. The presence of such activity in human aorta extracts was previously described in our laboratory (Hornebeck et al. 1975; Robert et al.

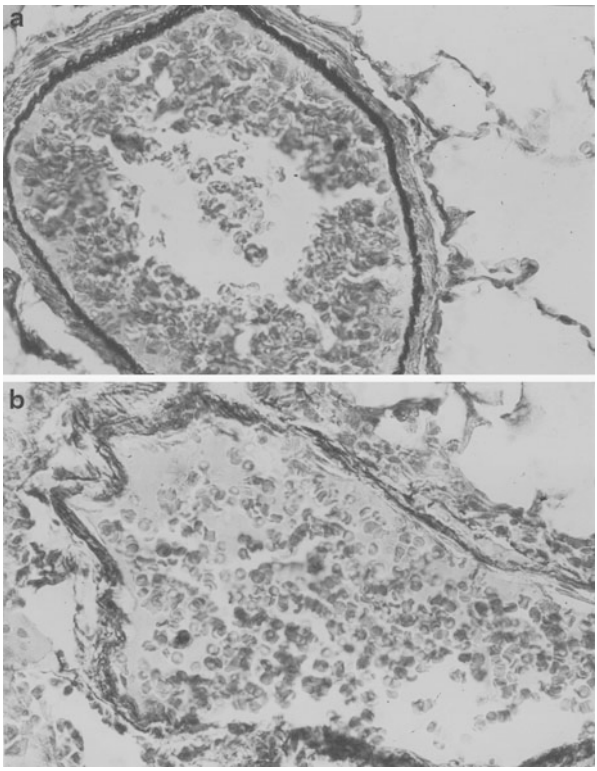

Fig. 5 Histological appearance of rabbit lung arterioles in control (**a**) and in κ-elastin immunized (**b**) rabbits (Modified from Jacob et al. 1984)

1974b) as will be detailed below. This activity could explain the presence of soluble elastin-peptides in the buffer-soluble aorta extracts, suspected in our first experiments. The biological properties of elastin peptides started to be studied in several laboratories and will be described in the following sections. These relatively recent results extended the horizon of our investigations and triggered a more complete search for pathological effects produced by elastin immunization as well as by elastin peptides. This time all rabbits immunized to κ-elastin developed typical vascular lesions at the macroscopic and microscopic level similar to those seen in our first experiments described in the previous section. The histological study of the lungs of the immunized rabbits revealed the presence of granulomatous lesions, absent in the control animals (Figs. 5 and 6). The most conspicuous modifications were, however, the lysis of elastic laminae in the large vessels as well as in the walls of small lung-vessels (Figs. 4 and 5). This time, the loss of continuity of elastic fibers was quantitatively assessed by computerized image-analysis. A pronounced fragmentation of elastin fibers could be demonstrated as shown on Fig. 4. No such fragmentation was seen in vessels of control animals injected only with complete Freund's adjuvant or with BSA in complete Freund's adjuvant. A curious observation was the more pronounced fragmentation of the elastic lamellae in the outer segment of the media than in its inner segment, near the intima. The SMCs of elastin-immunized animals showed striking morphological changes and random orientation,

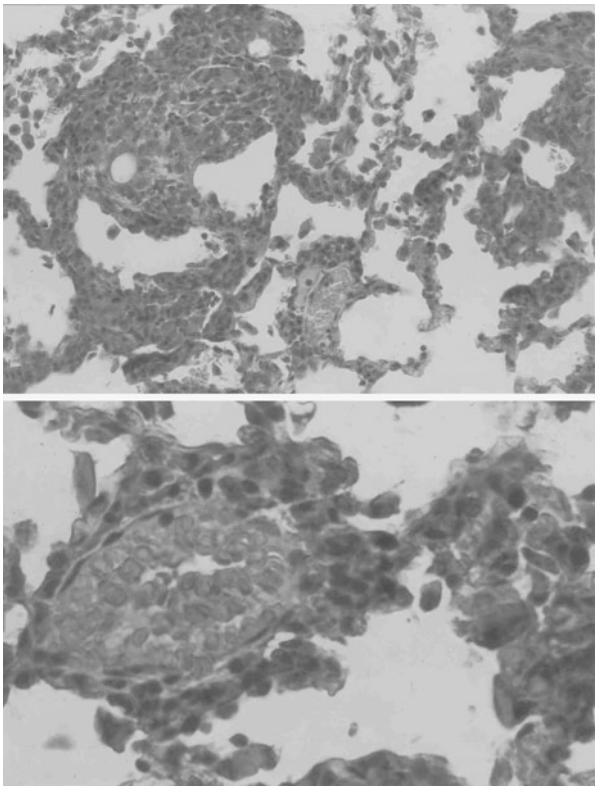

Fig. 6 Granulomatous lesion with vasculitis in the lungs of κ-elastin immunized rabbit (Unpublished results from Chantal Lafuma in the author's laboratory)

comparable to those seen in our first experiment (Figs. 2 and 3). Their number was also decreased. The average length of elastic lamellae were about 13-times shorter in elastin-immunized aortas as compared to controls receiving adjuvant injections with or without BSA.

This proportion when calculated separately for the internal part of the media gave a ratio of only four times shorter elastin segments for the inner part of the media and a much more pronounced fragmentation, 40-times as compared to controls in the outer half of the media. In the lungs of the elastin-immunized animals, intense granulomatous lesions were seen as well as a strong elastolysis in the walls of small lung-vessels (Figs. 5 and 6.). The granulomatous lesions contained giant cells and eosinophil leucocytes. On the ultrastructural level, the findings were similar to those described in our first experiments (Robert et al. 1971a). Electron microscopy confirmed the alterations of SMCs and elastic lamellae which were fragmented, disorganized with an increased density of the microfibrillar components (Fig. 3). Endothelial cells were enlarged, vacuolated, and calcified as in the first experiments (Fig. 2). One of the striking observations was the loss of continuity of elastic fibers or close contact between SMCs and elastic lamellae, (Fig. 7) clearly seen in control aortas. The increased titer of antielastin antibodies in the κ-elastin immunized rabbits could be confirmed by passive hemagglutination, the titers were, however, lower

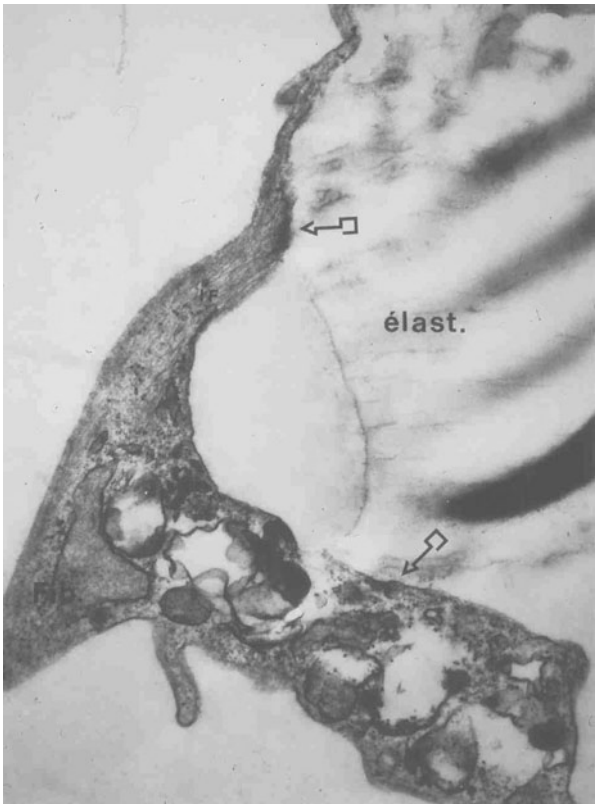

Fig. 7 Strong adhesion of a fibroblast to a purified and micronized elastic fibril under the electron microscope. No such adhesion with SMCs from elastin-immunized animals was observed (Modified from Perdomo et al. 1994)

than in the first experiments (10^{-2}–10^{-3}). The adsorption of these antielastin antibodies to purified, micronized elastin fibers could be confirmed by immunoperoxidase staining using sheep anti-rabbit Fb antibodies. No such reaction was seen with sera of animals immunized to BSA in Freund's complete adjuvant. The same reaction, adsorbed autologous IgG on aorta elastic fibers, could be demonstrated in the κ- elastin immunized rabbits also.

