Assessment of procollagen processing defects by fibroblasts cultured in the presence of dextran sulphate

John F. BATEMAN* and Suzanne B. GOLUB

University of Melbourne, Department of Paediatrics, Royal Children's Hospital, Parkville, Vic. 3052, Australia

The culture of skin fibroblasts in the presence of 0.01 % (w/v) dextran sulphate results in complete proteolytic processing of procollagen to collagen. Processing occurs predominantly via a pN-collagen intermediate, suggesting that C-propeptide cleavage occurs early during the processing pathway. The processed collagen is associated with the cell-layer fraction. This method of inducing procollagen processing was evaluated for use in detecting procollagen processing abnormalities in heritable connective-tissue diseases. Abnormal type I procollagen processing was clearly demonstrated in two cases with known defects of pN-propeptide cleavage. In one, the cleavage deficiency was due to diminished N-proteinase activity (dermatosparaxis) and in the other case (Ehler's–Danlos syndrome type VIIA) the cleavage site was deleted. In a case of osteogenesis imperfecta (type II) the slow electrophoretic migration of type I collagen α -chains due to over-modification of lysine was readily demonstrated. Inefficient procollagen processing was also evident in this patient, as had been previously reported [de Wet, Pihlanjaniemi, Myers, Kelly & Prockop (1983) *J. Biol. Chem.* **258**, 7721–7728]. Thus this method of culture in the presence of dextran sulphate provides a simple and rapid procedure for the detection of procollagen processing defects and electrophoretic abnormalities.

INTRODUCTION

The incorporation of types I and III collagen molecules into fibrils within the extracellular matrix requires the proteolytic processing of the procollagen precursors. This processing involves at least two specific proteinases, which cleave the N-propeptide and C-propeptide regions. The cleavages occur at the junction of the short non-helical telopeptide sequences with the propeptide domain, thus leaving the intact telopeptides at the N- and C-termini of the α -chain helix to fulfil the critical role of inter- and intra-molecular cross-linking [see reviews by Bornstein & Traub (1979) and Peltonen *et al.* (1985)].

The sequence of cleavage of type I procollagen has not been established, with reports of processing via initial N-propeptide removal (pC-collagen) (Fessler *et al.*, 1975; Davidson *et al.*, 1977; Limeback & Sodek, 1979; Leung *et al.*, 1979; Gerstenfeld *et al.*, 1984) or C-propeptide removal (pN-collagen) (Veis *et al.*, 1973; Fleischmajer *et al.*, 1981, 1983; Helseth & Veis, 1984). Fibroblast culture experiments have generally supported a pC-collagen processing intermediate. However, the results from these cell-culture studies are difficult to relate to the process occurring *in vivo* because of the relatively small amount of complete processing of type I and III procollagen that occurs (Goldberg & Sherr, 1973; Layman & Ross, 1973; Taubman & Goldberg, 1976; Limeback & Sodek, 1979).

Attempts have been made to induce full processing in cell culture, so that the order of propeptide processing could be defined in normal cells and in cells from patients with heritable connective-tissue diseases such as Ehler's–Danlos syndrome (EDS) and osteogenesis imperfecta (OI). Minor *et al.* (1986) used long-term culture of fibroblasts in the presence of ascorbate to increase procollagen processing *in vitro*. They identified apparent processing defects in cases of OI and EDS. However, even with these modified culture conditions processing was incomplete, as most of the procollagen was processed only to pC α 1(I)- and pC α 2(I)-chains and not fully processed to α 1(I)- and α 2(I)-chains as happens rapidly *in vivo*.

The addition of neutral polymers to cell-culture medium induces the complete processing of type I procollagen to collagen (Bateman *et al.*, 1986a). In most experiments this processing involved the normal N-proteinase and C-proteinase, but in some human fibroblast cultures a cell-layer-associated telopeptide-cleaving activity was identified that could also remove the propeptides (Bateman *et al.*, 1987). Although telopeptide cleavage could be overcome with inhibitors and pH adjustment, it would be desirable to have a culture system that would consistently allow correct processing without these inhibitors, which may also alter normal procollagen processing.

