FEBS Letters 351 (1994) 85–89

FEBS 14431

Catabolism of intact fibrillin microfibrils by neutrophil elastase, chymotrypsin and trypsin

Cay M. Kielty[a,*], David E. Woolley[b], Stephen P. Whittaker[a], C. Adrian Shuttleworth[a]

[a]*School of Biological Sciences, 2.205 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, UK*
[b]*Department of Medicine, University Hospital of South Manchester, Manchester M20 8LR, UK*

Received 12 July 1994

Abstract

We present ultrastructural and biochemical evidence for the turnover of intact fibrillin microfibrils by the serine proteinases, neutrophil elastase, chymotrypsin and trypsin. Rotary shadowing electron microscopy revealed that serine proteinase treatment of intact microfibrils isolated from foetal bovine skin resulted in extensive degradation. Microfibrils were destroyed by neutrophil elastase and effectively disrupted by chymotrypsin and trypsin, with no morphologically identifiable arrays remaining. Evidence of defined fibrillin degradation products was obtained by Western blotting of these enzyme-treated fibrillin assemblies. Fibrillin immunoprecipitated from dermal fibroblast culture medium was also comprehensively degraded by these enzymes. These observations demonstrate that serine proteinases are potent effectors for the physiological and pathological catabolism of microfibrils, and suggest a key role in elastic fibre degradation.

Key words: Fibrillin microfibril; Catabolism; Elastase; Serine proteinase

1. Introduction

The glycoprotein fibrillin is the major component of a class of extracellular microfibrils that are key determinants of connective tissue architecture and integrity [1–3]. Characteristically these microfibrils have an average 55 nm beaded periodicity and a diameter of 10–12 nm [4–6]. The importance of these microfibrils was recently brought into close focus by the linkage of fibrillin genes on chromosomes 15 and 5 to the heritable connective tissue diseases Marfan syndrome (MFS), ectopia lentis (EL) and congenital contractural arachnodactyly (CCA) [7–9].

Fibrillin microfibrils have a widespread distribution in connective tissues and are particularly abundant in elastic tissues such as aorta, ligament and skin where they form integral components of elastic fibres [1,10]. Ultrastructurally indistinguishable microfibrils are also present in many non-elastic tissues such as ocular zonular fibres, tendon and bone [4,5]. At present, the assumption is that fibrillin is the major component of all morphologically identical microfibrils in both elastic and non-elastic tissues.

Elastic fibres are deposited in tissues in a variety of structural forms [10]. Studies on the early stages of elastic fibrillogenesis have all indicated that the bundles of microfibrils which appear first within the matrix apparently define the development and organisation of elastic fibres [1]. They are retained in mature elastic fibres in the form of a peripheral mantle surrounding the amorphous elastin core.

Whilst details of microfibrillar involvement in elastin deposition are beginning to be unravelled, there is a paucity of information about the fates of both components in the extracellular matrix. In normal circumstances, elastin has an exceptionally long biological half-life, and it appears that elastic fibres are likely to function for exceedingly long periods. The physiological mechanisms of elastic fibre degradation is not well defined. In some pathological circumstances, such as pulmonary emphysema, more rapid breakdown is seen, but it is not clear if this occurs by upregulation of the mechanisms involved in normal turnover, or by additional factors. The principal enzyme involved in elastin turnover appears to be elastase, a serine proteinase secreted by neutrophils [11]. In addition, the matrix metalloproteinases (MMPs), stromelysin and gelatinase [12,13], produced by a variety of cell types, and the cysteine proteinase, cathepsin L [14,15], have all been implicated in elastin degradation.

Further information about the enzyme susceptibility of elastic fibres came when attempts were made to isolate and separate microfibrillar proteins from elastin [16]. It was clear that both components were resistant to bacterial collagenase digestion, but when tissues were treated with a range of enzymes it appeared that elastin-associated microfibrils and amorphous elastic tissue had different susceptibilities to enzymic digestion. Microfibrils could no longer be demonstrated after the tissue was digested with trypsin and chymotrypsin, although they appeared to be unaffected by elastase. In contrast, elastin was degraded by elastase but unaffected by trypsin and chymotrypsin [1,16].

*Corresponding author. Fax: (44) (61) 275-5082.

