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Original Paper

Ultrastructural Arguments for the Extracellular Location of Amyloid Deposits

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REZUMAT

Argumente ultrastructurale pentru localizarea extracelulara a depozitelor de amiloid

Obiective: Amiloidoza este o boală caracterizată prin apariția unor depozite mari sau mici, conținând un material fibrilar format din proteine insolubile, ce sunt identificate din ce în ce mai frecvent, mai recent și cu ajutorul microscopului electronic. Patogenia și mecanismele amiloidozei încă mai au multe semne de întrebare. Majoritatea specialiștilor consideră că depozitele de amyloid sunt acumulări interstițial. Unii autori consideră însă formarea acestor depozite atât intra cât și extracellular.

Metode: Pentru formarea unei opinii clare în acest domeniu, am cercetat cu ajutorul microscopului electronic apariția materialului amyloidic fibrilar în cardiomiocitele atriale în cazuri de amiloidoză atrială izolată, și în glomeruli în amiloidoza renală.

Concluzie: Chiar și depozitele minore invizibile cu microscopul optic, sau considerate intracitoplasmatice, sunt întotdeauna înconjurate de o citomembrană în continuitate cu membrană celulară care formează recese interstițial. Astfel, rezultatele cercetării sunt argumente că depozitele de amyloid sunt întotdeauna extracelulare.

Cuvinte cheie: amyloid, fibrile amiloidice, extracellular, rinichi, atriu, diabet zaharat

ABSTRACT

Objectives: Amyloidosis is a disease characterized by the occurrence of small and large deposits containing a fibrillar material of insoluble proteins, which is lately more and more frequently identified due also to electron microscopy. The pathogeny and mechanisms of amyloidosis have still many question marks. Most people consider the amyloid deposits as interstitial accumulations. Some authors are still considering formation of amyloid collections both intra and extracellular.

Methods: To assume a definite opinion in this matter we have investigated carefully with the electron microscope, the occurrence of amyloid fibrillar material in atrial cardiomyocytes in cases of isolated atrial amyloidosis, and in the glomeruli in renal amyloidosis.

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Conclusion: Even minute deposits invisible in light microscopy, or considered intracytoplasmic, are always surrounded by a cytomembrane in continuity with the plasmamembrane, forming interstitial recesses. Thus, our findings are arguments that amyloid deposits are always extracellular.

Key words: amyloidosis, amyloid fibrils, extracellular, kidney, atrium, diabetes mellitus

INTRODUCTION

Amyloidosis is a disease characterized by deposition and accumulation of insoluble proteins as small or large deposits of a fibrillar material. Despite the fact that these deposits are ultrastructurally identical, the basic proteins are chemically different, so far being recognized over 25 amyloidogenic precursors [1]. All these proteins can be immunohistochemically identified, thus leading to a more and more precise diagnosis and an appropriate treatment.

Amyloid fibrils have an 8 to 12 nm diameter, are extremely strong, highly ordered and organized and can be formed, as already mentioned, by a large number of proteins and peptides [2]. They are rigid, nonbranching, hollow-cored tubules randomly arranged. Examined in X-ray diffraction they have a characteristic β-pleated sheet configuration. This macromolecular helix of 100 nm periodicity, formed by two twisted β-pleated sheet micelles is responsible for the resistance of amyloid to solubilization or proteolytic digestion. The amyloid precursor peptides may be normal serum proteins, or abnormal degradation variants, which can be repetitively incorporated into a developing amyloid fibril. The P component of amyloid fibril is a pentagonal, 8 nm diameters, doughnut like glycoprotein, similar to complement component C1t and C-reactive protein. P component is probably the cause of amyloid deposit staining with iodine. All amyloids contain a proteoglycans matrix. Pure amyloid contains amyloid fibrils, P component and proteoglycans matrix. In tissue, the deposits are contaminated with varying amounts of plasma proteins and collagen. Variations in staining and density of amyloid result from different amounts of non-amyloid components attached to the fibrilar scaffold.

