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**Hiroyuki Morita · Toru Shinzato · Zhe Cai
Guido David · Akihiro Mizutani · Hiroko Habuchi
Masafumi Ito · Junpei Asai · Ken-ichi Isobe
Hideo Yamada · Kenji Maeda · Koji Kimata**

Basic fibroblast growth factor-heparan sulphate complex in the human dialysis-related amyloidosis

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Abstract A major constituent of the amyloid fibrils in dialysis-related amyloidosis is β_2-microglobulin (β_2-MG). Heparan sulphates (HS) co-localize with the amyloid fibrils and monocytes/macrophages are commonly found around amyloid deposits, but the role of HS in amyloidogenesis is not yet defined. HS have variable saccharide sequences and can interact specifically with basic fibroblast growth factor (bFGF), a potent chemotactic factor for the monocyte/macrophage. The present investigation was undertaken to look for a functional link between co-localized HS and the pathogenesis of dialysis-related amyloidosis. Using amyloid-enriched ligament, immunohistochemical localization was tested for β_2-MG, endogenous bFGF, and bFGF-binding portions of HS. For the detection of bFGF-binding portions of HS, the ligament sections were incubated with exogenous bFGF and then with anti-bFGF antibody. The specificity of the interaction between bFGF and HS was established by confirming a concomitant loss of immunoreactivity during selective removal of HS with heparitinase. β_2-MG, endogenous bFGF, and bFGF-binding portions of HS were detected between bundles of collagen. Endogenous bFGF and bFGF-binding portions of HS were not detected in more advanced amyloid lesions, whereas β_2-MG and other portions of HS were detected. We propose that β_2-MG, endogenous bFGF, and bFGF-binding portions of HS form a complex and localize in the early amyloid lesions of dialysis-related amyloidosis.

Key words Basic fibroblast growth factor · Heparan sulphate · Beta-2-microglobulin · Amyloidosis · Haemodialysis

H. Morita (✉) · T. Shinzato · Z. Cai · K. Maeda
Department of Internal Medicine,
Branch Hospital of Nagoya University, School of Medicine,
1-1-20 Daiko-Minami, Higashi-ku, Nagoya 461, Japan

M. Ito · J. Asai
First Department of Pathology, Nagoya University,
School of Medicine, Nagoya, Japan

A. Mizutani · H. Habuchi · K. Kimata
Institute for Molecular Science of Medicine,
Aichi Medical University, Nagakute, Japan

G. David
Center for Human Genetics, University of Leuven, Leuven,
Belgium

H. Morita · K. Isobe · H. Yamada
National Institute for Longevity Sciences, Obu, Japan

Introduction

Dialysis-related amyloidosis is one of the most common and serious complications of patients with chronic renal failure on long-term haemodialysis.. Biochemical and immunohistochemical studies have shown that a major constituent of amyloid fibres in dialysis-related amyloidosis is β_2-microglobulin (β_2-MG) [1, 2]. Several investigators have provided evidence that the structure of amyloidogenic β_2-MG is different from that of non-amyloidogenic β_2-MG; structural modifications include fragmentation [3, 4], substitution of an amino acid [5], and production of advanced glycation [6]. Apart from the importance of these molecular modifications, it is essential to study "common elements" that co-localize with amyloid fibres, because they are capable of altering local environments at a molecular level to play a part in amyloid deposition. Heparan sulphates (HS) are well-known common elements.

HS consist essentially of alterating D-glucuronate and *N*-acetyl-D-glucosamine units. They are modified through *N*-deacetylation/*N*-sulphation of the *N*-acetyl-D-glucosamine residues, C5 epimerization of D-gluconate (to L-iduronate), and *O*-sulphation at various positions [7]. Since these modifications are most often incomplete, HS have polymeric structures of enormous complexity. This property of HS appears to be involved in specific interactions with a variety of macromolecules that mediate biological activities [8]. An example is a specific binding of HS rich in iduronate ($2SO_4$)-*N*-sulphated-glucosamine [8–10] to basic fibroblast growth factor (bFGF).