Figure 8 shows the results of radioactive lysine and glucosamine incorporation by aorta explant cultures, results being expressed as cpm per mg DNA. Incorporation was strongly decreased in the aorta explants from the κ-elastin immunized animals as compared to control animals injected with the complete adjuvant alone. Another important observation was the increase of elastase-type endopeptidase activity of the aorta extracts of κ-elastin immunized animals (Table 2). *N*-succinyl-ala-PNA was used as the chromogenic substrate, relatively specific for elastase-type endopeptidases (Bieth, 1978). These experiments largely confirmed and extended our first observations (Robert et al. 1971a) on the pathological modifications of large and small elastic vessels in animals immunized to human, porcine, or bovine elastin-peptides. All treated animals showed characteristic macroscopic, microscopic, and ultrastructural modifications with conspicuous fragmentation of elastic lamellae, modifications of endothelial cells, and SMCs and also strong calcification. Further

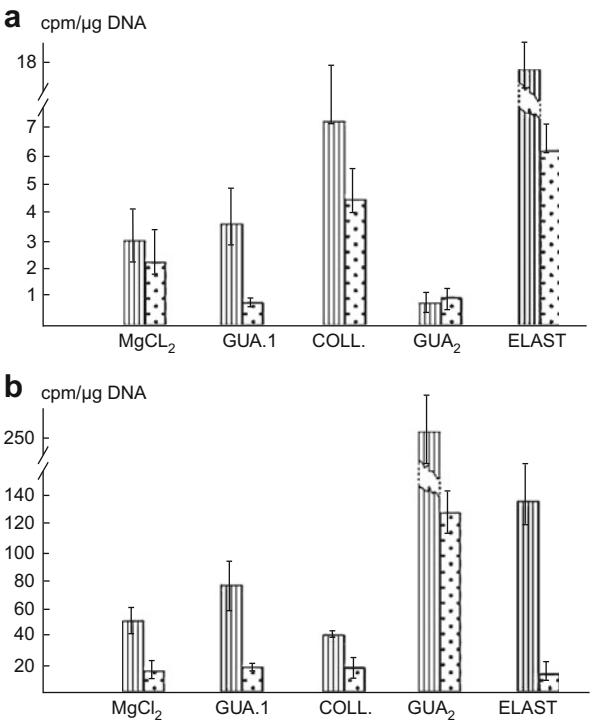

Fig. 8 Incorporation of ^{14}C–lysine (**a**) and ^{14}C–glucosamine (**b**) in proteins of arterial wall extracts. Bars with lines: control group (CFA); bars with points: κ1-elastin immunized rabbit group (κ1-CFA). Ordinates: radioactivity incorporated in each extract, expressed as cpm/μg DNA. Abscissa: *MgCl2* 2 M MgCl2 extract, GUA_1, first guanidinium chloride extract, COLL collagenase extract, GUA_2 second guanidinium chloride extract, *ELAST* elastase extract. The bars represent the SEM for the average value of each extract (For details *See* Jacob et al. 1984)

Table 2 Elastase-type endopeptidase activity of soluble extracts of aortas from rabbits immunized or not with κ-elastin

Nature of sera	Activity	Significance
Control	130 ± 23.4	
Immunized	434 ± 111.2	$p < 0.01$

The endopeptidase activity of sera was determined with *N*-suc-ala_3-PNa and expressed as nanograms of porcine pancreatic elastase equivalents per mg of DNA. The figures of the table are averages of 5–7 animals. The statistical significance of the results was determined according to the Mann and Whitney distribution free test (Modified from Jacob et al. 1984)

experiments were carried out in order to specify the individual aspects of these modifications at the cellular and molecular levels.

Cellular-Molecular Mechanisms Involved in the Immune-Inflammatory Vascular Pathology

The above-described experiments confirmed the possibility of the induction in rabbits of a macro and microvascular pathology reminiscent of arterio-atherosclerosis by immunization with elastin. Peptides derived from highly purified fibrous elastin, κ-elastin was shown to be the most efficient (if not the only) inducing antigen. The

humoral immune reaction could be confirmed by the passive hemagglutination test and by immunodiffusion. There was, however, an inverse relationship between antibody titers and efficiency of lesion-induction when consecutive aorta extracts were used. The ultrastructural studies revealed severe modifications of cells and ECM components, mainly of elastin. Among the most conspicuous modifications was intra- and extracellular calcification, obvious even at macroscopic examination of longitudinally opened aortas of elastin-immunized rabbits. It remained to elucidate the underlying cellular and molecular mechanisms. Some of these experiments will now be described.

Degradation of Elastic Fibers

One of the most conspicuous modifications produced by immunization with elastin was the pronounced degradation of elastic fibers of the large and small blood vessels. It could be shown that this is at least partially the result of an autoamplifying vicious circle mediated by the action of elastin peptides on the ER. As shown on Fig. 9, the addition of elastin peptides at concentrations shown to be present in the blood serum (*See* later) produced a pronounced upregulation of the production of elastase-type endopeptidases (Robert et al. 1986) essentially of MMP-2 and MMP-9 (Archilla-Marcos and Robert 1993). This increase of elastase production could be inhibited by lactose and melibiose, antagonists of the ER. It could also be shown during these studies that aging, both chronological and in vitro (increasing passage number), produced an increased expression of elastase-type endopeptidases (Fig. 10). These experiments, repeated over the years, suggested a mechanism for the progressive degradation of vascular and pulmonary elastic fibers. Using an ELISA-procedure, the concentration of circulating elastin peptides could be determined in a large number of normal and pathological human sera (Bizbiz et al.