Amyloid fibrils can be formed by abnormal processing of protein components such as acute-phase proteins, immunoglobulins, and endocrine secretions. The key of this process is the presence of a circulating, soluble precursor protein that has an amyloidogenic primary structure and assembles into a β-pleated sheet configuration. This form is resistant to enzymatic digestion and accumulates in tissues.

Amyloid is inefficiently removed by macrophages. It is resistant to phagocytosis by neutrophils and monocytes. If the cause of disease is removed, amyloid disappears from organs with active macrophages, such as spleen, but may not from the renal glomerulus [3].

It was recommended that amyloid and amyloidosis should be classified by the fibrillar protein forming the amyloid deposits. The current nomenclature is based on the name of the major fibrillar protein, which is designated protein A, followed by an abbreviation of the protein name [4]. The mechanisms involved in tissue damage can imply disturbance of tissue architecture, local cytotoxicity, free radicals, etc.

The immunohistochemical classification of amyloid deposits is a useful tool with increasing importance in diagnosis, and a large number of anti-amyloid antibodies are commercially available. Electron microscopy can detect unmistakably small amyloid deposits when histochemical stains such as Congo red are still negative. The fibrils are usually found in extracellular spaces and identified upon their specific dimensions, aspect and orientation. Nevertheless, some authors are still claiming that the assembly of amyloid fibrils is taking place in the intracellular area also. These findings have been collected mostly from experimental, either animal models [5] or cultured pancreatic islets, in connection with diabetes mellitus [6] Alzheimer's disease [7] [8], and other biopsy or necropsy specimens of human origin [9]. The present work was intended to clarify this equivocal location of amyloid deposits by comparison with our biopsies from renal and isolated atrial amyloidosis patients.

MATERIALS AND METHODS

The subjects we have investigated belonged to one group of patients suffering of isolated atrial amyloidosis (IAA) and to a second group with renal amyloidosis. An additional third group of known diabetic patients investigated for diabetic nephropathy proved to be associated in a small percentage with renal amyloidosis. The study was performed with the Ethics Committee of "Victor Babes" Institute of Pathology approval.

Human cardiac tissue biopsies were obtained from 37 patients undergoing coronary artery bypass, grafting or valvular surgery (11 females and 26 males). Tissue samples were collected from patients who had given informed consent before surgery. The basic cardiac condition of these 37 patients was the following: 16 had coronary diseases, 14 suffered of valvular heart diseases and 7 had both coronary and valvular diseases [10].

Atrial biopsies collected from these patients were processed for electron microscopy. Additional tissue samples fixed in formaldehyde and embedded in paraffin were available from only ten patients. From thirteen patients three different cardiac samples were harvested: left and right atrial appendages, and atrial sleeves of the pulmonary veins. As for the other twenty-four patients the surgical unit provided tissue samples only from the left atrial appendages.

A second group of 46 patients with nephritic syndrome (severe proteinuria over 5g/day) have been diagnosed as renal amyloidosis on per-cutaneous kidney biopsies, using 16 G guillotine needles.

The third group of 64 patients with diabetes mellitus type II (48% males) and diabetic nephropathy, was also submitted to renal biopsy for kidney evaluation. Four of these diabetic patients proved to have also associated renal amyloidosis [11].

Each kidney biopsy sample was divided in two parts: one middle fragment for direct immunofluorescence (IF), and the couple of 1 mm^3 end fragments for light microscopy (LM) and electron microscopy (EM). For immunostaining samples were immediately snap frozen and then cryosectioned. The 4 μm thick cryo-sections were processed using ten FITC conjugated antibodies: anti-IgA, IgG, IgM, C1q, C3, Kappa, Lambda, anti-albumin, and anti-fibrinogen.

For LM and EM fixation was performed with 4% buffered glutaraldehyde followed by one hour in 1% buffered osmic acid. After dehydration in ethanol, the 1mm^3 fragments were embedded in Epon. One micron thick sections stained with 1% toluidine blue were used for light microscopy, and 70 nm thin sections, double stained with uranyl acetate and lead citrate, were performed for electron microscopy.