SSDI 0014-5793(94)00818-3

The ability to isolate intact microfibrillar assemblies [6,17] has allowed us to examine in more detail the susceptibility of elastic microfibrils to serine proteases. This report provides information about the action of neutrophil elastase, trypsin and chymotrypsin on intact microfibrillar assemblies, and implicates elastase as a key enzyme in the turnover of the fibrillin microfibrils.

2. Materials and methods

Second trimester foetal calves were obtained from the local abattoir within 1 h of maternal death. Human neutrophil elastase was purified to homogeneity as previously described [18]. Bacterial collagenase (type 1A), chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), pancreatic elastase (EC 3.4.21.7), phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), *p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), diaminobenzamidine, dithiothreitol, gelatine, Tween 20, peroxidase-conjugated goat anti-rabbit IgG and prestained molecular weight markers were obtained from the Sigma Chemical Co., Poole Dorset, UK. CNBr-activated Sepharose CL-4B, protein A-Sepharose, and Sepharose CL-2B were supplied by Pharmacia-LKB, Milton Keynes, Bucks, UK. Enhanced chemiluminescence kits were supplied by Amersham International, Bucks, UK.

Intact fibrillin microfibrils were isolated from foetal bovine skin as previously described [6,17]. This procedure involves disruption of tissue with mild bacterial collagenase digestion and isolation of high-M_r microfibrils by gel filtration chromatography on Sepharose CL-2B.

Enzyme: microfibril incubations were carried out in 0.05 M Tris-HCl, pH 7.4 containing 0.4 M NaCl and 0.01 M $CaCl_2$ for 1 h, 2 h and 16 h at 22°C and 37°C. Microfibril preparations (diluted to a final concentration of 200 μg/ml) were incubated with 10 μg/ml, trypsin containing 1 μg/ml TPCK, 10 μg/ml chymotrypsin containing 1 μg/ml TLCK, or with 4 μg neutrophil elastase in a final volume of 100 μl. Enzyme reactions were stopped with PMSF (final concentration 2 mM). Aliquots (10 μl) were removed for ultrastructural analysis. Control and enzyme-treated microfibril preparations were analysed ultrastructurally by rotary shadowing electron microscopy as previously described [6,17]. The remainder of each treated aliquot was electrophoresed on 8% SDS-PAGE gels under non-reducing conditions prior to Western blotting using a polyclonal anti-fibrillin [17,19–21]. The primary antiserum and a peroxidase-conjugated goat anti-rabbit IgG secondary antibody were used at 1:10,000 dilutions and detection was by enhanced chemiluminescence.

Dermal fibroblasts (passage 4) were maintained in Dulbecco's minimum essential medium supplemented with 10% foetal calf serum, penicillin (400 U/ml), streptomycin (50 mg/ml), and glutamine (200 mg/ml). Confluent cells were labelled with [^{35}S]TranSlabel in medium containing 0.5% foetal calf serum. Fibrillin was immunoprecipitated from medium as previously described [19]. In view of the similar electrophoretic mobilities on SDS-PAGE of fibronectin and fibrillin, fibronectin was removed before immunoprecipitation of fibrillin by two sequential incubations of 100 μl 1:1 (v/v) solution of gelatin-Sepharose. Samples were then incubated for 1 h at 20°C with a 1:100 dilution of polyclonal anti-fibrillin serum [17,19–21] before the addition of 60 μl of a 1:1 solution of protein A-Sepharose in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl and 1% (v/v) Nonidet P40. Immunoprecipitates were incubated for 16 h at 37°C in the presence or absence of 10 μg/ml trypsin, chymotrypsin, or elastase, and then analysed directly by electrophoresis on 8% SDS-PAGE gels under non-reducing conditions, and fluorography.

3. Results

3.1. Ultrastructural analysis

Microfibrils incubated for 16 h at 22°C and 37°C remained intact, with abundant extensive fibrillin microfibrillar arrays clearly evident (Fig. 1A). Intact fibrillin microfibrils in solution were subjected to treatment at 22°C and 37°C with neutrophil elastase, chymotrypsin and trypsin for 1 h, 2 h, 6 h and 16 h. Rotary shadowing electron microscopy demonstrated conclusively that all three enzymes were capable of degrading intact fibrillin microfibrils (Fig. 1). Elastase proved particularly disruptive to mcirofibrils. Although microfibrils were detected after 1 h and 2 h incubations with elastase at 37°C, it was clear that substantial disruption had already occurred (Fig. 1B). After short treatment at 37°C, degradation was more advanced than at 22°C. After 16 h at 22°C or 37°C, few morphologically identifiable microfibrils remained, although extensive intact collagen fibres and type VI collagen microfibrils were evident (Fig. 1C,D). Such incubations highlighted the susceptibility of the interbeaded regions to cleavage.