Basic FGF is a 18 kDa polypeptide that mediates mitogenesis, differentiation and chemotaxis [11–13]. The interaction between bFGF and HS in the extracellular matrix stabilizes biological activities of bFGF by protecting it from proteolytic degradation and diffusion [8, 10]. Furthermore, the three-demensional structural changes of bFGF induced by HS binding have been suggested to be important for the activation of polypeptide receptor on the cell surface [14]. Thus, bFGF/HS complex may have a significant potential to change local environments and regulate amyloid formation in dialysis-related amyloidosis.

Attention paid to the studies on co-localization of glycosaminoglycans with amyloid fibrils seems to have been concentrated on identification of the types of glycosaminoglycans. Our knowledge of the distributional pattern of functional domains of HS in amyloid-enriched tissues is still incomplete. In the present study, we attempted to identify and localize bFGF-binding domains of HS and endogenous bFGF in amyloid-enriched carpal tunnel ligaments using immunohistochemical techniques. This approach may provide important clues as to the mechanism involved in amyloid formation and/or further progression of the deposition in dialysis-related amyloidosis.

Materials and methods

The present study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Carpal tunnel ligaments were obtained from 16 patients with chronic renal failure on maintenance haemodialysis at operative decompression for symptomatic carpal tunnel syndrome. Informed consent was obtained from each patient prior to his/her inclusion in the study. Samples were snap frozen in liquid nitrogen or fixed in 15% buffered formalin or Bouin's fixative (80% (v/v) ethanol saturated 2,4,6-trinitrophenol/15% (v/v) formalin/5% (v/v) acetic acid) for 24 h and embedded in paraffin.

For detection of amyloid deposits, the sections were stained by the Congo red method [15]. For immunohistochemistry, the streptavidin-biotin complex procedure was carried out. When peroxidase-conjugated streptavidin was used, the endogenous peroxidase was inactivated by exposing the sections to 0.3% H_2O_2 in absolute methanol prior to the application of the first antibodies and the final products were detected with 3,3'-diaminobenzidine. To explore the possibility of epitope masking, the sections were preincubated with or without trypsin. When necessary, the sections were treated with a mixture of heparitinase/heparinase or chondroitinase ABC (Seikagaku, Tokyo, Japan). Biotinylated antibodies against rat, mouse or rabbit immunoglobulins were used as second antibodies.

In situ detection of bFGF-binding domains of HS

HS interact specifically with bFGF and can act as a reservoir from which bFGF is released in response to triggering events. There is no antibody that recognizes the binding domains of HS. To detect it in situ, ligament sections were exposed to exogenous bFGF and then to a monoclonal antibody against bFGF designated as 05–118 [16]. Detailed procedures have been described elsewhere [17]. To establish the specificity of the interaction between exogenous bFGF and endogenous HS, some sections were digested with heparitinase before or after exposure to exogenous bFGF or incubated with an excess amount of HS prepared from human kidneys.

In situ detection of endogenous bFGF

Frozen sections embedded in OCT compound were used. Cryostat sections of 5 μm thickness were mounted on slides, air-dried and fixed in cold acetone for 5 min. A monoclonal antibody against bFGF (05–118) was used as the first antibody. The rest of the streptavidin-biotin complex immunohistochemical procedure was carried out as described above except that fluorescein isothiocyanate-conjugated streptavidin was used instead of peroxidase-conjugated streptavidin. To examine whether the endogenous bFGF is bound to HS in the ligaments, sections were treated with heparitinase before or after exposure to the first antibody.

Antibodies against other HS epitopes

Two different monoclonal antibodies against HS, designated as HK249 and 10E4, were used. They do not react with chondroitin sulphate or other forms of glycosaminoglycans. Although *N*-sulphate-enriched and *O*-sulphate-scarced portion of HS [18, 19] are involved in both epitopes, detailed structures seem to be different.

HK249 (IgM): Rats were immunized with a mixture of reduced and unreduced perlecan fractions prepared from Engelbreth-Holm-Swarm tumour (EHS tumour) [20] for production of this antibody. The spleen cells were harvested and fused with NS-1 mouse myeloma cells according to the standard procedure with minor modifications [20].