When available, atrial and kidney samples (10 atrial, 15 diabetic and 26 nephrotic patients) were fixed in 7% buffered formaldehyde and paraffin embedded. Serial sections at 3–5 μm were stained with haematoxylin and eosin, van Gieson and Congo red.

Diabetic nephropathy was diagnosed on morphologic ground (glomerular sclerosis, either nodular or diffuse), immunostaining criteria such as linear fluorescent deposits for anti-IgG and anti-albumin antibodies on the glomerular basement membranes (GBM), and thickened glomerular and tubular basement membranes in electron microscopy.

Amyloid deposits have been identified on Congo red stained sections when available, both in light microscopy and polarized light, and in electron microscopy upon the specific features of amyloidal fibrils.

RESULTS

Under destabilizing conditions proteins can self-aggregate into insoluble, fibrillar assemblies. The morphology of amyloid fibrils has been well investigated by several authors [12] [13]. In the form of amyloid fibrils or fibril precursors, the proteins loose their biological function and may become harmful to the organism, causing pathologies such as Alzheimer's and prion diseases, isolated atrial or renal amyloidosis. Although amyloid precursor proteins do not share any structural homology, amyloid fibrils are all typically unbranched, randomly distributed, protease-resistant filaments approximately 10 nm in diameter and composed of ´2.0-3.5 nm wide protofilaments, which are arranged around an electron lucent core [14]. The general morphology of amyloid aggregates depends on the conditions in which fibrillogenesis takes place. The structural variation can be caused by fibrils with a variable number and arrangement of protofilaments. Studies using X-ray diffraction showed a characteristic cross-β structure with β-strands of the precursor protein arranged perpendicular to, and ribbon-like β-sheets parallel, to fibril axis [15] [16] [17].

Our results showed fibrillar material in both atrial wall and kidney glomeruli. In the atrial wall

amyloid deposits were found in the interstitium as well as in the cardiomyocytes. In light microscopy these deposits looked like polymorphic spots placed either in the middle or in the periphery of myocardial fibers in cross sections (**Fig. 1**). Deposits were stained lighter compared with the intense blue of the cardiomyocytes on our toluidine blue stained sections. Some of the peripheral spots showed open connections with the interstitial space. These light stained spots showed more or less a similar shape in electron microscopy both in cross (**Fig. 2**) and longitudinal sections (**Fig. 3**). The ultrastructure of the deposits content was typical for amyloid material: randomly arranged filaments about 10 nm thick, unbranched and more or less crowded. At a higher magnification these spot-like areas were always limited by cytomembranes no matter if they were placed peripherally or in the centre of muscular fibers (**Fig. 4**). These membranes showed a constant lamina on the deposit side, and from place to place we found clusters of caveolae on the myofilaments side (**Fig. 4**). The surrounding membranes of

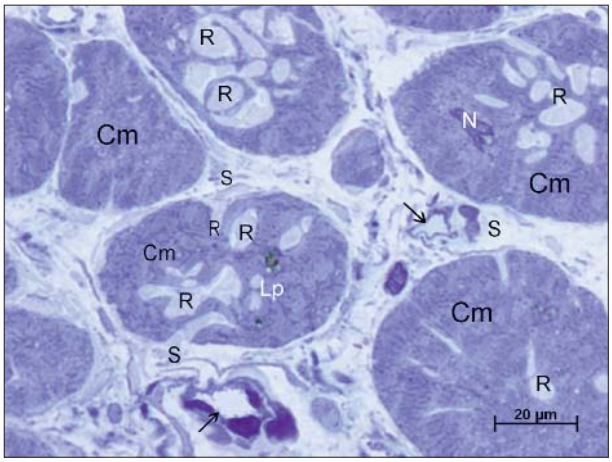

Figure 1. Atrial wall showing cardiomyocytes in cross section. Cm, cardiomyocytes; N, nucleus; Lp, lipofuscin bodies; R, lightly stained spot deposits of amyloid filaments, some opened in the interstitium (S). The interstitial area contains fibroblasts, blood vessels (arrows) and extracellular matrix. Epon thick section of 1○m, toluidine blue staining

