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Biosynthesis and secretion of procollagenase by rabbit synovial fibroblasts

Inhibition of procollagenase secretion by monensin and evidence for glycosylation of procollagenase

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Monolayer cultures of rabbit synovial fibroblasts stimulated with phorbol myristate acetate to produce large amounts of collagenase (EC 3.4.24.7) were used to study the biosynthesis and secretion of this enzyme. [^{3}H]Leucine was added to cell cultures for pulse–chase and continuous-labelling experiments. The labelled procollagenase synthesized was identified by immunoprecipitation followed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and fluorography. The amounts of intracellular and extracellular proenzyme were quantified by measuring radioactivity incorporated into the proteins. Procollagenase was synthesized as doublet proteins of M_r 57000 and M_r 61000. Immunoprecipitable proenzyme proteins were first detected in culture medium 35 min after [^{3}H]leucine was added to the cells. Monensin treatment of the cells inhibited procollagenase secretion and led to intracellular accumulation of the proenzyme. Cells treated with tunicamycin produced only the 57000-M_r form, indicating that in rabbit synovial cells the 61000-M_r form was post-translationally modified by addition of oligosaccharides to asparagine residues. The ratios of glycosylated to unglycosylated forms in cell lysates and in culture medium were 0.22 : 1 and 0.07 : 1 respectively.

Mammalian collagenases (EC 3.4.24.7) are metallo-proteinases that are capable of cleaving interstitial collagen types I, II and III at specific sites to give characteristic three-quarter and one-quarter fragments of the native polypeptides (McCroskery *et al.*, 1975; Woolley *et al.*, 1975; Welgus *et al.*, 1981). The involvement of collagenase in pathological breakdown as well as in normal remodelling of connective tissues has been described by many workers [see Harris & Krane (1974*a*,*b*,*c*) and Woolley & Evanson (1980) for reviews]. Local collagenolysis in tissues may be regulated by (1) biosynthesis and secretion of procollagenase into the extracellular matrix (Harper *et al.*, 1971; Jeffrey *et al.*, 1975; Birkedal-Hansen *et al.*, 1976; Dayer *et al.*, 1976; Wahl *et al.*, 1977; Vaes *et al.*, 1978; McMillan *et al.*, 1981), (2) activation of procollagenase by limited proteolysis (Harper *et al.*, 1971; Birkedal-Hansen *et al.*, 1976; Horwitz *et al.*, 1976; Eeckhout & Vaes, 1977; Woessner, 1977; Werb *et al.*, 1977; Nagase *et al.*, 1982) and (3) inhibition of the activated enzyme by various serum and/or tissue-derived inhibitors (Werb *et al.*, 1974; Kuettner *et al.*, 1976; Woolley *et al.*, 1976; Hiti-Harper *et al.*, 1978; Vater *et al.*, 1979; Welgus *et al.*, 1979; Cawston *et al.*, 1981; Murphy *et al.*, 1981).

In our previous studies with a translation system used *in vitro* with mRNA extracted from rabbit synovial fibroblasts, we demonstrated that a precursor of collagenase was synthesized as a pre-proenzyme (M_r 59000) in the absence of microsomal fraction (Nagase *et al.*, 1981). In the presence of microsomal membranes, the nascent protein was co-translationally translocated into the lumen of microsomal vesicles and processed to a polypeptide chain of M_r 57000, which was identical in mobility on SDS/polyacrylamide-gel electrophoresis with the major collagenase secreted from fibroblasts in culture (Nagase *et al.*, 1981). In the present study we have investigated early events of biosynthesis and secretion of procollagenase from rabbit synovial cells treated with phorbol myristate acetate, a compound known to stimulate collagenase production (Brinckerhoff *et al.*, 1979, 1982). As previously shown for secretory proteins such as immunoglobulins (Tartakoff & Vassalli, 1977), enzymes from pancreas and macrophages (Tartakoff &

Abbreviation used: SDS, sodium dodecyl sulphate.

Vassalli, 1978), acetylcholinesterase (Smilowitz, 1979), procollagen (Tartakoff & Vassalli, 1978; Uchida *et al.*, 1979) and fibronectin (Uchida *et al.*, 1979), the secretion of procollagenase was prevented by the univalent ionophore monensin, without impairing the biosynthesis of procollagenase.

In culture medium procollagenase is usually secreted as a doublet protein of M_r 61000 and M_r 57000 (Nagase *et al.*, 1981; Vater *et al.*, 1981). A doublet form of procollagenase has also been reported for the human skin-fibroblast enzyme (Stricklin *et al.*, 1977; Valle & Bauer, 1979). Since the 61000-M_r species bound to concanavalin A–Sepharose (Nagase *et al.*, 1981), post-translational glycosylation of the procollagenase seemed to be a likely mechanism. We have further tested and confirmed this possibility by using in our cultures the antibiotic tunicamycin, which inhibits the formation of *N*-acetylglucosaminyl pyrophosphate polyisoprenol (Tkacz & Lampen, 1975; Takatsuki *et al.*, 1975; Lehle & Tanner, 1976).