10E4 (IgM): Mice were immunized with liposome-incorporated membrane heparan sulphate proteoglycans and heparitinase-digested medium heparan sulphate proteoglycans for the production of this antibody [19]. Detailed protocols for hybridoma production and selection have been described elsewhere [21].

Results

In the present investigation, antibodies against various molecules were applied to both fresh frozen and paraffin-embedded sections. Except for the endogenous bFGF, the patterns of staining was similar.

In situ detection of β_2-MG

Beta-2-MG was immunohistologically detected where there was massive amyloid deposition (Fig. 1A). These sites were positive for Congo red staining (data not shown). β_2-MG was also detected at various sites that were not necessarily congophilic. Immunolocalization was summarized in the following way: (1) fibrous (and/or small nodular) localization between wavy bundles of collagen, (2) vascular localization (Fig. 1B), (3) localization in the vicinity of inflammatory cells (arrows in Fig. 1C), and (4) amorphous localization in the extravascular areas (arrowheads in Fig. 1C). Pretreatment with 7 M urea and other solutions did not alter the staining patterns. The above patterns of immunolocalization have been reported in principle by other investigators [2]. Although interrelations among the different forms of β_2-MG accumulation remain to be clarified, it is possible that some of them are consecutive lesions.

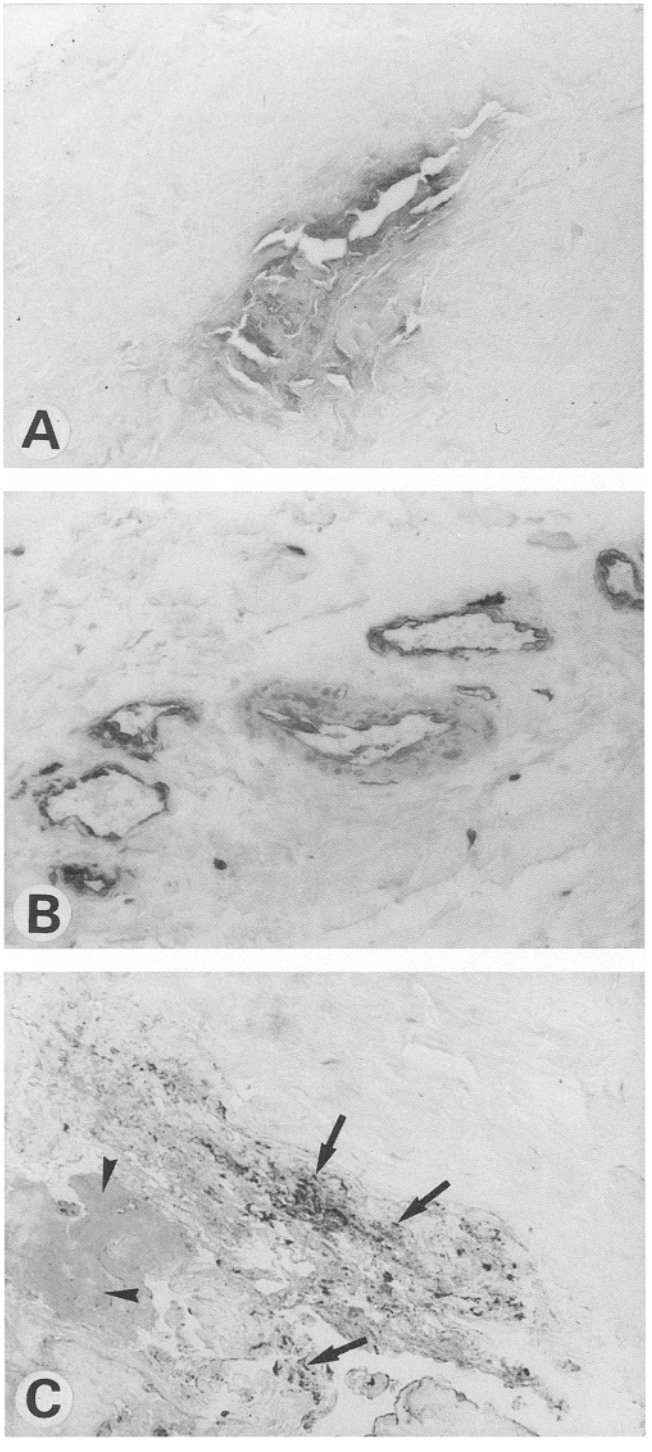

Fig. 1A–C Various forms of β_2-microglobulin (β_2-MG) expression in amyloid-enriched carpal tunnel ligament. Expression was determined by immunohistochemical examination of paraffin-embedded sections with anti-β_2-MG antibodies. **A** Nodular β_2-MG expression where massive accumulation is indicated. **B** β_2-MG expression in blood vessels. **C** β_2-MG expression around inflammatory and/or resident cells (*arrows*), and amorphous β_2-MG expression (*arrowheads*)

In situ detection of bFGF-binding domains of HS