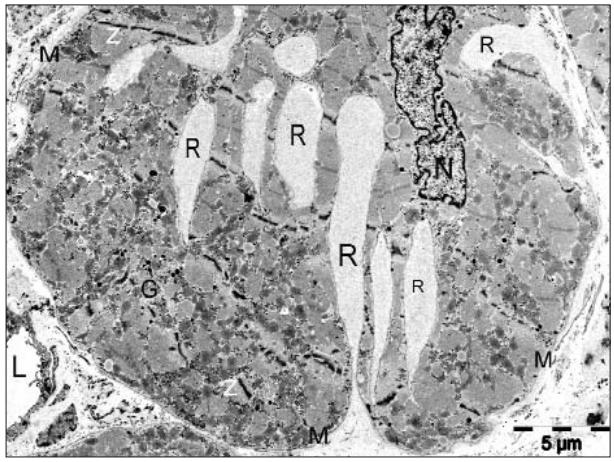

Figure 2. Cross section of an atrial cardiomyocytes. N, nucleus; Z, z bands of sarcomers; M, myocyte plasmamembrane; G, atrial natriuretic peptide dense granules; L, blood vessel in the interstitial area; R, interstitial recesses containing fibrillar amyloid deposits

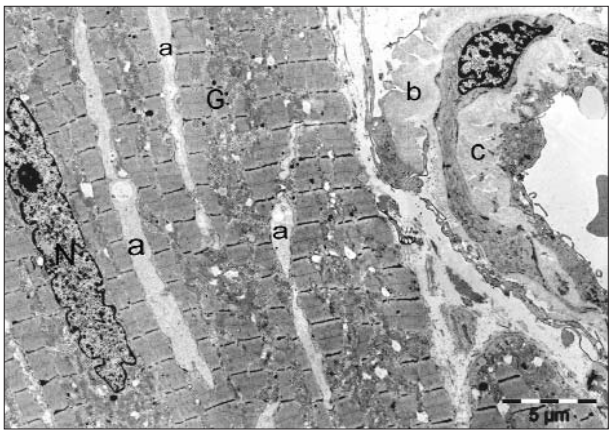

Figure 3. Longitudinal section of an atrial myocyte. N, nucleus; G, atrial natriuretic peptide dense granules. Amyloid deposits: a-intramyocyte recesses, b-periarteriolar deposits, c-subendothelial deposits

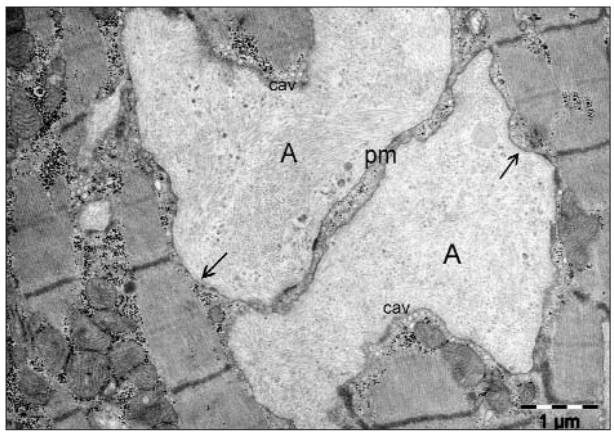

Figure 4. Atrial cardiomyocyte showing two (A) central recesses loaded with amyloid filaments arranged randomly, and admixed with a few dense granules. Both recesses are surrounded by plasmamembranes (pm) with associated caveole clusters (cav). On the outer aspect of membranes, namely on the amyloid recess side, a thin lamina of basal membrane material can be noticed (arrows)

