Evidence for Intracellular Amyloid Formation in Myeloma

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Evidence for intracellular formation of amyloid fibrils in a patient with kappa light-chain myeloma is described. Amyloid fibrils were seen as intracytoplasmic inclusions within plasma cells, histiocytes, renal tubule cells, and possibly in hepatocytes. Extracellular amyloid was also present. The electron microscopic studies suggest that amyloid fibrils may form in the Golgi apparatus and within lysosomes.

The relationship between multiple myeloma and amyloidosis has been the subject of extensive investigations and controversy for many years. Recent studies have demonstrated that the major constituent of amyloid deposits associated with myeloma is a fibrillar protein related to the light chains of immunoglobulins.

The conclusion that amyloid fibrillar protein in multiple myeloma is derived primarily from an immunoglobulin is supported by the following data: immunocchemical crossreactivity has been demonstrated between amyloid fibrils and light chains; amino acid sequence analysis has shown areas of homology between some amyloid fibrillar proteins and the variable region of the immunoglobulin light chain; fibrils showing staining, electron microscopic, and crystallographic characteristics of amyloid can be produced by proteolytic digestion of some light chains; and the β-pleated sheet conformation of amyloid fibrillar protein is present in the variable region of the immunoglobulin light chain.

Isobe and Osserman, in a study of sera from 100 cases of amyloidosis, found light chains in the serum of virtually all patients with myeloma-associated amyloidosis and in the majority of patients with secondary amyloidosis.

However, there is still considerable controversy regarding the site of formation of amyloid fibrils. This report presents evidence for intracellular sites of formation for amyloid fibrils in plasma cells and histiocytes of a patient with kappa light-chain multiple myeloma.

Materials and Methods

Case Report

A 60-yr-old white male presented to his physician in June 1975 complaining of anorexia, weight loss, fatigue, and pallor of approximately 6-mo duration. He had experienced no infections or bleeding problems. His previous health had been excellent.
Physical examination showed a pale man appearing older than his stated age. His spleen was palpable 1 cm below the costal margin. He had no bone tenderness. There were no ecchymoses or petechiae.

Initial laboratory evaluation revealed a pancytopenia, hemoglobin 9.2 g/dl, platelets 74 x 10^9/liter, and white blood cell count 2.2 x 10^9/liter with 3% bands, 50% neutrophils, 45% lymphocytes, and 2% monocytes on differential count. A bone marrow aspirate showed infiltration with immature lymphoid cells, many containing "needlelike crystals." An axillary lymph node biopsy showed reactive hyperplasia.

He was felt to have malignant lymphoma involving the marrow and was treated over the next 5 mo with vincristine and cyclophosphamide. He required frequent transfusions throughout this time and did not show any clinical improvement.

The patient was first seen at the University of Utah Medical Center in October 1975, and the bone marrow aspirate and biopsy were repeated. The bone marrow smears and sections showed a diffuse infiltrate of plasma cells and plasmacytoid lymphocytes. Approximately 60% of these cells had large, needle-shaped, intracytoplasmic inclusions which on Wright's stained smears had a purple color (Fig. 1). Many extracellular needlelike crystals were also seen.

Serum immunodiffusion study showed reduced levels of all normal immunoglobulins: IgG 280 mg/dl, IgA 8 mg/dl, and IgM 30 mg/dl. Serum immunoelectrophoresis revealed a bulging, scooping deformity of the kappa arc. No abnormalities were noted with tests for IgD and IgE. Urine immunoelectrophoresis failed to show the presence of light chains. On the basis of this finding, the diagnosis of kappa light-chain myeloma was made.

With the diagnosis of light-chain multiple myeloma and the apparent failure to respond to vincristine and cyclophosphamide, it was recommended that Adriamycin and prednisone be added to his therapy. Despite three courses of this therapy over the next 2 mo, he continued to deteriorate and was hospitalized in late November 1975 with fever and gastrointestinal bleeding. He was treated with antibiotics and packed red blood cell and platelet transfusions, but expired 3 days after admission.
Autopsy Findings

At autopsy the bone marrow appeared almost completely replaced by plasma cells. These cells and histiocytes contained crystallike inclusions. Similar inclusions were also seen extracellularly. Plasma cell infiltration was present in lungs, liver, spleen, kidneys, and lymph nodes.