In the present study the use of dextran sulphate to induce processing is assessed. The accuracy and route of propeptide cleavage is determined and the method is evaluated for use in determining procollagen-cleavage abnormalities in heritable connective-tissue diseases.

EXPERIMENTAL

Materials

Dextran sulphate (sodium salt, M_r 500 000) was purchased from Pharmacia, Uppsala, Sweden. L-[5- 3 H]Proline (30 mCi/mmol) and L-[14 C]proline (285 mCi/mmol) were obtained from Amersham Australia Pty., Sydney, N.S.W., Australia. Other chemicals used were commercially available analytical-grade reagents. Cell-culture media, including those containing dextran sulphate, were sterilized by filtration before use.

Dermal fibroblast culture and biosynthetic labelling

Control dermal fibroblast cultures were established from skin biopsies taken at autopsy of adults and children who had died of diseases not affecting the connective tissues. The biopsies were obtained with the approval of the Ethics Committee of this hospital. Fibroblasts were also grown from biopsies obtained from a patient with Ehler's–Danlos syndrome (EDS) type VIIA (Cole *et al.*, 1986) and from a case of lethal perinatal osteogenesis

Abbreviations used: N-propeptide, N-terminal propeptide; C-propeptide, C-terminal propeptide; pN-collagen, processing intermediate in the conversion of procollagen into collagen that contains the N-propeptide but not the C-propeptide; pC-collagen, intermediate that contains the C-propeptide but not the N-propeptide; OI, osteogenesis imperfecta; EDS, Ehler's–Danlos syndrome.

* To whom correspondence should be addressed.

imperfecta (OI) (I.M.R. 2962; Institute of Medical Research, Camden, NJ, U.S.A.). Sheep dermal fibroblasts established from a 2-year-old Merino ewe with a mild form of dermatosparaxis (Ramshaw, 1984) and from two age-matched control animals were generously supplied by Dr. J. A. M. Ramshaw (Commonwealth Scientific and Industrial Research Organization, Division of Biotechnology, Melbourne, Vic., Australia).

Cell cultures between passages 4 and 10 were grown in 60 cm² culture dishes and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-calf serum, 2 mM-L-glutamine and buffered with 20 mM-Hepes/22-NaHCO₃, pH 7.3, as previously described (Bateman *et al.*, 1984). At 2–4 days post-confluency, growth medium was replaced with 5 ml of fresh medium containing 0.15 mM-sodium ascorbate and 10% (v/v) dialysed foetal-calf serum either with or without 0.01% (w/v) dextran sulphate. After preincubation for 4 h, 50 μ Ci of L-[5-³H]proline was added and the incubation was continued for 18 h (Bateman *et al.*, 1984, 1986a). In some experiments 50 mM-L-arginine was included in the labelling medium to inhibit C-propeptide cleavage (Leung *et al.*, 1979). For pulse-chase experiments cultures were labelled for 30 min in 3 ml of medium containing 10% (v/v) dialysed foetal-calf serum, 0.15 mM-sodium ascorbate, 0.1 mM-CaCl₂, 0.2 mM- β -aminopropionitrile and 15 μ Ci of L-[U-¹⁴C]proline either with or without 0.01% (w/v) dextran sulphate. After the incubation the labelling medium was removed and replaced with fresh chase medium pre-warmed to 37 °C containing no isotopic label but supplemented with 50 mM-L-proline (Bateman *et al.*, 1986a). Under these conditions no further incorporation of [¹⁴C]proline into protein occurred. Incubation with the chase medium was performed for 0.5, 0.75, 1, 1.5, 1.75, 2, 3, 6 and 24 h.