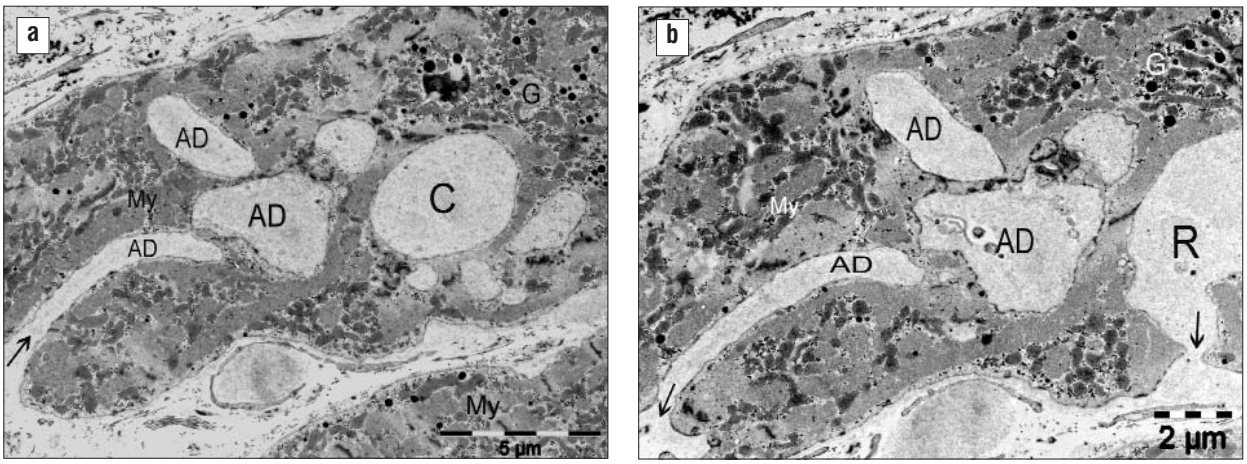

Figure 5 (a, b). Two distinct sections of the same atrial cardiomyocyte showing "intracellular" amyloid deposits (recesses) (AD). The fibrillar deposit from the right side of fig. 5a (C) became an open recess in fig. 5b (R). These successive images prove the ubiquitous connection of amyloid deposits with the extracellular space; G, several atrial natriuretic peptide dense granules; My, myocytes. Extracellular openings (arrows)

deposits were in fact similar to the plasma membrane of cardiomyocytes. Among the thin filaments of deposits we also found dense small granules measuring between 25 and 50 nm. Although chaotically distributed, in some points the filaments were gathered perpendicularly on the surrounding plasma membrane (**Fig. 4**). We found no connection between these points and the membrane attached caveolae.

Depending on the level of sections some of the amyloid deposits had connections with the interstitial space through strait strips suggesting a "neck-like" segment of deposits. A careful analysis on serial sections proved the fact that every one of these deposits has somewhere an open communication with the extracellular matrix (**Fig. 5a** and **5b**).

We found similar aspects in the kidney samples from patients with renal amyloidosis. The bulk of amyloid deposits in kidneys has been found in the glomeruli. The most affected area was the mesangium, namely the matrix. Beside this location we found fibrillar deposits in the cytoplasm of podocytes, between the glomerular basement membrane and podocytes, and also in the urinary space. In the podocyte cytoplasm these deposits were also polymorphic and clearly surrounded by a cytomembrane (**Fig. 6**). The amyloid deposits contained typical filaments of 8-12 nm diameters and showed some small zones of higher or lesser density (**Fig. 6, 7**). The surrounding membrane was continuous, but in some segments where it was discontinuous like the foot processes in contact with the lamina rara externa of glomerular basement membrane (**Fig. 7**).

Further examination of the mesangial amyloid deposits revealed a few mesangial cells spread in the filamentous mass. These cells had a stellate shape. Their cytoplasm was split by bunches of amyloid filaments. Some of these filament strands had their tip in the perinuclear envelope (**Fig. 8**). These bunches were bordered by a cytomembrane continuous with the plasma membrane of the corresponding mesangial cell. These structures looked like a continuity between the perinuclear cistern and the extracellular matrix.