The lungs showed edema, acute congestion, and hemorrhagic infarcts. Multifocal sheets of gram-negative bacteria were seen in the alveoli without any associated inflammatory reaction. The spleen exhibited moderate enlargement, reduction in white pulp, and numerous plasma cells within the sinusoids. The liver was found to be moderately enlarged and congested and showed mild fatty metamorphosis. Portal tracts and sinusoids contained a moderate number of plasma cells. The renal cortex was diffusely infiltrated by plasma cells, and many hyalinized glomeruli were seen. Proximal tubules showed degenerative changes and many contained eosinophilic, laminated casts with a waxy, granular appearance. There was no giant cell reaction associated with these casts. Several small mediastinal lymph nodes were diffusely infiltrated by plasma cells.

Special Studies

Smears of bone marrow aspirate made antemortem were stained with Wright Giemsa, PAS, methyl green pyronine, and acid phosphatase. Histologic sections of bone marrow, lungs, liver, spleen, and kidneys were stained with hematoxylin and eosin, PAS, methyl green pyronine, Congo red, Thioflavine-T, and crystal violet. Sections from these organs were also embedded in plastic (Epon) and semithin sections were stained with toluidine blue. Histologic sections were viewed by bright field and fluorescent microscopy. The Congo red stained sections were viewed with polarized and fluorescent light.

For electron microscopy, bone marrow aspirate was fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer. The material was then fixed in 2% osmium tetroxide with 0.1 M cacodylate buffer for 2 hr, dehydrated in graded ethanol, and embedded in Epon. Formalin-fixed autopsy

Fig. 2. Spleen diffusely infiltrated with plasma cells containing many intracytoplasmic inclusions. Semithin plastic section. Toluidine blue. × 560.
tissue from liver, spleen, kidney, and lungs was handled in a similar fashion. Then 75-nm sections were cut on an LKB Ultratome III and were stained with uranyl acetate and lead citrate. The specimens were examined with an RCA EMU-4 electron microscope.

Smears of washed cell suspensions of bone marrow aspirate were used for immunofluorescent studies. The slides were air dried and fixed for 10 min in absolute ethanol with 5\(\%\) acetic acid. They were then examined by a direct immunofluorescent method using fluorescein-conjugated, monospecific antisera to IgG, IgA, IgM, and kappa and lambda light chains (from Cappel Laboratories, Downingtown, Pa.). The specificity of staining was ascertained by immunoelectrophoresis in which the fluoresceinated antisera were reacted against normal human sera. In addition, the antisera were tested against Sepharose beads that had been complexed with known myeloma proteins.

RESULTS

Light Microscopy

In the bone marrow aspirate, many plasma cell inclusions had a pink color, though others were colorless with Wright’s stain. The inclusions were PAS, methyl green pyronin, and acid phosphatase negative. Intracytoplasmic inclusions in plasma cells, histiocytes, hepatocytes, Kupffer cells, and proximal tubule cells were readily seen in the semithin plastic sections (Fig. 2). Sections of the spleen also showed numerous inclusions within the lining cells of the venous sinuses. In the histologic sections of bone marrow, liver, spleen, and kidneys, the intracytoplasmic inclusions were stained intensely by Thioflavine-T and weakly by Congo red (Figs. 3 and 4). Crystal violet, Thioflavine-T, and Congo red staining material was seen in the walls of small- and medium-size
Fig. 4. Proximal renal tubule containing numerous inclusions staining strongly with Thioflavine-T. × 400.

Fig. 5. Plasma cells showing immunofluorescent staining with kappa light-chain antisera. Needle-shaped nonstaining intracytoplasmic defects correspond to the inclusions. × 1000.
arteries, in bone marrow, liver, spleen, and kidneys, in the walls of some alveoli, and focally in skeletal muscle and renal glomeruli. The renal casts showed strong positive staining with all amyloid stains.

Different tinctorial properties of amyloid deposits are common, and intracytoplasmic amyloid may be difficult to detect with Congo red stain. The Thioflavine stain is thought to be more sensitive for amyloid, but perhaps less specific. It is also possible that the chemical composition of the intracellular amyloid is different from that seen extracellularly.