The cell-layer and medium fractions of the labelled cultures were analysed separately as previously described (Bateman *et al.*, 1984, 1986a,b). Medium was removed and the proteinase inhibitors 0.1 mM-phenylmethanesulphonyl fluoride, 10 mM-N-ethylmaleimide and 25 mM-EDTA were added to prevent procollagen degradation during isolation. The cell layer was scraped into 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.5, containing the proteinase inhibitors and sonicated. Procollagen and collagens were precipitated by 25%-saturated (NH₄)₂SO₄. The precipitates were redissolved in 0.15 M-NaCl/1 mM-EDTA/50 mM-Tris/HCl buffer, pH 7.5, and portions were quantitatively loaded on to polyacrylamide gels for electrophoretic analysis.

SDS/PAGE

Samples were prepared and analysed by gel electrophoresis as described previously (Laemmli, 1970; Bateman *et al.*, 1984, 1986a). Procollagen and collagen chains were separated by electrophoresis on 5% (w/v) polyacrylamide gels after reduction with 10 mM-dithiothreitol. The procollagen pro α 1(III)-, pro α 1(I)- and pro α 2(I)-chains and the various processing intermediates pC α 1(I)-, pC α 2(I)-, pC α 1(III)-, pN α 1(I)- and pN α 2(I)-chains and the α 1(I)- and α 2(I)-chains were positively identified by Western blotting and two-dimensional CNBr-cleavage peptide mapping (results not shown). The migration positions of pN α 1(I)- and pN α 2(I)-chains were also confirmed by comparison of the migration of these components electrophoresed under non-reducing and reducing conditions (results not shown).

Radioactively labelled collagen bands were detected by fluorography (Bonner & Laskey, 1974). The individual procollagen and collagen species were quantified by careful excision of the bands from the dried gels, hydrolysis on Protosol (New England Nuclear, Boston, MA, U.S.A.) and scintillation counting (Bateman *et al.*, 1988). For pulse-chase experiments the individual procollagen processing intermediates were quantified

by densitometry of the pre-flashed fluorograms (Laskey & Mills, 1975).

RESULTS AND DISCUSSION

In cell-culture experiments, ample evidence has been obtained that newly synthesized type I and type III procollagens are efficiently and rapidly secreted into the surrounding cell-culture medium, where they largely remain as procollagen or as partially processed pC-collagen or pN-collagen intermediates (Goldberg & Sherr, 1973; Layman & Ross, 1973; Taubman & Goldberg, 1976; Limeback & Sodek, 1979). Only a small fraction of the type I collagen is fully processed to collagen α 1(I)- and α 2(I)-chains (Fig. 1a, lanes 1 and 2). However, when the charged polymer dextran sulphate was added to the culture medium during the labelling period at a concentration of 0.01% (w/v) the procollagens were completely processed to collagen, which accumulated in the cell-layer fraction (Fig. 1a, lanes 3 and 4). A range of dextran sulphate concentrations was examined for this effect, and, although processing occurred at concentrations as low as 0.005% (w/v), most efficient processing occurred at 0.01–0.05% (w/v). The processing of procollagen and deposition of collagen in the cell layer induced by dextran sulphate was similar to that induced by neutral uncharged polymers such as poly(ethylene glycol), polyvinylpyrrolidone, Ficoll and dextran (Bateman *et al.*, 1986a). The concentrations of these neutral polymers that were required were much higher, in the 2–5% (w/v) range, and this was also the concentration range of these polymers necessary to precipitate type I collagen and procollagen from fibroblast culture medium *in vitro* (Ramshaw *et al.*, 1984). This suggests that the molecular mechanism may involve the retention of the procollagen in the pericellular environment and consequent exposure to the N-proteinase and C-proteinase.

The presence of dimeric β -components in collagen processed in the presence of dextran sulphate (Fig. 1a, lane 3) suggested that the short telopeptide regions adjoining the propeptide were intact, as they contain the major cross-linking sites (Eyre *et al.*, 1984). Furthermore, limited pepsin digestion of the processed collagen resulted in increased electrophoretic mobility (Fig. 1b, lane 2), providing additional evidence for the presence of the proteolytically sensitive non-helical telopeptide regions. CNBr-cleavage peptide mapping experiments confirmed the presence of C-terminal telopeptide sequences (results not shown).