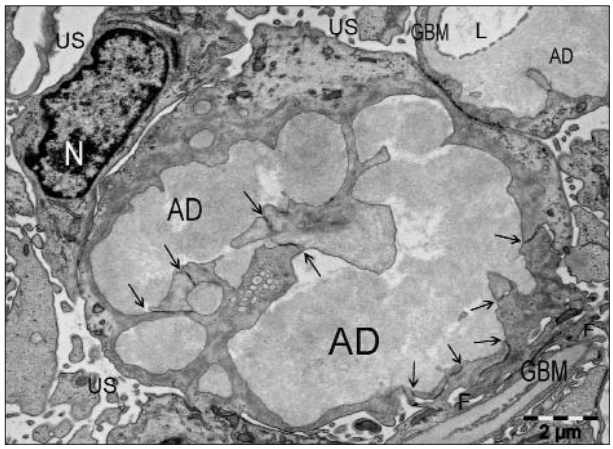

Figure 6. Podocyte containing several amyloid deposits (recesses) with polymorphic shapes, variable densities and integrally surrounded by the cell plasmamembrane but a few points were diaphragm slits occur (arrows). GBM, glomerular basement membrane; N, podocyte nucleus; US, urinary space; L, glomerular capillary lumen; AD, amyloid deposits; F, flattened foot processes

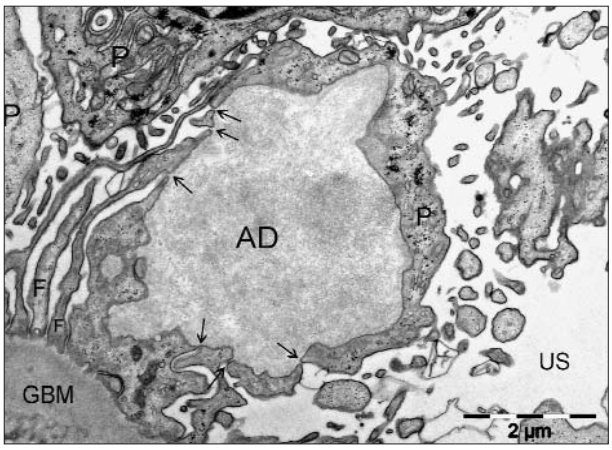

Figure 7. Podocyte containing a cytoplasmic deposit of amyloid, not yet opened in the extracellular space, and still contained by some slit diaphragms (arrows). At these points the deposit surrounding membrane is continuous with the cell membrane of podocyte. AD, amyloid deposit; P, podocyte cytoplasm; GBM, glomerular basement membrane; US, urinary space; F, foot processes

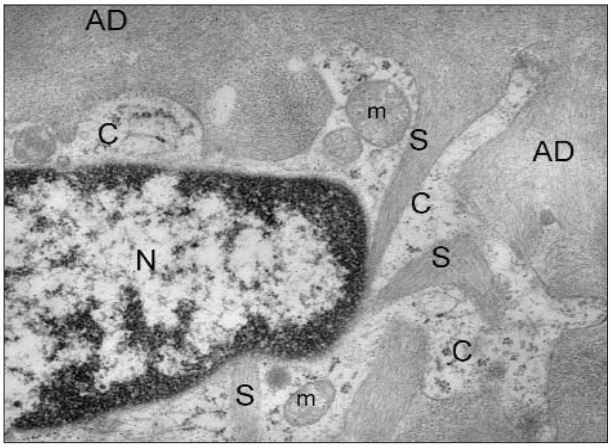

Figure 8. Kidney sample. Glomerular mesangial matrix full of amyloid fibril deposition (AD). N, mesangial cell nucleus; C, mesangial cytoplasm; S, strands of amyloid filaments springing from the perinuclear endoplasmic cistern; Mitochondria (m). X25,000

DISCUSSION

Both investigated tissues, kidney and atrial wall, in light microscopy provided features suggesting the development of amyloid deposits either in extracellular or intracellular spaces, or both. The point of the present work was not to assert the diagnosis already established with the classic methods (Congo red, polarized light, electron microscopy), but to clarify the precise location of the minute deposits of amyloidal filaments. The light microscopy highest magnification emphasized in atrial cardiomyocytes intracellular round or elongated spots filled with a differently stained material, which proved to be filamentous in electron microscopy (EM). Thin sections oriented either longitudinally or transversally, showed in EM equivalent small bags, some closed, some having narrow openings communicating with the interstitial space. In electron microscopy we could establish that these intracellular amyloid deposits were surrounded by cytomembranes equivalent to the plasmamembrane, and their majority possessed open contacts with the extracellular compartment. Analyzing these deposits on multiple sections it became obvious that all of them had communications with the interstitial area.