Immunofluorescent Microscopy

The majority of the plasma cells in the bone marrow aspirate exhibited diffuse homogenous cytoplasmic staining only with antisera to kappa light chain. Similar cytoplasmic staining was also seen in a few histiocytes. There were, however, needle-shaped unstaining defects that corresponded to the inclusions seen by light microscopy (Fig. 5). Tissue from other organs was not available for immunofluorescent studies.

Electron Microscopy

Bone marrow: The cells in the bone marrow varied from typical plasma cells to plasmacytoid lymphocytes. Most contained multiple cytoplasmic inclusions that usually occupied a major portion of the cytoplasm (Fig. 6). The inclusions varied in size and shape. Some were similar in size and shape to small mito-

![Fig. 6. Transmission electron micrograph of bone marrow aspirate showing several plasma cells and plasmacytoid lymphocytes containing multiple large, intracytoplasmic inclusions (In). Also seen is a histiocyte with several small and a large crystalline inclusions (Cln). × 3700.](from)
The inclusions were composed of tightly packed, regular arrays of fibrils cut in both longitudinal and cross section. The fibrils were always oriented in parallel to the long axis of the inclusions and were 80-90 Å in diameter. On cross section, the fibrils showed an electrolucent center (Fig. 8). The fibrils in most inclusions were tightly packed; in others, they were more loosely arranged. The inclusions varied in electron density. In the more electron-dense inclusions, the fibrillar structure was not readily apparent. The single smooth membrane surrounding the inclusions resembled that of Golgi vacuoles but could not be differentiated from the rest of the α-cytomembrane system in appearance. The inclusions were prominent within and adjacent to the Golgi zone and in a few cells appeared to be in continuity with the Golgi apparatus.

Histiocytes containing multiple, often large, intracytoplasmic inclusions bounded by a single smooth membrane or within vacuoles were also seen in the bone marrow aspirate. The inclusions were, for the most part, considerably more electron dense than the inclusions within the plasma cells and resembled crystals. However, distinct fibrillar inclusions were also seen (Fig. 9). A few...
Fig. 8. Electron micrograph of inclusions (In) in longitudinal and cross section. The fibrils are oriented in parallel to the long axis of the inclusions. On cross section the fibrils show an electronlucent center. × 67,500.

Fig. 9. Electron micrograph of a portion of a macrophage in the bone marrow containing electron-dense crystallike inclusions (CIn) as well as a conglomeration of fibrillar inclusions (In). Immediately adjacent on the left-hand side of the figure is a plasmacytoid lymphocyte with several fibrillar inclusions. × 15,700.
macrophages showed lysosomelike structures in which the granular electron-dense matrix exhibited an apparent transition to fibrillar structures (Fig. 10).

Spleen and liver. Inclusions similar to those described above were seen within plasma cells, histiocytes, and hepatocytes. However, because of considerable autolytic change the morphologic demonstration was less than satisfactory.

Kidney. In spite of considerable autolysis, multiple inclusion-containing plasma cells and plasmacytoid lymphocytes, occurring in sheets, were seen in the cortex. Identical inclusions were also seen in the proximal renal tubule cells, and these were often more electron dense than those seen in the plasma cells. However, under high magnification, a fibrillar structure could be identified.

Extracellular fibrils similar to those seen in the intracytoplasmic inclusions were seen within the wall of blood vessels. However, these fibrils were arranged in a less organized fashion.

DISCUSSION

This report describes a patient with kappa light-chain multiple myeloma and amyloidosis. On the basis of cytochemical, immunofluorescent, and electron microscopic studies, intracytoplasmic fibrillar inclusions having all the characteristics of amyloid were demonstrated in plasma cells, reticuloendothelial cells, hepatocytes, and renal tubule cells. The precise site(s) of formation of amyloid fibrils is controversial, and this morphologic study adds to our understanding of the possible locations of amyloid fibril formation.
Since amyloidosis is often associated with plasmacytosis and production of light chains, a relationship between plasma cells and production of amyloid seems logical. Other cells that have been reported to be associated with the formation of amyloid fibrils include fibroblasts,\textsuperscript{11,12} endothelial cells,\textsuperscript{13,14} and histiocytes.\textsuperscript{15,16} In addition to finding amyloid fibrils within plasma cells and histiocytes, we saw similar fibrils within renal tubule cells and hepatocytes.