In the presence of 50 mM-arginine, a specific inhibitor of the C-proteinase (Leung *et al.*, 1979), dextran sulphate-induced processing was inhibited, with an accumulation of the intact pC α 1(I)- and pC α 2(I)-chains (Fig. 1c, lane 2). This provided strong evidence that C-propeptide cleavage was achieved by the specific C-proteinase. The presence of the non-helical telopeptides and the involvement of the arginine-sensitive C-proteinase in dextran sulphate-induced processing indicated that the procollagen processing was not due to non-specific cleavage. Further degradation of the telopeptides by cell-layer-associated proteolytic activities, such as sometimes occurred with neutral polymers (Bateman *et al.*, 1987), was not observed in any of these cell cultures incubated with dextran sulphate.

To examine the order of propeptide removal in more detail, pulse-chase experiments were performed (Fig. 2). After a 30 min pulse with isotopically labelled precursor all of the type I procollagen was present as pro α -chains, which remained as the sole procollagen species until at least 1 h into the chase period. As the chase period was continued the amount of intact pro α -chains diminished and processing intermediates appeared. The first major intermediate was the pN α -chain components, which gradually accumulated as the major processing intermediate. There was a small amount of pC-collagen, but this represented a

Fig. 1. Dextran sulphate-induced fibroblast procollagen processing

Human dermal fibroblast cultures were labelled with [3 H]proline for 18 h and samples were prepared and analysed by SDS/PAGE as described in the Experimental section. All samples were reduced with 10 mM-dithiothreitol before analysis. The migrations of pro α -chains, pC α -chains, pN α -chains, fully processed α -chains and cross-linked α -chain dimers (β -components) are shown. (a) Cell-layer (Cell) and medium (Med) samples from cultures labelled in the absence (lanes 1 and 2) and in the presence (lanes 3 and 4) of 0.01% (w/v) dextran sulphate. (b) Cell-layer collagen labelled in the presence of 0.01% (w/v) dextran sulphate. Portions were acidified, then subjected to digestion with pepsin (100 μ g/ml) for 6 h (Bateman *et al.*, 1986b). Lane 1, undigested collagen; lane 2, collagens after pepsin digestion. The arrows indicate the faster-migrating pepsin-digested collagen chains. (c) [3 H]Collagen from the cell layer of fibroblasts labelled in the presence of 0.01% (w/v) dextran sulphate and in the absence (lane 1) and in the presence (lane 2) of 50 mM-L-arginine.

Fig. 2. Pulse-chase analysis of procollagen processing

Fibroblast cultures were pulse-labelled with [14 C]proline for 30 min in the presence of 0.01% (w/v) dextran sulphate and chased with medium containing 50 mM-proline and 0.01% (w/v) dextran sulphate for periods up to 24 h (see the Experimental section for details). (a) SDS/PAGE of portions of the cell layer at different chase times as indicated. Samples were reduced before electrophoresis. The migration positions of pro α -chains, pC-collagen and pN-collagen intermediates and fully processed α -chains are shown. (b) Quantification of procollagen processing. Processing intermediates of pro α 1(I)-chains were quantified by densitometry of the fluorograms and calculation of the peak areas. The amount of each component is expressed as a percentage of the total of α 1(I)-, pN α 1(I)-, pC α 1(I)- and pro α 1(I)-chains present at each chase time point. ●, Pro α 1(I)-chains; △, pN α 1(I)-chains; ○, pC α 1(I)-chains; ▲, α 1(I)-chains.

minor fraction and did not accumulate during the chase (Figs. 2a and 2b). These experiments were repeated and the pN-collagen intermediates were always apparent whereas pC-collagens were only seen in some experiments.