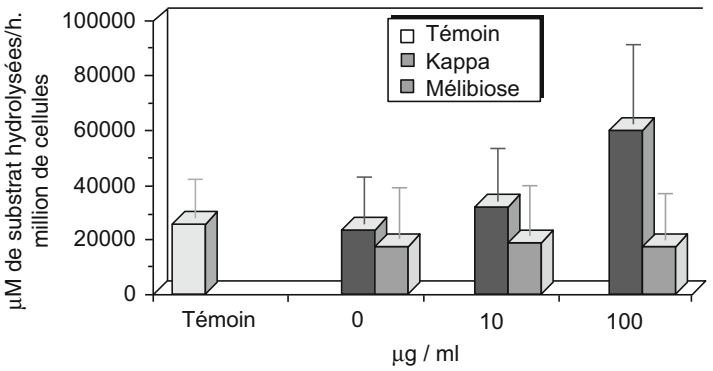

Fig. 9 Increase of elastase-type endopeptidase activity in fibroblast cultures in presence of increasing concentrations of κ-elastin (0, 10 and 100 µg/ml). Addition of melibiose, an ER antagonist inhibits this upregulation of elastase activity (Modified from Archilla-Marcos and Robert 1993)

1997; Fülöp et al. 1989a). Their level followed a Gaussian curve with an average concentration in the μM range, largely exceeding the KD value of the ER, shown to be in the nanomolar range (Fülöp et al. 1989b; Robert et al. 1989). In elastin-immunized animals, these processes appear to be exaggerated by the action of soluble immune complexes on monocytes and lymphocytes able to trigger an increased release of elastin degrading endopeptidases. The presence of antielastin

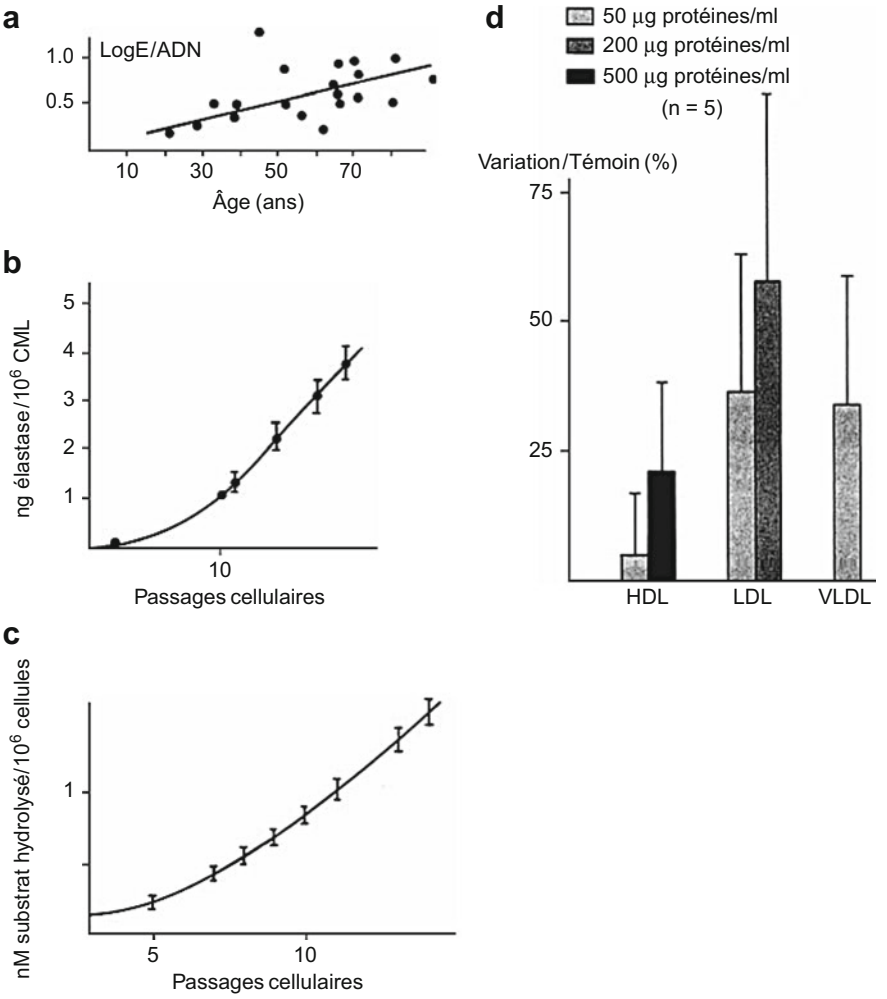

Fig. 10 Elastase type endopeptidase activity determined with *N*-suc-ala -$_3$Pna as substrate. (**a**) in human aorta extracts. Abscissa: age in years, ordinates: log elastase activity per cell (DNA); (**b**) in successive passages of aorta smooth muscle cells. Abscissa: passage number, ordinates: elastase activity given as ng equivalent of pancreatic elastase; (**c**) in successive passages of human skin fibroblast cultures. Abscissa: passage numbers, ordinates: nM substrate hydrolyzed/106 cells; (**d**) effect of human lipoproteins added to SMC-cultures (For other details *See* text and Robert et al. 1986)

antibodies in all human sera tested suggests a similar mechanism in humans also (Fülöp et al. 1989a, b). The selective and irreversible adsorption of elastases on the surface of elastic fibers largely limits the possibility to counteract this process by elastase inhibitors (Robert et al. 1974a).

Calcification of Elastin

The intense calcification of elastin fibers in the immunized animals, seen macroscopically and on the electron-microscopic preparations, could be confirmed by specific histochemical methods (Van Kossa staining) and direct chemical determinations. A partial explanation of this strong affinity of elastin for calcium came from physicochemical studies of Dan Urry (1980) showing that the β-turns of elastin represent high affinity fixation sites for calcium. Earlier studies by Max Burger (1947) and Lansing (1959) revealed the progressive Ca-fixation in elastic blood vessels in a diffuse fashion, and still present in elastin purified by heating to 100 °C in 0.1 N NaOH, the standard procedure for purifying elastin (Robert et al. 1985 for review). Moreover it was shown that calcium fixation on elastin strongly potentiates its affinity for lipids (determined by using 14C-cholesterol (Jacob et al. 1983; Hornebeck and Partridge 1975)) and vice versa, lipid fixation potentiates Ca-fixation. As β-lipoproteins (LDL) increased during the first phase of elastin-immunization, this also could contribute to Ca-retention in elastin. We have to mention here the demonstration by the team of J.L. Beaumont the role of anti-LDL antibodies in the development of atherosclerosis (Beaumont 1965, 1969, 1970; Beaumont et al. 1965; Beaumont and Beaumont 1968). This team demonstrated also the importance of anti-heparin antibodies in atherogenesis (Beaumont and Lemort 1974; Buxtorf et al. 1981; Lorenzelli-Edouard et al. 1980). The work of Bihari-Varga and Gero pointed also on the potential role of LDL and anti-LDL antibodies on the atherogenic process (1966; Bihari- Varga et al. 1984, 1986). This team pioneered also the recognition of the acid polysaccharides (glycosaminoglycans, proteoglycans) of the vascular wall in the retention of lipoproteins (Bihari-Varga and Gero, 1966). These two, essentially postsynthetic processes, calcification, lipid fixation confirmed by direct analysis of lipid classes in purified human aorta elastin by Claire et al. (1976) largely explained the progressive loss of elasticity and increased susceptibility to degradation of vascular elastic fibers.