In a similar study on patients with cardiac amyloidosis Kaye et al [18] demonstrated, by labeling with immunogold, the connection between isolated atrial amyloidosis and atrial natriuretic peptide preset as neurosecretory granules exclusively in the atrial cardiomyocytes. The authors mentioned the presence of intracellular amyloid in two cases of IAA and concluded to be in agreement with similar features in endocrine tumors. We consider this imprecise interpretation as a consequence of immunogold labeling which covered the cardiomyocytes also.

In the renal glomeruli, by electron microscopy the fibrillar deposits of podocytes were also surrounded by plasma membranes which frequently showed some fine slits suggesting the slit diaphragms of foot processes. Through these podocyte slits, the amyloid deposits were in fact in contact with the glomerular basement membrane and the virtual space in between, which is usually stuffed with amyloidal fibrils.

Using a cell culture system, Friederich et al [7] showed that the formation of amyloid plaque represents a template dependent process that critically involves the presence of endocytosis or phagocytosis competent cells. Thus, internalized Aβ peptide are stored in multivesicular bodies where they grow out and penetrate through the vesicular membrane in the cytoplasm. Eventually the affected cells undergo cell death, and intracellular amyloid components are released into the extracellular space. The authors suggest the necessity of further works to ascertain the consistency of these pathways.

By the help of a model with transfected COS-1 cells, O'Brien TD. et al [19] claimed to have demonstrated that high level expression of hIAPP results in

intracellular IAPP-derived amyloid formation. In an experimental model using cultured human islet amyloid polypeptide (hIAPP), de Koning et al [7] also found immunoreactive fibrils at both intra- and extracellular sites of β cell cytoplasm cultured at 11.1 mM glucose. These intracellular fibrils were of similar dimensions and appearance as the extracellular ones. Zucker-Franklin D et al [9] also mentioned the presence of amyloid-like cytoplasmic fibrils located in the perinuclear and Golgi regions of human specimens, but we consider that most probably they were intermediate filaments. Nevertheless the same authors emphasized a series of cell invaginations loaded with amyloid fibrils which are very much in agreement with our findings, namely the interstitial recesses of atrial cardiomyocytes.

In disagreement with the above mentioned authors, we appreciate our findings as strong arguments for considering the apparently intracellular amyloid deposits to be, as a matter of fact, interstitial recesses belonging to the extracellular area and penetrating deep in the cytoplasm of affected cells. Although the amyloid precursor proteins could be synthesized in the cytoplasm of podocytes or cardiomyocytes, the final characteristic assemblage of cross-β structured amyloid filaments happens in these extracellular recesses.

Concerning the features depicting mesangial cells in connection with bunches of amyloid filaments we consider this aspect in accordance with the process of formation and accumulation of amyloid in interstitial recesses. These features are endorsing the findings of Gueft B. et al [5] referred to amyloid strands in contact with the nuclear membrane and the suggested "star" formation. In the instance of mesangial cells the endoplasmic reticulum has been restricted to the perinuclear cistern, and the largely opened recesses of these exhausted cells became filamentous strands, pouring out to the extracellular area. Thus, it seems that the only and last activity of these mesangial cells was production of amyloid fibrils.

CONCLUSIONS

As a conclusion, the amyloid material, at least in its fibrillar ultrastructural appearance, is always assembled in locations belonging to the extracellular space. In spite of the fact that in light microscopy and immunohistochemistry it can be supposed to be in the cytoplasm of certain cells, by electron microscopy the deposits are placed inside plasmamembrane bounded interstitial recesses, in direct connection with the extracellular space.

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