Fibrous or slit-like immunostaining of β_2-MG was observed between wavy bundles of collagen in the amyloid-enriched ligament (Fig. 2A). This form of immunostaining was also observed when the ligament sections were exposed to exogenous bFGF and then to a monoclonal antibody directed against bFGF (Fig. 2B). Positive staining was not observed when the ligament section was digested with heparitinase prior to exposure to exogenous bFGF (Fig. 2C), excess amount of HS was added to a solution containing exogenous bFGF (Fig. 2D), or prior to exposure to anti-bFGF antibody (data not shown). We therefore thought that specificity of the interaction between exogenous bFGF and endogenous HS was confirmed.

In situ detection of endogenous bFGF

In paraffin-embedded ligament sections fixed in either 20% formalin or Bouin's fixative, endogenous bFGF was not detected with a monoclonal antibody directed against bFGF (data not shown). In frozen sections, it was detected with the same monoclonal antibody. Fibrous or slit-like immunostaining that was similar to that of bFGF-binding domains of HS was observed (Fig. 3A). Concomitant loss of immunostaining during selective removal of HS in the ligament sections with heparitinase indicated that endogenous bFGF interacted with endogenous HS (Fig. 3B). Summarizing, we propose that endogenous bFGF forms a complex with amyloid β_2-MG and bFGF-binding domains of HS that are localized fibrously between wavy bundles of collagen.

In situ detection of other portions of HS

N-Sulphate-enriched and *O*-sulphate-scarced portions of HS were detected with HK-249 and 10E4 antibodies. In the ligament sections, immuno-staining pattern with these antibodies was very similar. Massive accumulation of HS along collagen bundles was indicated (Fig. 4A). A similar form of β_2-MG immunostaining was observed (Fig. 4B), indicating that this form of amyloid accumulation occurs in dialysis-related amyloidosis. Interestingly, bFGF-binding portions of HS did not show this pattern.

Discussion

The present study has shown for the first time that bFGF-binding portions of HS, endogenous bFGF (the ligand for these portions of HS), and β_2-MG co-localize along the bundles of collagen in the amyloid-enriched carpal tunnel ligament. This might be an early form of amyloid deposition. Expression of bFGF-binding portions of HS was not detected in more advanced lesions. Instead, other HS portions were detected there.