Microfibrillar structure was also profoundly disrupted by chymotrypsin and trypsin (Fig. 1E–H). The disruptive effects of these enzymes was already apparent on examination of preparations after 2 h enzyme incubations at 37°C (Fig. 1E,G). After 16 h at 37°C, very few short assemblies were still detectable within the treated samples (Fig. 1F,H).

3.2. Biochemical analysis of effects of enzyme treatments

The process of fibrillin degradation was investigated by Western blotting of high-M_r assemblies from foetal bovine skin treated with elastase, trypsin or chymotrypsin in time course experiments (Fig. 2). This approach highlighted the occurrence of several defined intermediates of fibrillin degradation in the M_r range 40–65 kDa as well as small fragments which migrated at or near the dye front.

Immunoprecipitated fibrillin from dermal fibroblast culture medium was treated at 37°C with elastase, chymotrypsin and trypsin for 16 h and then analysed by SDS-PAGE and fluorography (Fig. 3). Intact fibrillin monomers (M_r 300,000) had been comprehensively degraded by all three enzymes, and after 16 h no intact fibrillin remained. Enhanced levels of a smaller fibrillin-immunoreactive component (M_r 55,000) apparent in the undigested control, were detected in the trypsin- and chymotrypsin-treated immunoprecipitates, but this polypeptide was degraded by elastase. In addition, small fragments migrating with the dye front were detected in the three enzyme treated samples.

4. Discussion

Fibrillin microfibrils are essential structural components of connective tissues, and play a key role in maintaining tissue integrity and functionality. Despite their importance in matrix organisation, nothing is known to date about microfibril turnover in vivo. We present the