Materials and methods

Materials

Chemicals and isotopically labelled compounds were purchased from the following sources: L-[4,5-^{3}H]leucine (specific radioactivity 130Ci/mmol) from New England Nuclear, Boston, MA, U.S.A.; NCS tissue solubilizer and PCS (phase-combining scintillant) from Amersham/Searle, Arlington Heights, IL, U.S.A.; di-isopropyl phosphorofluoridate, protein A–Sepharose CL-4B and tunicamycin from Sigma Chemical Co., St. Louis, MO, U.S.A.; phorbol myristate acetate from Consolidated Midland, Brewster, NY, U.S.A.; protein standards and chemicals for SDS/polyacrylamide-gel electrophoresis from Bio-Rad Laboratories, Richmond, CA, U.S.A.; cell-culture reagents (Dulbecco's Modified Eagle's Medium, Hanks Balanced Salt Solution, foetal calf serum, penicillin, streptomycin and trypsin) from Grand Island Biological Co., Grand Island, NY, U.S.A. Sodium monensin was generously given by Eli Lilly Laboratories, Indianapolis, IN, U.S.A. Monospecific sheep anti-(rabbit fibroblast collagenase) serum was prepared as described by Vater *et al.* (1981). Compound Ep-475 [L-*trans*-epoxysuccinyl-leucylamido-(3-methyl)butane] (Tamai *et al.*, 1981) was generously given by Dr. K. Hanada of Taisho Pharmaceutical Co., Omiya, Japan.

Cell cultures

Monolayer cultures of rabbit synovial fibroblasts were established from explants of synovium as described previously (Brinckerhoff *et al.*, 1979). All experiments were performed with confluent cultures of cells in Dulbecco's Modified Eagle's Medium (Vogt & Dulbecco, 1960) containing antibiotics. Procollagenase production was stimulated maximally 48–72h after addition of phorbol myristate acetate (final concn. 10ng/ml), at which time the quantity of translatable mRNA and amount of procollagenase secreted into culture medium appeared to be at a steady state (Brinckerhoff *et al.*, 1979, 1982). At this point, over a 24h period cells secreted procollagenase into culture medium sufficient to degrade between 200 and 300μg of collagen/h per mg of cell protein. At this time, the cells were employed for experiments.

Radioisotopic labelling of cells

Cells (2.5×10^6) were grown in 100mm-diameter dishes. When cells were stimulated to produce maximal amount of procollagenase they were rinsed three times with Hanks Balanced Salt Solution and cultured in leucine-free Dulbecco's Modified Eagle's Medium. Cells were incubated in this medium for 20min to decrease the intracellular leucine pool before radioisotopic labelling of cells. The labelling medium (10ml) was the same leucine-free Dulbecco's Modified Eagle's Medium supplemented with 0.5mCi of [^{3}H]leucine.

For pulse–chase experiments, the labelling was terminated by an immediate change of medium supplemented with 1mM-leucine. At the desired chase time the medium was collected and subjected to immunoprecipitation to quantify extracellular procollagenase. At the same time, cells were harvested from the culture dishes with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (1.5mM-KH_2PO_4/8.2mM-Na_2HPO_4/2.7mM-KCl/137mM-NaCl) containing 0.5mM-EDTA by quick scraping with a rubber 'policeman' and then were pelleted by brief centrifugation. After being washed three times with phosphate-buffered saline containing proteinase inhibitors (1mM-di-isopropyl phosphorofluoridate, 0.1mM-compound Ep-475 and 5mM-EDTA), the cells were suspended in 1ml of the above buffer containing 1% Triton X-100 and proteinase inhibitors at the same concentrations and homogenized thoroughly in a Dounce homogenizer. Cell disruption was examined by using phase-contrast microscopy. No detectable intact cells were observed. The cell homogenate was transferred to a 1.5ml conical Microfuge tube and centrifuged in a Beckman Microfuge B for 4min. The supernatant was subjected to immunoprecipitation to quantify intracellular radiolabelled procollagenase.

For continuous-labelling experiments, duplicate cultures were exposed to 0.5mCi of [^{3}H]leucine in 7ml of leucine-free Dulbecco's Modified Eagle's Medium without depletion of intracellular leucine. A 0.5ml portion of culture medium was removed from each dish at specific intervals. Duplicate samples at each time point were combined and subjected to

immunoprecipitation. The amounts of [^{3}H]leucine-labelled procollagenase secreted into the culture medium were calculated according to the following equation:

$$\text{d.p.m.}_{Tn} = \left(\frac{A}{B} - (n-1)\right) \cdot \text{d.p.m.}_{n} + \text{d.p.m.}_{T(n-1)} \cdot \frac{1}{\frac{A}{B} - (n-2)}$$

where d.p.m._{Tn} is the accumulated radiolabelled procollagenase secreted from the cells by the time of the nth removal of culture medium ($\text{d.p.m.}_{T0} = 0$, however) and d.p.m._{n} is the radiolabelled procollagenase in a portion removed at the time of the nth removal. A is the original volume of culture medium and B is the fixed volume of a portion to be removed at the desired time.