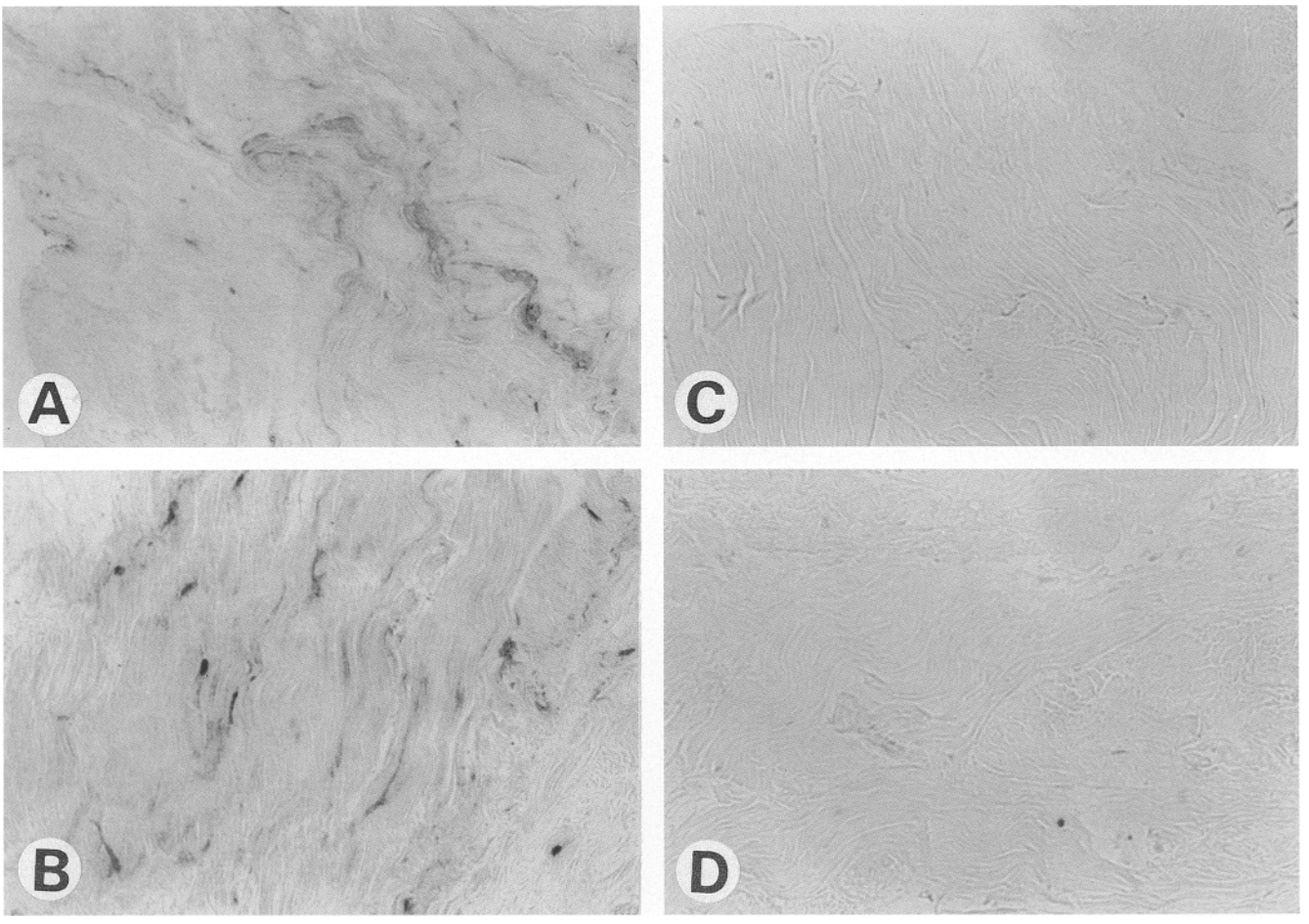

Fig. 2A–D Fibrous or slit-like expression of β_2-MG and basic fibroblast growth factor (bFGF)-binding domains of heparan sulphates (HS). **A** A part of amyloid-enriched ligament obtained from a patient on long-term haemodialysis shows fibrous immunolocalization of β_2-MG. **B** A ligament also shows fibrous immunolocalization when it was exposed to exogenous bFGF and then stained with anti-bFGF antibody. **C** Immunoreactivity of exogenous bFGF disappears following selective removal of HS with heparitinase. **D** Loss of immunoreactivity is also evident in the presence of excess HS added to a solution containing exogenous bFGF