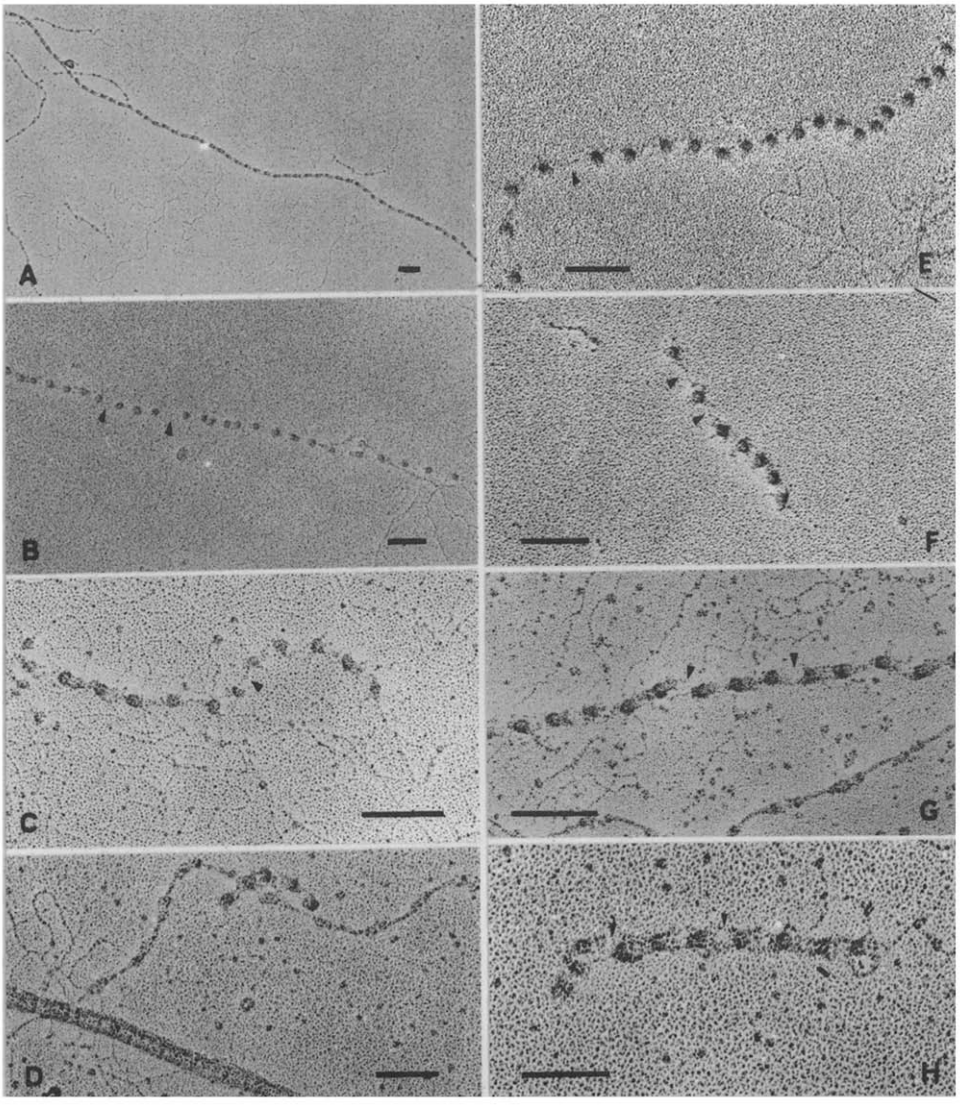

Fig. 1. Electron micrographs after rotary shadowing of fibrillin microfibrils isolated from foetal bovine skin [6,17]. Fibrillin assemblies were clearly recognised by their distinctive beaded appearance. Microfibril preparations were treated with neutrophil elastase (B,C) or pancreatic elastase (D), trypsin (E,F) and chymotrypsin (G,H) for 2 h or 16 h at 37°C. Arrows highlight disrupted interbeaded domains. The micrographs shown are representative of all fields examined. Bars = 100 nm.

first evidence that intact microfibrils are effectively catabolised in vitro by the serine proteases neutrophil elastase, chymotrypsin and trypsin.

Neutrophil elastase is a serine proteinase of the primary (azurophilic) granules of neutrophils. It is released upon activation and is purported to be essential for migration of neutrophils through connective tissues [22]. It is a major degradative enzyme of neutrophils and is known to compromise the integrity of the extracellular matrix especially at sites of ulceration or inflammation.

Tissue integrity is dependent upon an appropriate balance between proteolytic activities required for normal tissue remodelling, and their inhibitors. The array of matrix-degrading proteinases and inhibitors is extensive. The most abundant plasma serine protease inhibitor is α_1-antitrypsin [23], and it is interesting to note that α_1-antitrypsin deficiency is associated with abnormally rapid degradation of lung elastin leading to emphysema [24]. It has recently been shown that thrombospondin is a potent inhibitor of neutrophil elastase, and may serve as a regulator of enzyme activity in localised inflammatory reactions [25]. Inappropriate control of elastase activity is believed to be integral to the pathogenesis of pulmonary emphysema, acute respiratory disease, rheumatoid arthritis, Crohn's disease and other inflammatory conditions [22].

The observation that fibrillin microfibrils and monomers are degraded by neutrophil elastase has major implications for elastic fibre stability, function and turnover in physiological and pathological conditions. It is not clear whether the elastase-susceptible immunoreactive

M_r 55,000 component represents a stable product of normal fibrillin turnover or a microfibril-associated molecule. In inflammatory conditions where high levels of neutrophil elastase activity are found, fibrillin microfibrils which form the outer mantle of elastic fibres are likely to be rapidly removed, thereby exposing the elastin core to the further degradative effects of neutrophil elastase. Such a sequence of events possibly underlies the extensive elastic fibre damage classically associated with many inflammatory conditions.

The demonstration that three serine proteinases are capable of degrading fibrillin microfibrils suggests a general susceptibility to this class of enzyme. In addition to degrading defined elements of the matrix, the serine proteases are also implicated as key components of an interdependent mechanism leading to activation of the matrix metalloproteinase precursors [26]. The presence of neutrophils, macrophages and mast cells in early inflammatory lesions provides a source of serine proteinases necessary for the breakdown of fibrillin and elastin, as well as contributing to the activation of metalloproteinases involved in the degradation of other matrix components.

Acknowledgements: This work was supported by the Medical Research Council.