Role of the Elastin Receptor

The demonstration of an elastin-recognizing cell-membrane receptor started in our laboratory with the observations on the capacity of cells to strongly adhere to micronized elastin fibrils, as shown by time-lapse video microscopy (Hornebeck et al. 1986). Addition of elastin peptides to cells (fibroblasts and vascular SMCs) was supposed to compete with fibrous elastin and inhibit elastin fiber fixation on cells. The opposite effect was observed; low concentrations of κ-elastin strongly increased the speed of adherence of cells to elastic fibers (Groult et al. 1998). This

effect could be attributed to the induced synthesis of a membrane glycoprotein of 120 kDa termed elastonectin (Hornebeck et al. 1986). These initial experiments were followed by a series of other experiments aimed to the exploration of the physiopathological roles of the elastin-receptor. Using immune-histochemical procedures, the presence of the elastin-receptor could be demonstrated on vascular cells, endothelial cells, SMCs, fibroblasts, as well as on WBC-s, monocytes, PMN-s, and lymphocytes (Faury et al. 1995, 1998a; Jacob et al. 1987a; Perdomo et al. 1994; Péterszegi et al. 1997c). Most importantly, the elastin-receptor could be demonstrated on monocytes and lymphocytes inside the human atherosclerotic plaques obtained by endarterectomy (Péterszegi et al. 1997a). A variety of tumor cells did also exhibit the ER (Timar et al. 1995). All these cells, mobile and sessile, found in the vascular wall might therefore contribute to the ER-mediated upregulation of elastolytic protease production. It could be shown also during a long collaboration with T. Fülöp and his team that the activation of the ER contributes by still other mechanisms to the progression of vascular lesions. One of these mechanisms is the elastin-peptide triggered release of superoxide (Fülöp et al. 1989b) suggesting a free-radical (ROS-mediated) contribution to the vascular-cellular lesions. Another significant observation was the demonstration of accelerated oxidation of LDL by activation of the ER (Fülöp et al. 2005). It also could be shown that aging accelerated these harmful effects mediated by the ER. In cells (monocytes, PMN-s) obtained from old individuals, the normal transmission pathway of ER, as shown to function on young cells (Fülöp et al. 1990a; Varga et al. 1988, 1989) did no more function as shown by the inefficiency of pertussis toxin to block the transmission pathway mediated by a Gi protein in young cells. Free-radical release was, however, maintained and even amplified. These observations suggested an uncoupling of ER in old cells from its normal transmission pathway. This uncoupling results in the exacerbation of the harmful effects mediated by the receptor and loss of its physiologically useful functions, as NO-mediated vasodilation (Faury et al. 1997; Fülöp et al. 1992) and inhibition of cholesterol synthesis by monocytes (Varga et al. 1997). Most of these experiments as well as others pertaining to the role of ER in the malignant process were reviewed recently (Labat-Robert and Robert 2007). ER-mediated mechanisms play an important role in the inflammatory process also, which accompanies the immune-induced pathologies. Elastin peptides were shown to be chemotactic to WBC-s, monocytes especially (Antonicelli et al. 2007 for review). Activation of the ER on PMN-s and monocytes triggers the release of proinflammatory enzymes and cytokines (Antonicelli et al. 2007 for a review). ER-triggered reactions include endothelial iNOS upregulation and NO-dependent vasodilation (Faury et al. 1998a, b). As mentioned above, ER-NOS coupling decreases with age with a steady age-dependent loss of vasodilation by elastin peptides (Faury et al. 1997). Besides the ER-triggered proinflammatory reactions, soluble immune-complexes formed by the reaction of antielastin antibodies and circulating elastin peptides might well contribute also to the harmful effects produced by immunization with elastin. These rapidly summarized experiments substantiated the claim for an important pathogenetic role of the elastin–antielastin system in the genesis of athero-arteriosclerosis (Fülöp et al. 2001; Robert 1999a, b).

Extension to Human Pathology

The first experiments on immune-atherosclerosis were performed on human sera with the demonstration of antielastin antibodies present in all sera tested, using the passive hemagglutination method (Stein et al. 1965). Although the titers were relatively low (from 1/2 dilution to 1/512 in these first experiments), it could be shown that sera from 68 atherosclerotic persons were in the lower range (1/2–1/32 dilution still giving agglutination) attributed to the enhanced adsorption of antielastin antibodies on degrading elastin fibers offering a larger surface for antigen–antibody complex formation. Such complexes were demonstrated on fibrous elastin (Jacob et al. 1984, 1987b). The anti-elastin antibodies appeared at about 20 years and disappeared after 80 years (Stein et al. 1965). Surface area measurements on purified elastin using radioactive Krypton (Robert et al. 1970a, 1971c) revealed a relatively large specific surface of elastin fibers, favoring interaction (adsorption) with soluble molecules. These results were further confirmed by microcalorimetric adsorption studies (Robert et al. 1971c). Later experiments (Fülöp et al. 1989a) confirmed the presence of antielastin antibodies, by direct titration of IgG and IgM type antibodies, in a larger number (265) of human sera, normal and pathological, mainly atherosclerosis of the legs, ischemic heart disease, stroke, diabetes, hyperlipidemia type II/b and IV, and hypertension. No obvious correlation was found with age. IgG-type antielastin antibodies were elevated in obliterative arteriosclerosis of the legs and ischemic heart disease. No such modifications were seen in the IgM-type antibodies. Both types of antibodies were, however, decreased in type IV hyperlipidemia. Figure 11 shows the age-dependent evolution of the above-summarized determinations of antielastin antibodies in human sera. More details on the specificity and sensitivity of the ELISA method used can be found in the original publication (Fülöp et al. 1989a). Circulating elastin peptides were also determined by an ELISA-procedure on about 1,500 human sera (Bizbiz et al. 1997; Fülöp et al. 1990b). These last experiments performed in collaboration with epidemiologists on a study on the role of vascular aging on brain aging (the EVA-study, Bizbiz et al. 1996) confirmed also the correlation between carotid artery wall thickness and plaques with the elastin-elastase parameters. Elastase-type endopeptidases were also demonstrated in a large number of human sera (Table 3; Hornebeck et al. 1983; Bihari-Varga et al. 1984; Bizbiz et al. 1996). Monocyte-macrophage elastase-type activity was shown to be upregulated by cholesterol and by proinflammatory cytokines (Rouis et al. 1990). It should be mentioned, however, that after screening a large number of synthetic elastin peptides by monoclonal and polyclonal antibodies, it could be shown that according to their structure they presented a variable reactivity to antibodies used for their detection and quantification (Wei et al. 1993). Further confirmation of the role of the elastin-immune system and of the central importance of ER-mediated processes came from the above-mentioned demonstration of ER-exhibiting mononuclear cells in freshly excised human endarterectomy samples (Péterszegi et al. 1997a). It also could be shown that human helper (CD4) and memory (CD45R+) lymphocytes when cultured in presence of elastin peptides exhibit inducibly the ER as shown by flow-immuno-cytofluorimetry (Péterszegi et al. 1997c). Addition of increasing concentrations of elastin peptides upregulated

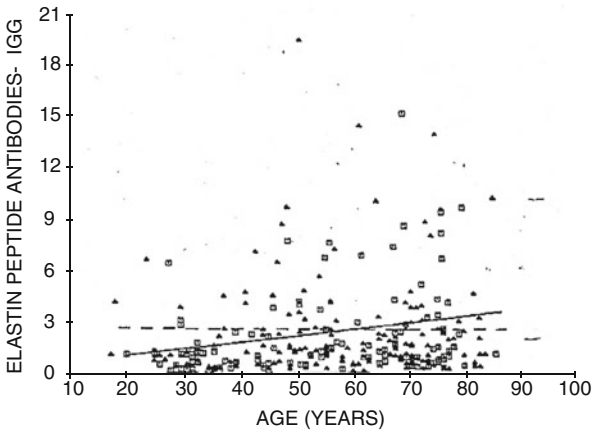

Fig. 11 Correlation between anti-elastin peptide antibody concentration (1-g/ml) of IgG type and the age of subjects. Males ($r = 0.19$, $n = 117$)(-), females ($r = 0.01$, $n = 166$)(- -)