Previous studies have demonstrated a close relationship between glycosaminoglycan and β_2-MG amyloid deposition. Aruga et al. [22] detected HS epitopes that were closely related to the *O*-sulphated and *N*-acetylated glucosamine linked to glucuronic acid in amyloid-enriched carpal synovium and showed that the epitopes were localized at sites of β_2-MG accumulation. We showed *N*-sulphate-enriched and *O*-sulphate-scarced portions of HS in the advanced lesions of amyloid-enriched carpal tunnel ligament. Although the functions of the HS epitopes that the above antibodies recognize remain to be clarified, these observations correspond to several reports indicating that HS co-localize with amyloid deposits [23]. There are other reports, however, indicating that dialysis-related amyloidosis is unique in that chondroitin sulphate and/or hyaluronate are the major glycosaminoglycan forms. Ohishi et al. [24] demonstrated, in their analysis of amyloid-enriched preparation from carpal synovium, that predominant types of glycosaminoglycans were chondroitin sulphate and hyaluronate. Glycosaminoglycans are anionic carbohydrates capable of altering local environments either by influencing the manner in which the amyloidogenic molecules fold and interact with each other to form β-pleated sheets or by contributing to the stability of amyloid fibres in tissues by protecting them from proteolytic degradation. Glycosaminoglycans might also influence amyloid fibres to deposit at specific sites in tissues. Although preferential co-localization of monocytes and macrophages was often observed histologically in the vicinity of amyloid deposits [25] in dialysis-related amyloidosis, none of the above hypotheses provides a satisfactory explanation for this phenomenon.

Three principle roles for the macrophage in the amyloidogenic process of dialysis-related amyloidosis have been suggested. Firstly, macrophages are capable of secreting tissue-degradating cytokines such as tumour necrosis factor-α [26], interleukin-1β [26], and interleukin-6 [27] as well as proteolytic enzymes such as collagenase [26]. Secondly, macrophages can secrete amyloid enhancing factor [28]. Thirdly, macrophages might supply the precursor proteins of the amyloid [25]. All of the

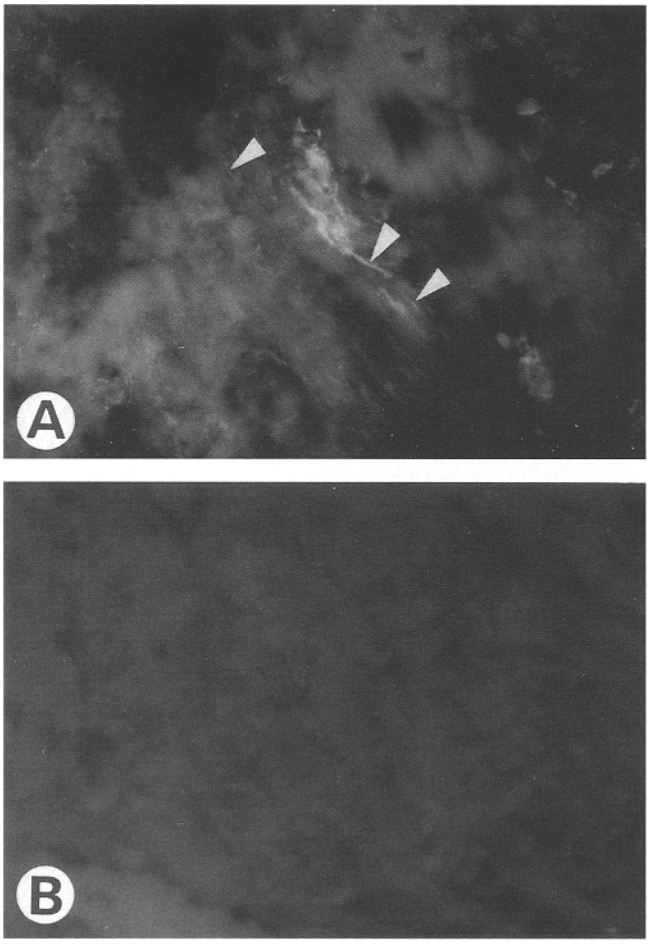

Fig. 3A, B Fibrous or slit-like expression of endogenous bFGF bound to endogenous HS. Cryostat secions of a ligament were immunostained as described in the Methods section. **A** Endogenous bFGF was demonstrated in fibrous form (*arrowheads*). **B** In contrast, immunoreactivity disappeared following selective removal of HS with heparitinase