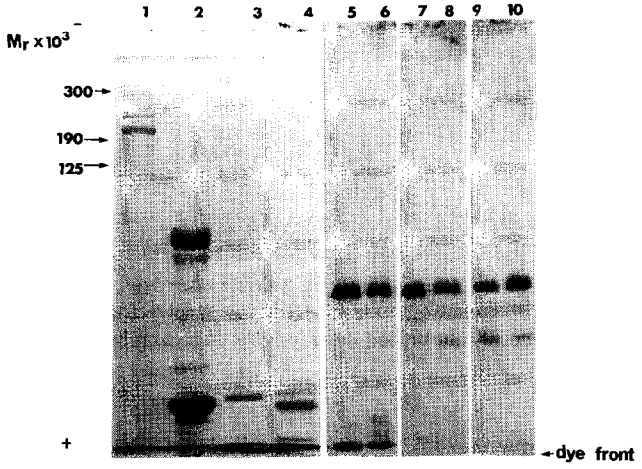

Fig. 2. Western blotting of enzyme-treated fibrillin assemblies isolated from foetal bovine skin. High-M_r assemblies were incubated at 37°C for 2 h or 6 h in the presence or absence of elastase, chymotrypsin or trypsin, prior to electrophoresis on 8% SDS-PAGE gels under non-reducing conditions. Western blotting was carried out using a polyclonal anti-fibrillin serum [17,19–21] and detection was by enhanced chemiluminescence. The electrophoretic mobilities of molecular weight markers α_2-macroglobulin (M_r 190,000) and β-galactosidase (M_r 125,000) are indicated. Tracks 1–4 are stained with Coomassie brilliant blue; tracks 5–10 are Western blots. Track 1, untreated high-M_r fraction isolated from foetal bovine skin after bacterial collagenase digestion and size-fractionation. Fibrillin is present within this fraction. Tracks 2–4 contain the enzymes used in the digestions (track 3, elastase; track 4, chymotrypsin; track 5, trypsin). Tracks 5, 7 and 9 are 2 h digestions and tracks 6, 8 and 10 are 6 h digestions. Tracks 5 and 6, elastase-treated; tracks 7 and 8, chymotrypsin-treated; tracks 9 and 10, trypsin-treated. Defined fibrillin degradation products of all three enzymes were detected.

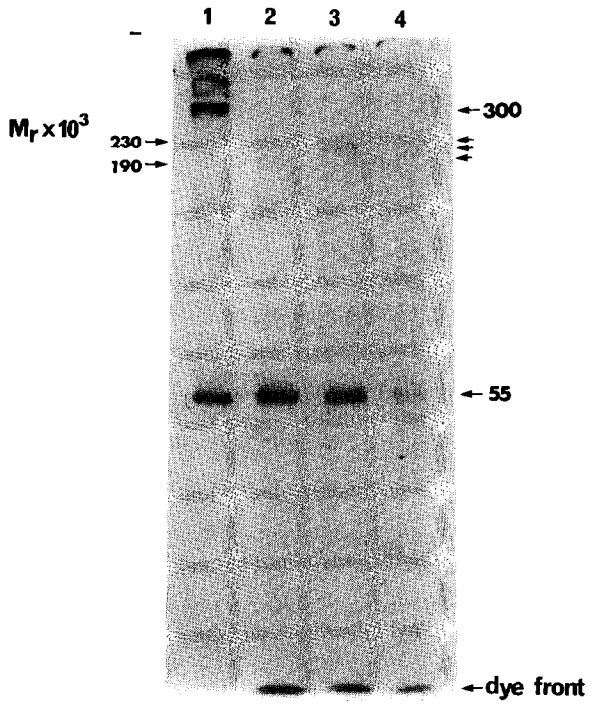

Fig. 3. Electrophoretic analysis of fibrillin immunoprecipitated from medium of normal human dermal fibroblasts. Cells were labelled with [^{35}S]TranSlabel for 16 h. Immunoprecipitates were incubated at 37°C overnight in the presence or absence of trypsin (10 μg/ml), chymotrypsin (10 μg/ml) or elastase (10 μg/ml). Samples were analysed by SDS-PAGE on 8% gels under non-reducing conditions and by fluorography. The electrophoretic mobilities of molecular weight markers catalase (M_r 232,000) and α_2-macroglobulin (M_r 190,000) are indicated. Track 1, untreated control; track 2, trypsin-treated; track 3, chymotrypsin-treated; track 4, elastase-treated. Arrows highlight degradation products of trypsin and chymotrypsin. An M_r 55,000 fibrillin-immunoreactive component was present in untreated immunoprecipitates and in increased amounts after trypsin and chymotrypsin treatment, but was clearly degraded by elastase. Low-M_r fragments migrating with the dye front were also apparent in all the enzyme-treated samples.

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