Table 3 Elastase-type endopeptidase activity of normal and pathological human sera

Nature of sera	Activity	Significance
Control	78.1 ± 41.9	
Atheroma	91.1 ± 63.8	N.S.
Emphysema	237,2 ± 133.8	$p < 1 \times 10^{-8}$

The endopeptidase activity of sera was determined with *N*-suc-ala$_3$ -PNa and expressed as μg/ml pancreatic elastase ±SD. The statistical significance of the results was determined according to the Mann and Whitney distribution free test (Modified from Hornebeck et al. 1983)

in a dose-dependent fashion the expression of a serine-elastase identical to PMN-elastase (shown by inhibition with monoclonal anti-PMN-elastase antibody) followed by cell death (Péterszegi et al. 1999; Fig. 12). The crucial role of the ER could be demonstrated in these experiments also by the inhibition of cell death in presence of the antagonists of the ER, lactose, and melibiose (Robert 1999a, b). It also could be shown that circulating lymphocytes isolated from the blood of elderly patients suffering from denutrition and dementia exhibited an increased elastase and cathepsin G activation mediated by the ER (Péterszegi et al. 1997b). Another finding in favor of the above hypothesis, connecting the immune-hypothesis to the lipid-theory of atherogenesis, came from the demonstration of an increased concentration of elastase-type endopeptidases in aorta-extracts from cholesterol-fed animals (Jacob et al. 1982) confirmed by in vitro experiments showing that addition of human LDL and VLDL (but not HDL) to vascular SMC-cultures strongly increased the production of elastase-type endopeptidases (Fig. 10; Bourdillon et al. 1984).

The pharmacological consequences of the above-described immuno-atherosclerotic process were also investigated. It could be shown that immunization of rabbits with elastin strongly increased SMC-membrane permeability, as shown by the increase of ouabaine-insensitive 22Na+ efflux, the 86Rb efflux (indicator of K+ efflux), and the 45Ca++ influx. Passive permeability to Na+ and K+ as well as the sodium pump were enhanced. Administration of porcine calcitonine largely prevented these modifications, as well as the development of the athero-arteriosclerotic plaques (Jacob et al. 1987b). Treatment with calcitonin largely prevented also the calcification of

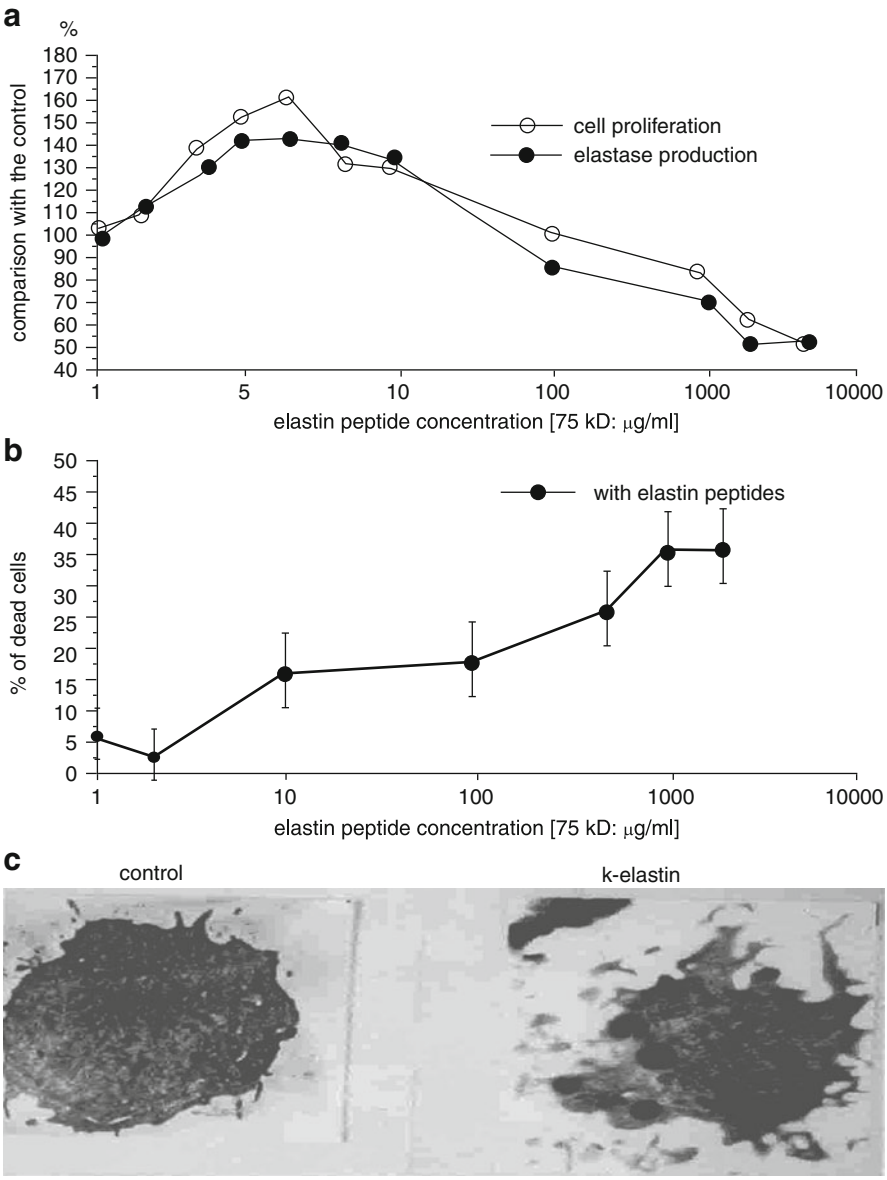

Fig. 12 Upper figure (**a**) Human lymphocytes cultured in presence of increasing concentrations of κ-elastin (in log conc.μg/ml on the abscissa). —○—: modification of cell proliferation and of elastase production —●— as a function of κ-elastin concentration. (**b**) increase of cell death with increasing κ-elastin concentration, as % of total cells. (**c**) Electron microscopy of a normal lymphocyte from the above cultures (control) and a lymphocyte with apoptotic bodies (κ-elastin) (Modified from Péterszegi et al. 1999)

elastin fibers in these animals (Jacob et al. 1987b). The inhibition of Ca-influx by calcitonine might well be the key factor in these experiments.

Among the not completely elucidated consequences of the above-summarized immune mechanisms remain the effect of possible age-dependent modifications of immune functions on elastin induced athero-arteriosclerosis. Age-dependent modifications of the human and animal immune systems were extensively investigated over the second half of the twentieth century (Robert and Robert 1973 for a review). The described age-dependent modifications of the immune systems might well influence the outcome of the immune-atherosclerotic process also. As the emphasis of our research and the interest of other teams shifted to the age-dependent modifications of receptor function (Joseph and Roth 1990; Robert 1998; Roth 1979 for reviews), the interpretation of the observed pathological modifications shifted progressively towards postgenetic (epignetic, posttranscriptional) mechanisms (Robert and Labat-Robert 2000 for review). Several experimental facts pleaded in favor of the progressive preponderance of such mechanisms. Although the selective uptake of cholesterol by the vascular elastin fibers were first demonstrated in vivo in animals (Jacotot et al. 1973; Robert et al. 1984b; Szigeti et al. 1972), qualitative and quantitative determinations of lipid classes strongly associated with purified human elastin carried out on human aorta samples (Claire et al. 1976) confirmed the same strong affinity of human elastin also to lipids, attributed to factors inherent to the hydrophobic nature of elastin. Circulating elastin peptides present in all human sera examined were shown to exhibit a number of relevant pharmacological properties (Robert et al. 1984a) important also for lipid-mediated processes during atherogenesis.