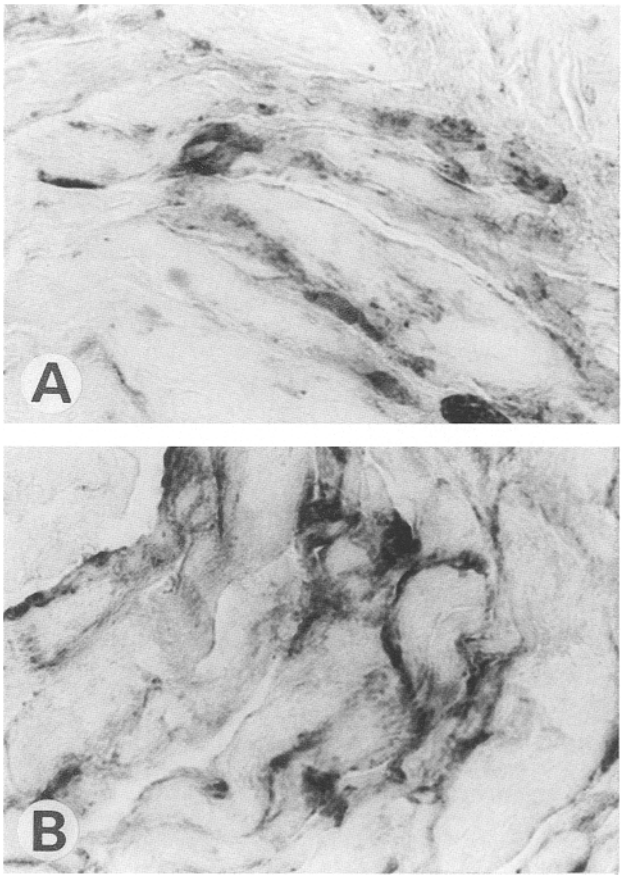

Fig. 4A, B Expression of β_2-MG and other domains of HS in more advanced amyloid lesions. Immunohistochemical examination of (**A**) *N*-sulphate-enriched and *O*-sulphate-scarced portions of HS and (**B**) β_2-MG shows extracellular areas along bundles of collagen

assumed mechanisms may act in concert to accelerate β_2-MG amyloid formation and/or deposition in dialysis-related amyloidosis.

In conclusion, we propose the hypothesis that bFGF-heparan sulphate complex that co-localizes in the early amyloid lesions might function as a chemotactic and/or growth factor for the monocyte/macrophage. However, one should refrain from speculating too much on the potential effects of bFGF in the β_2-MG amyloid-enriched tissues. Although bFGF and interleukin-1 are examples of the cytokines demonstrated immunohistochemically in these tissues [29], the monocyte/macrophage can secrete a rich variety of cytokines. At this point we do not know which of these cytokines are responsible for transendothelial chemotaxis, differentiation, proliferation, and secretion of proteases in the β_2-MG amyloid-enriched tissues. Furthermore, one cannot exclude the possibility that the pathogenesis of β_2-MG amyloidosis is multifactorial [25, 30]. Clearly, the absence of bFGF-heparan sulphate complex in advanced lesions indicates the involvement of different mechanisms in the further progression of amyloid deposition. Nevertheless, it may well be that bFGF bound specifically to bFGF-binding portions of HS contributes to some parts of the pathogenesis of β_2-MG amyloidosis in its early stages, since bFGF has become much less susceptible to proteolytic degradation and much more effective for the activation of intra-cytoplasmic signal transduction than intact bFGF [8, 10, 14]. Finally, further immunohistochemical analysis, in situ hybridization, and in vitro studies (e.g. using Boyden chambers) should be carried out to clarify the cytokine network and elucidate the involvement of each cytokine in the pathogenesis of dialysis-related amyloidosis.

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