Inclusions within the cytoplasm of proximal convoluted tubule cells that have been considered to be Bence-Jones proteins have been described in patients with multiple myeloma.\textsuperscript{17} Clyne et al.\textsuperscript{20} demonstrated that $80\%$ of human kappa light chains injected into rats were reabsorbed in the proximal tubules. It was therefore proposed that Bence-Jones proteins are filtered in the glomeruli and reabsorbed in the renal tubules where amyloid fibrils occasionally may be formed.

When amyloid involves the liver, it is usually present between the columns of liver cells and the sinusoidal wall in the space of Disse. Although the liver in this case showed significant autolytic change, making examination difficult, several intracellular fibrillar inclusions identical to those seen in plasma cells were demonstrated. It is possible, but unlikely, that these represent artifacts.

The fibrillar inclusions seen in this patient appeared to be intracellular and not, as some authors have indicated, indentations of the cell membrane by extracellular structures.\textsuperscript{21} The multiple inclusions occupied a major portion of the cytoplasm and we were unable to identify a continuity of intracellular fibrils with extracellular amyloid.

A variety of inclusion bodies have been described in plasma cells both in myeloma and in benign conditions.\textsuperscript{22} These have been described as dense bodies (Russell bodies) or crystalline structures. In most cases the inclusions have been within RER and have been thought to represent condensed or crystallized immunoglobulin. In the present case, most inclusions had a distinct fibrillar appearance, were located outside the RER, and were surrounded by a smooth membrane, and thus were not Russell bodies.

Investigators have questioned whether amyloid fibrils are formed intracellularly or if the cells in question produce a precursor which polymerizes after its secretion into the extracellular space. Zucker-Franklin and Franklin,\textsuperscript{10} using electron microscopy and fluorescent antibodies to amyloid fibrils, have shown evidence for intracellular localization of amyloid fibrils within plasma cells and histiocytes. They have postulated that amyloid or a precursor is elaborated through the RER and that polymerization of the fibrils takes place in the Golgi apparatus. Suzuki et al.\textsuperscript{23} using electron microscopy and peroxidase-labeled antibody in a patient with kappa light-chain myeloma, have demonstrated kappa light chains in the cisternae of RER but not in the Golgi zone.

Another theory of amyloid fibril formation is that proposed by Shirahama and Cohen.\textsuperscript{26,27} They suggest that amyloid fibril formation occurs in lysosomes. They have shown inclusions within histiocytes with transition from the dense body of a primary lysosome to less dense fibrillar inclusions, and finally, to smooth membrane-bounded amyloid fibrils. Similar observations were made in our case in histiocytes. This theory is supported by the finding that amyloid fibrils can be made in vitro by action of hydrolytic enzymes on some light
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chains, as well as with lysosome enzymes extracted from human kidneys. Ranlov and Wanstrup have suggested that amyloid fibrils are normally formed extracellularly, and that intracellular formation occurs only when the excretory mechanism has been exhausted.

From the data presented, we find evidence supporting formation of amyloid fibrils both in the Golgi apparatus and in lysosomes. One may question whether it is possible to deduce the precise location of amyloid fibril formation on the basis of morphologic findings in a dynamic situation. The light chains produced by plasma cells may have precipitated as amyloid fibrils in the extracellular space and then been phagocytosed by plasma cells, reticuloendothelial cells, and hepatocytes. This process might be possible with histiocytes, but is extremely unlikely with plasma cells and hepatocytes. The hepatocytes are not thought to be phagocytic and plasma cells are only weakly so. Furthermore, it seems unlikely that the cells involved could be capable of secreting such large bundles of fibrillar material. This material might attain an extracellular site through destruction of the involved cells.

It seems quite probable that several physical and chemical mechanisms operate in amyloid formation from immunoglobulin light chains. In this study, the presence of amyloid in plasma cells, reticuloendothelial cells, renal tubule cells, and hepatocytes indicated that many areas in the body may have the capacity to form amyloid fibrils. We also offer morphologic evidence to support the participation of the Golgi apparatus as well as lysosomal structures in the intracellular formation of amyloid.

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