Some remarks on circulating elastase-type endopeptidases. Their upregulation could be, at least partially, attributed to circulating elastin peptides as described in previous sections of this review. The presence of identical or very similar peptide sequences in elastin of several species might also contribute to the explanation of the incomplete elimination of elastin-recognizing immunocompetent cells during human development. And finally, the demonstration of increased elastin mobilization with cholesterol feeding alone (Jacob et al. 1982, 1983) might have justified to some extent the neglection of the immune-mediated mechanisms in human atherogenesis. With the rapid increase of human longevity, this approach might, however, reach its limits, producing a revival in the interest of immune-mediated processes in human vascular diseases.

Conclusion

The above summarized experiments and conceptions support the contention for an immuno-inflammatory mechanism involved in the development of athero-arteriosclerosis. Although most studies were conducted on animal models, the findings on humans reviewed in the preceeding sections strongly support the validity of the elastin-antielastin antibody-based process as of importance also for human pathology (Robert et al. 1967, 1968, 1970b, 1974c, 1984c; Robert and Robert 1975).

The connection of this process to the ER-mediated pathways via elastin degradation and elastin peptide liberation in the circulation is of importance for the rationalization of the role for both immune-mediated and receptor-mediated mechanisms. There is also the connection to the lipid-mediated processes, essentially by the upregulation of elastase production by the atherogenic lipoproteins, increasing therefore the local liberation of elastin peptides. Not all elastin peptides are, however, equivalent in this respect. Synthetic hexapeptides made on the pattern of several exons of the elastin-gene were used for screening with monoclonal antibodies (Wei et al. 1993) and for triggering the ER on endothelial cells (Faury et al. 1998b). These and other studies revealed the sequences recognized by the ER, corresponding to the pattern GXXPG comprising the most studied peptide, VGVAPG. Using the endothelial cell model, it could be shown, however, that even tripeptides as VGV can already trigger the ER (Faury et al. 1998b). Although all the above-cited studies enlarged the scope of the initially proposed immune-theory of atherogenesis, its essential features remained valid, close to the original hypothesis (Robert et al. 1967, 1968). Further experiments are clearly needed essentially to explore the relevance of notions acquired during the study of the ER and their physiopathological consequences as well as their connection to known risk factors of atherogenesis. It seems probable that such studies could open new vistas for original pharmacological innovations.

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References

Antonicelli F, Bellon G, Debelle L, Hornebeck W (2007) Elastin-elastases and inflamm-aging. Curr Top Dev Biol 79:99–155

Archilla-Marcos M, Robert L (1993) Control of the biosynthesis and excretion of the elastase-type protease of human skin fibroblasts by the elastin receptor. Clin Physiol Biochem 10:86–91

Beaumont J-L (1965) Hyperlipidemia caused by anti-beta-lipoprotein antibodies. A new pathological entity. C R Acad Sci 261:4563–4566

Beaumont J-L (1969) Gamma globulins and hyperlipemia. Hyperlipemia caused by autoantibodies. Ann Biol Clin 27:611–635

Beaumont J-L (1970) Auto-immune hyperlipidemia. An atherogenic metabolic disease of immune origin. Rev Eur Etud Clin Biol 15:1037–1041

Beaumont V, Beaumont J-L (1968) Experimental hyperlipemia induced by immunization in the rabbit. Pathol Biol 16:870–876

Beaumont J-L, Lemort N (1974) Anti-heparin immunoglobulins, a factor of thrombosis, hyperlipidemia and atherosclerosis. Pathol Biol 22:67–76

Beaumont J-L, Jacotot B, Vilain C, Beaumont V (1965) Presence of an anti-beta-lipoprotein autoantibody in a myeloma seum. C R Acad Sci 260:5960–5962

Bieth J (1978) Elastases: structure, function and pathological role. In: Robert L (ed) Frontiers in matrix biology, vol 6. Karger, Basel, pp 1–82

Bihari-Varga M, Gero S (1966) Role of intimal mucoid substances in the pathogenesis of atherosclerosis. Investigations on the components of the mucopolysaccharide-beta-lipoprotein complex formation in vitro. Acta Physiol Acad Sci Hung 29:273–281

Bihari-Varga M, Keller L, Landi A, Robert L (1984) Elastase-type activity, elastase inhibitory capacity, lipids and lipoproteins in the sera of patients with ischemic vascular disease. Atherosclerosis 50:273–281

Bihari-Varga M, Kadar A, Jacob M-P, Robert L (1986) Physicochemical and ultrastructural properties of cholesterol esters bound to elastin. Connect Tissue Res 15:43–55

Bing DK, Weygand JGM, Stavitsky AB (1967) Hemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. Proc Soc Exp Biol Med 124:1168

Bizbiz L, Bonithon-Kopp C, Ducimetière P, Berr C, Alperovitch A, Robert L (1996) Relation of serum elastase activity to ultrasonographically assessed carotid artery wall lesions and cardiovascular risk factors. The EVA study. Atherosclerosis 120:47–55

Bizbiz L, Alperovitch A, Robert L, the EVA group (1997) Aging of the vascular wall: serum concentration of elastin peptides and elastase inhibitors in relation with cardiovascular risk factor. The EVA study. Atherosclerosis 131:73–78

Bourdillon M-C, Soleilhac J-M, Crouzet B, Robert L, Hornebeck W (1984) Influence of lipoproteins on elastase-type activity of arterial smooth muscle cells in culture. Cell Biol Int Rep 8:415–421

Bürger M (1947) Altern und Krankheit. Georg Thieme, Leipzig

Burstein M, Samaille J (1959) Nouvelle méthode de séparation et de dosage des lipoprotéines de faible densité. Ann Biol Clin 17:23

Buxtorf J-C, Lorenzelli-Edouard L, Beaumont J-L (1981) Immunoglobulins and hyperlipoproteinemias. Biomedicine 35:90–93

Claire M, Jacotot B, Robert L (1976) Characterisation of lipids associated with macromolecules of the intercellular matrix of human aorta. Connect Tissue Res 4:61–71

Comper WD (ed) (1996) Extracellular matrix, vol I–II. Harwood Academic Publishers

Faury G, Ristori MT, Verdetti J, Jacob M-P, Robert L (1995) Effect of elastin peptides on vascular tone. J Vasc Res 32:112–119

Faury G, Chabaud A, Ristori MT, Robert L, Verdetti J (1997) Effect of age on the vasodilatatory action of elastin peptides. Mech Aging Dev 95:31–42

Faury G, Usson Y, Robert-Nicoud M, Robert L, Verdetti J (1998a) Nuclear and cytoplasmic free calcium level changes induced by elastin peptides in human endothelial cells. Proc Natl Acad Sci 95:2967–2972

Faury G, Garnier S, Weiss AS, Wallach J, Fülöp T Jr, Jacob M-P, Mecham RP, Robert L, Verdetti J (1998b) Action of tropoelastin and synthetic elastin sequences on vascular tone and on free calcium level in human vascular endothelial cells. Circ Res 82:328–336

Fülöp T Jr, Jacob M-P, Robert L (1989a) Determination of anti-elastin antibodies in normal and atherosclerotic human sera by ELISA. J Clin Lab Immunol 30:69–74