From 2 h chase onwards the fully processed $\alpha 1(I)$ - and $\alpha 2(I)$ -chains were detected, and after 24 h chase were the only significant type I collagen components (Fig. 2b). Similar results were obtained when fibroblasts were cultured in the presence of neutral polymers (Bateman *et al.*, 1986a).

These data strongly suggest that the dextran sulphate-induced processing occurred by rapid cleavage of the C-propeptide followed by a slower cleavage of the N-propeptide, although the presence of some pC-collagen species indicated that initial cleavage of the N-propeptide may occur to a minor extent. Initial C-propeptide removal is consistent with the finding of pN-collagens in normal collagen fibrils *in vivo* (Veis *et al.*, 1973; Fleischmajer *et al.*, 1981, 1983). Studies performed *in vitro* have also demonstrated that pN-collagen can form fibrils (Miyahara *et al.*, 1983), but that pC-collagen does not (Miyahara *et al.*, 1982). Since pN-collagen forms thin fibrils (Miyahara *et al.*, 1983), it has been suggested that N-terminal propeptide cleavage during fibrillogenesis may act as a regulatory mechanism of collagen fibril diameter (Hulmes, 1983).

The rapid removal of the C-propeptide suggests that the C-proteinase is located largely in the immediate pericellular environment. This proposed route of processing disagrees with a number of other studies performed *in vitro* that have concluded that either N-propeptide cleavage precedes C-propeptide removal (Fessler *et al.*, 1975; Davidson *et al.*, 1977; Limeback & Sodek, 1979; Leung *et al.*, 1979; Gerstenfeld *et al.*, 1984) or that N-propeptide and C-propeptide cleavage may occur independently (Sonohara *et al.*, 1981). However, in these cell culture experiments the absence of a substantial extracellular matrix and the consequent rapid dilution of the procollagen into the culture medium may have affected the order of propeptide cleavage. It is also possible that changes in the cleavage pattern may occur in different tissue and cell types or at different stages of development. If N-terminal cleavage is a regulatory step in fibrillogenesis (Hulmes, 1983), this would provide a convenient mechanism by which cells could readily regulate the functional characteristic of the type I collagen fibrils.

Because of the rapid and extensive processing of type I procollagen to collagen in this culture system containing dextran sulphate, we applied this method to the identification of procollagen-processing abnormalities in hereditary connective-tissue diseases (Fig. 3). Fibroblasts from two diseases with previously characterized defects in N-propeptide cleavage were studied. These were a mild case of sheep dermatosparaxis, a fragile skin disease resulting from abnormally low levels of pN-proteinase activity (Ramshaw, 1984), and a patient with EDS VIIA, in which the N-proteinase cleavage site is deleted in the product of one allele of the $\text{pro}\alpha 1(I)$ -chain (Cole *et al.*, 1986). In both cases (Fig. 3a, lane 2, and Fig. 3b, lane 2) there was a clear deficiency of procollagen processing during continuous labelling experiments. The diminished processing to $\alpha 1(I)$ -chains was quantified in the case of EDS VII (Fig. 4a) and represented about half that of control cells. The pN-collagen intermediates were accumulated in both these cases (Fig. 4b) compared with age- and species-matched control cell lines. This accumulation of the pN-collagen intermediate closely resembled that seen in tissue samples from these cases (Cole *et al.*, 1986; Ramshaw, 1984). The diminished cleavage in these cases with well-defined defects in pN-propeptide cleavage was also strong evidence that specific N-proteinase was responsible for the N-propeptide cleavage in the fibroblast cultures containing dextran sulphate.