Fülöp T Jr, Jacob M-P, Robert L (1989b) Biological effects of elastin peptides. In: Robert L, Hornebck W (eds) Elastin and elastases, vol I. CRC Press, Boca Raton, pp 201–210

Fülöp T Jr, Varga Z, Csongor J, Jacob M-P, Robert L, Leovey A, Foris G (1990a) Altered phosphatidylinositol breakdown in polymorphonuclear leukocytes with aging. In: Goldstein AL (ed) Biomedical advances in aging. Plenum Publishing, New York

Fülöp T Jr, Wei SM, Robert L, Jacob M-P (1990b) Determination of elastin peptides in normal and atherosclerotic human sera by ELISA. Clin Physiol Biochem 8:273–282

Fülöp T Jr, Barabas G, Varga Z, Csongor J, Hauck M, Szücs S, Seres I, Mohacsi A, Kekessy D, Despont J-P, Robert L, Penyige A (1992) Transmembrane signaling changes with age. Ann N Y Acad Sci 673:165–171

Fülöp T Jr, Douziech N, Jacob M-P, Hauck M, Wallach J, Robert L (2001) Age-related alterations in the signal transduction pathways of the elastin-laminin receptor. Pathol Biol 49:339–348

Fülöp T Jr, Larbi A, Fortun A, Robert L, Khalil A (2005) Elastin peptides induced oxidation of LDL by phagocytic cells. Pathol Biol 53:416–423

Gardner DL (1965) Pathology of the connective tissue diseases. Edward Arnold Publishers, London

Gero S (1967) Some data on the influence of cholesterol atherosclerosis by immunological means. Rev Atheroscler (Paris) 9(Suppl 1):194–119

Gero S, Gergely J, Jakab L, Székely J, Virag S, Farkas K, Czuppon A (1959) Inhibition of cholesterol atherosclerosis by immunisation with beta-lipoprotein. Lancet 2(7088):6–7

Gero S, Gergely J, Dévényi T, Jakab L, Székely J, Virag S (1960) Role of mucoid substances of the aorta in the deposition of lipids. Nature 187:152–153
Groult V, Hornebeck W, Robert L, Pouchelet M, Jacob M-P (1998) Interactions of elastic fibers with fibroblasts – a time-lapse cinematographic study. Pathol Biol 46:507–516
Hornebeck W, Partridge M (1975) Conformational changes in fibrous elastin due to calcium ions. Eur J Biochem 51:73–78
Hornebeck W, Derouette J-C, Robert L (1975) Isolation, purification and properties of aortic elastase. FEBS Lett 58:66–70
Hornebeck W, Potazman JP, De Cremoux H, Bellon G, Robert L (1983) Elastase-type activity of human serum. Clin Physiol Biochem 1:285–292
Hornebeck W, Tixier J-M, Robert L (1986) Inducible adhesion of mesenchymal cells to elastic fibers: elastonectin. Proc Natl Acad Sci 83:5517–5520
Jacob M-P, Brechemier D, Robert L, Hornebeck W (1982) Variation of elastase-type protease activity and elastin biosynthesis in rabbit aorta induced by cholesterol diet. Artery 10:310–316
Jacob M-P, Hornebeck W, Robert L (1983) Studies on the interaction of cholesterol with soluble and insoluble elastins. Int J Biol Macromol 5:275–278
Jacob M-P, Hornebeck W, Lafuma C, Bernaudin JF, Robert L, Godeau G (1984) Ultrastructural and biochemical modifications of rabbit arteries induced by immunisation with soluble elastin peptides. Exp Mol Pathol 41:171–190
Jacob M-P, Fülöp T Jr, Foris G, Robert L (1987a) Effect of elastin peptides on ion fluxes in mononuclear cells, fibroblasts and smooth muscle cells. Proc Natl Acad Sci 84:995–999
Jacob M-P, Moura AM, Tixier J-M, Lafuma C, Robert AM, Robert L, Worcel M (1987b) Prevention by calcitonin of the pathological modifications of the rabbit arterial wall induced by immunisation with elastin peptides: effect on vascular smooth muscle permeability. Exp Mol Pathol 46:345–356
Jacotot B, Beaumont J-L, Monnier G, Szigeti M, Robert B, Robert L (1973) Role of elastic tissue in cholesterol deposition in the arterial wall. Nutr Metab 15:46–58
Joseph JA, Roth GS (1990) Loss of agonist-receptor efficacy in senescence: possible decrements in second messenger function and calcium mobilisation. In: Bergener M, Ermini M, Stähelin HB (eds) Challenges in aging. Academic, New York, pp 167–184
Labat-Robert J, Robert L (2007) The effect of cell-matrix interactions and aging on the malignant process. In: Advances in cancer research, vol 98. Elsevier, Amsterdam, pp 221–259
Lansing AI (ed) (1959) The arterial wall. Williams & Wilkins, Baltimore
Lorenzelli-Edouard L, Marie F, Beaumont J-L (1980) Antilipoprotein autoimmune hyperlipidemia. The Ig-Lp test. Biomedicine 33:160–163
Minick CR, Murphy GE, Campbell WG Jr (1966) Experimental induction of athero- arteriosclerosis by the synergy of allergic injury to arteries and lipid rich diet. J Exp Med 124:635
Olsson AG (ed) (1987) Atherosclerosis. Biology and clinical science. Churchill Livingstone, Edinburgh
Perdomo JJ, Gounon P, Schaeverbeke M, Schaeverbeke J, Groult V, Jacob MP, Robert L (1994) Interaction between cells and elastin fibers: an ultrastructural and immunocytochemical study. J Cell Physiol 158:451–458
Péterszegi G, Mandet C, Texier S, Robert L, Bruneval P (1997a) Lymphocytes in human atherosclerotic plaques exhibit the elastin-laminin receptor: potential role in atherogenesis. Atherosclerosis 135:103–107
Péterszegi G, Texier S, Robert AM, Moulias R, Robert L (1997b) Increased elastase and cathepsin G activity in activated lymphocytes from aged patients. Role of denutrition and dementia. Arch Gerontol Geriatr 25:285–298
Péterszegi G, Texier S, Robert L (1997c) Human helper and memory lymphocytes exhibit an inducible elastin-laminin receptor. Int Arch Allergy Immunol 114:218–223
Péterszegi G, Texier S, Robert L (1999) Cell death by overload of the elastin-laminin receptor on human activated lymphocytes: protection by lactose and melibiose. Eur J Clin Investig 29:166–172
Renais J, Groult N, Scebat L, Lenegre J (1968) Pouvoir antigénique et pathogène du tissu artériel. In: Le rôle de la paroi artérielle dans l'atherogénèse. Colloque International CNRS, Paris