Efficient processing of procollagen was evident in all control

Fig. 3. Procollagen processing in fibroblasts from patients with connective-tissue diseases

Fibroblasts were labelled with [^3H]proline for 18 h in the presence of 0.01 % (w/v) dextran sulphate, and the cell-layer fractions were analysed by SDS/PAGE (see the Experimental section for details). (a) Human skin fibroblast collagen from control (C) (lane 1), a patient with EDS VII (ED) (lane 2) and a patient with lethal perinatal OI (OI) (lane 3). (b) Sheep skin fibroblast collagen from a control animal (C) (lane 1) and an animal with dermatosparaxis (DS) (lane 2). pN $\alpha 1(I)$ -, pN $\alpha 2(I)$ -, $\alpha 1(I)$ - and $\alpha 2(I)$ -chains are indicated. Slowly migrating $\alpha 1(I)$ - and $\alpha 2(I)$ -chains in the case of OI are designated by arrowheads ($\blacktriangleleft$).

Fig. 4. Quantification of procollagen processing in connective-tissue diseases

Cell cultures were labelled in the presence of 0.01 % (w/v) dextran sulphate, and the collagenous components were resolved by SDS/PAGE. Gel bands corresponding to the intermediates in procollagen processing were identified and quantified by excision from the gel and scintillation counting (see the Experimental section for details). Separate control cell lines were studied ($n = 6$), and each determination was made in duplicate and the results are expressed as the mean with the range indicated by a vertical bar. Each processing value obtained in disease-cell cultures represents the average of duplicate determinations in separate labelling experiments. (a) The amount of fully processed $\alpha 1(I)$ -chain was calculated as a percentage of total unprocessed $\text{pro}\alpha 1(I)$ -chains and the partially processed pC-collagen and pN-collagen intermediates. $\circ$, Control human fibroblasts; $\bullet$, EDS VII fibroblasts; $\blacksquare$, lethal perinatal OI fibroblasts. (b) The proportion of unprocessed pN $\alpha 1(I)$ -chains accumulated during labelling is expressed as a ratio of pN $\alpha 1(I)$ -chains to the fully processed $\alpha 1(I)$ -chains. $\circ$, Control human fibroblasts; $\bullet$, EDS VII fibroblasts; $\triangle$, control sheep fibroblasts; $\blacktriangle$, dermatosparactic sheep fibroblasts.

cells studied, with $75 \pm 9\%$ pro $\alpha 1$ (I)-chains fully processed to $\alpha 1$ (I)-chains during the labelling period (Fig. 4a). The only measurable partially processed pro $\alpha 1$ (I)-chain intermediate was pN $\alpha 1$ (I)-chains (Fig. 4b).

Poor processing of procollagen was also observed in cells from a patient with lethal perinatal OI (Fig. 3a, lane 3) with an accumulation of the pN-collagen intermediates. A defect in procollagen processing in cells from this patient has been reported previously (Minor *et al.*, 1986). The mutation has been characterized as a deletion in the $\alpha 2$ (I)-chain (de Wet *et al.*, 1983; Tromp & Prockop, 1988). Since this mutation does not involve the propeptide-cleavage site, it is not known whether the mutation leads directly to the decreased processing, or whether there is also a reduced N-proteinase activity in this case.

In common with other cases of lethal perinatal OI (Bateman *et al.*, 1984, 1986b; Bonadio & Byers, 1985), the $\alpha 1$ (I)- and $\alpha 2$ (I)-chains had a decreased electrophoretic mobility due to increased extents of post-translational modification of lysine. This increase in lysine modification is a consequence of delayed helix formation due to the structural mutation (Bonadio & Byers, 1985). The slow migration of the α -chains is clearly evident (Fig. 3a, lane 3), indicating that electrophoretic abnormalities of collagen are also expressed when cells are labelled in the presence of dextran sulphate. This is of considerable importance, since the use of dextran sulphate obviates the need to perform pepsin digestions on the procollagens, as has been the previous common practice (Bateman *et al.*, 1984, 1986b; Bonadio & Byers, 1985), to enable the demonstration of collagen structural abnormalities.

The present paper describes a fibroblast culture procedure for the rapid detection of procollagen processing defects and structural defects. This simple procedure should be of considerable value in studies on collagen biosynthesis in connective-tissue diseases and for the further analysis of the detailed mechanisms of procollagen processing *in vitro*.

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