Robert L (1996) Aging of the vascular wall and atherogenesis: role of the elastin-laminin receptor. Atherosclerosis 123:169–179
Robert L (1998) Mechanisms of aging of the extracellular matrix. Role of the elastin-laminin receptor. Novartis Price Lecture. Gerontology 44:307–317
Robert L (1999a) Aging of the vascular wall and atherosclerosis. Exp Gerontol 34:491–501
Robert L (1999b) Interaction between cells and elastin, the elastin receptor. Connect Tissue Res 40:75–82
Robert L, Labat-Robert J (2000) Aging of connective tissues, from genetic to epigenetic mechanisms. Biogerontology 1:123–131
Robert L, Parlebas I (1965) Biosynthèes in vitro des glycoprotéines de la cornée. Bull Soc Chim Biol 47:1853–1866
Robert L, Poullain N (1963) Étude sur la structure de l'élastine et le mode d'action des élastases. I. Nouvelle méthode de préparation des dérivés solubles de l'élastine. Bull Soc Chim Biol 45:1317–1326
Robert L, Robert B (1973) Immunology and aging. Gerontologia 19:330–335
Robert B, Robert L (1975) Aortic elastase, its role in the degradation of arterial elastin. In: Peeters H (ed) Protids of biological fluids. Pergamon Press, pp 413–418
Robert L, Stein F, Pezess MP, Poullain N (1967) Propriétés immunochimiques de l'élastine. Leur importance dans l'atherosclerose. Arch Mal Coeur 60(Suppl 1):233–241
Robert L, Robert AM, Moczar M, Moczar E (1968) Constituants macromoléculaires de la paroi artérielle. Antigénicité et rôle dans l'atherosclérose. In: Le rôle de la paroi artérielle dans l'atherosclérose. Colloque CNRS, Paris, pp 395–424
Robert L, Robert B, Medema J, Houtman JPW (1970a) Surface areas of elastin samples determined by krypton-85 adsorption. Biochim Biophys Acta 214:235–237
Robert L, Robert B, Robert AM (1970b) Molecular biology of elastin as related to aging and atherosclerosis. Exp Gerontol 5:339–356
Robert AM, Grosgogeat Y, Reverdy V, Robert B, Robert L (1971a) Lésions artérielles produites chez le lapin par immunisation avec l'élastine et les glycoprotéines de structure de l'aorte. Études biochimiques et morphologiques. Atherosclerosis 13:427–449
Robert B, Szigeti M, Derouette J-C, Robert L, Bouissou H, Fabre M-T (1971b) Studies on the nature of the microfibrillar component of elastic fibers. Eur J Biochem 21:507–516
Robert L, Robert B, Houtman JPW, Stack MV (1971c) Flow calorimetry of the sorption of butanol to elastin preparations and comparison with surface areas determined by Krypton-85 adsorption. Biochim Biophys Acta 251:370–375
Robert B, Hornebeck W, Robert L (1974a) Cinétique hétérogène de l'interaction élastine-élastase. Biochimie 56:239–244
Robert B, Derouette J-C, Robert L (1974b) Mise en évidence de protéases à activité élastolytique dans les extraits d'aortes humaines et animales. C R Acad Sci Paris 278:3251–3254
Robert B, Robert L, Robert AM (1974c) Elastrine, élastase et artériosclérose. Pathol Biol 22:661–669
Robert L, Jacob M-P, Szemenyei K, Robert AM (1984a) Pharmacological properties of elastin peptides, their action on serum and aorta lipids and on the atherosclerotic process. In: Carlson LA, Olsson AG (eds) Treatment of hyperlipoproteinemia. Raven Press, New York, pp 185–188
Robert L, Chaudière J, Jacotot B (1984b) Interaction between lipids and the intercellular matrix of the arterial wall: its role in the evolution of atherosclerotic lesions. In: Malinow R, Blaton VH (eds) Regression of atherosclerotic lesions. Plenum Publishing Corporation, pp 145–173
Robert L, Jacob M-P, Frances C, Godeau G, Hornebeck W (1984c) Interaction between elastin and elastases and its role in the aging of the arterial wall, skin and other connective tissues. A review. Mech Aging Dev 28:155–166
Robert L, Moczar M, Moczar E (eds) (1985) Methods of connective tissue research, Frontiers in matrix biology, vol 10. Karger, Basel
Robert L, Labat-Robert J, Hornebeck W (1986) Aging and atherosclerosis. Atherosclerosis Rev 14:143–170, Raven Press, New York

Robert L, Jacob M-P, Fülöp T Jr, Timar J, Hornebeck W (1989) Elastonectin and the elastin receptor. Pathol Biol 37:736–741

Roth GS (1979) Hormone receptor changes during adulthood and senescence: significance for aging research. Fed Proc 38:1910–1914

Rouis M, Nigon F, Lafuma C, Hornebeck W, Chapman J (1990) Expression of elastase activity by human monocyte-macrophages is modulated by cellular cholesterol content, inflammatory mediators and phorbol myristate acetate. Arteriosclerosis 10:246–255

Scebat L, Renais J, Iris L, Groult N, Lenegre J (1966) Lésions artérielles produites chez le lapin par des injections de broyat d'aorte homologue et hétérologue. Archives Des Maladies Du Coeur 59 (Suppl 1):56–72

Scebat L, Renais J, Groult N, Iris L, Lenegre J (1967) Arterial lesions produced in rabbits by injections of pulverized rat aorta. Preliminary study. Rev Atheroscler (Paris) 9:249–262

Stein F, Pezess M-P, Robert L, Poullain N (1965) Anti-elastin antibodies in normal and pathological human sera. Nature 207:312–313

Szigeti I, Ormos J, Jako J, Toszegi A (1960) The atherogenic effect of immunisation with homologous complex great vessel wall in rabbit. Acta Allergol (Suppl VII):374–387

Szigeti I, Jako I, Doman J (1968) Study of the antigenic and immunopathological effects of structural proteins of the mammalian arterial wall and their role in the pathogenesis of atherosclerosis. In: colloque CNRS N° 169: Le rôle de la paroi artérielle dans l'athérogénèse. Paris, pp 387–394

Szigeti M, Monnier G, Jacotot B, Robert L (1972) Distribution of ingested 14C-cholesterol in the macromolecular fractions of rat connective tissue. Connect Tissue Res 1:145–152

Timar J, Diczhazi CS, Ladanyi A, Raso E, Hornebeck W, Robert L, Lapis K (1995) Interaction of tumor cells with elastin and the metastatic phenotype. In: The molecular biology and pathology of elastic tissues. Wiley, Chichester, pp 321–333

Trentham DE (1984) Immunity to type II collagen in rheumatoid arthritis: a current appraisal. Proc Soc Exp Biol Med 176:85–104

Urry DW (1980) Sequential polypeptides of elastin. Structural properties and molecular pathologies. In: Frontics in Matrix Biology. Robert AM, Robert L (eds) Biology and pathology of elastic tissues, vol 8. Karger, Basel, pp 78–103

Varga Z, Kovacs EM, Paragh G, Jacob M-P, Robert L, Fülöp T Jr (1988) Effect of elastin peptides and *N*-Formyl-methionyl-Leucyl-Phenylalanine on cytosolic free calcium in polymorphonuclear leucocytes of healthy middle-aged and elderly subjects. Clin Biochem 21:127–130

Varga Z, Jacob M-P, Robert L, Fülöp T Jr (1989) Identification and signal transduction mechanism of elastine peptide receptor in human leucocytes. FEBS Lett 258:5–8

Varga Z, Jacob M-P, Robert L, Csongor J, Fülöp T Jr (1997) Age-dependent changes of k-elastin stimulated effector functions of human phagocytic cells: relevance for atherogenesis. Exp Gerontol 32:653–662

Wei SM, Erdei J, Fülöp T Jr, Robert L, Jacob M-P (1993) Elastine peptide concentration in human serum: variation with antibodies and elastin peptides used for the enzyme-linked immunosorbent assay. J Immunol Methods 164:175–187

White FN, Grollman A (1964) Experimental periarteritis nodosa in the rat. Arch Pathol 78:31