At the end of experiments the cells were harvested, washed with phosphate-buffered saline containing 1mM-di-isopropyl phosphorofluoridate, 0.1mM-compound Ep-475 and 5mM-EDTA, and homogenized as described above. The cell lysates were then subjected to immunoprecipitation.

Treatment of cells with monensin and tunicamycin

Monensin was dissolved in ethanol at a concentration of 1mM, and 5μl of this solution was added to the culture medium at a final concentration of 0.5μM. Control cells received the same amount of ethanol without monensin. The cells were incubated at 37°C in air/CO_2 (19:1) for 5h before the labelling with [^{3}H]leucine. Tunicamycin was dissolved in 25mM-NaOH at a concentration of 1mg/ml and was added to the culture medium at a final concentration of 1μg/ml. The cells were incubated at 37°C for 4h or 24h before the labelling. Control cells received an equal volume of 25mM-NaOH without tunicamycin.

Immunoprecipitation

Intracellular and extracellular procollagenase was identified by immunoprecipitation with monospecific sheep anti-(rabbit fibroblast collagenase) serum by a modification of the procedure described previously (Nagase *et al.*, 1981). To 1ml of culture medium or cell lysate, 0.5ml of 0.5M-arginine hydrochloride, pH8.0, containing 0.04% NaN_3, 50μl of 1M-Tris/HCl buffer, pH8.6, 30μl of 0.2M-EDTA, 10μl of 0.2M-di-isopropyl phosphorofluoridate, 50μl of 20% (v/v) Triton X-100 and 20μl of antiserum were added. The mixture was incubated for 16h at 23°C. Antigen–antibody complexes were isolated by constant rotation of the samples with 50μl of protein A–Sepharose CL-4B for 2h at 23°C. After washing of the Sepharose beads four times with 1ml of 0.1M-Tris/HCl buffer, pH8.6, containing 250mM-arginine and 1% (v/v) Triton X-100, and once with water, the antigen–antibody complex was dissociated by boiling with 50μl of SDS/polyacrylamide-gel electrophoresis sample buffer [40mM-2-amino-2-methylpropane-1,3-diol/31mM-HCl/2% (w/v) SDS/1% (v/v) 2-mercaptoethanol/0.01% Bromophenol Blue/40% (v/v) glycerol] for 2min. Under these conditions about 65% of the [^{3}H]leucine-labelled procollagenase–antibody complexes was bound to protein A–Sepharose. The second immunoprecipitation of the supernatant obtained after this procedure with 20μl of antiserum showed no immunoprecipitable procollagenase. Thus the method is adequate for the determination of the relative amounts of intracellular and extracellular procollagenase.

To obtain intact procollagenase from the cell lysate, treatment of intact cells with proteinase inhibitors (di-isopropyl phosphorofluoridate, compound Ep-475 and EDTA) before cell disruption and homogenization of the cells in the presence of these inhibitors were absolutely essential. It was found that, without proteinase inhibitors present, the procollagenase was degraded into several fragments after cell disruption.

SDS/polyacrylamide-gel electrophoresis and fluorography

[^{3}H]Leucine-labelled procollagenase isolated by immunoprecipitation, was subjected to electrophoresis in SDS/polyacrylamide gels (7.5% total acrylamide concentration). The buffer system used was a modification of the 2-amino-2-methylpropane-1,3-diol/glycine/HCl discontinuous system of Wyckoff *et al.* (1977) as described by Barrett *et al.* (1979). After the staining of proteins with Coomassie Brilliant Blue R-250, the gel was impregnated with 2,5-diphenyloxazole (Laskey & Mills, 1975) and exposed to Kodak X-Omat (XRP-1) film (Eastman Kodak Co.) at −70°C. The film was developed with a Kodak RPX-Omat processor. To quantify procollagenase, bands located by fluorography were excised and hydrated with 200μl of water for 1h at 23°C. Then 2ml of NCS tissue solubilizer/water (9:1, v/v) was added and the whole was incubated at 50°C for 2h. After cooling, the radioactivity of the sample was counted with 15ml of PCS scintillant and 50μl of 100% acetic acid. To determine the ratio of 61000-M_r and 57000-M_r forms of procollagenase, fluorograms were scanned at 800nm with a Gilford 2400-S spectrophotometer attached to a Gilford 2410-S

Linear Transport (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.). Absorption patterns were photocopied, and the areas corresponding to M_r 61000 and M_r 57000 were excised and weighed to determine the ratio of one to the other.

Results

Synthesis and secretion of procollagenase

Treatment of monolayer cultures of rabbit synovial fibroblasts with phorbol myristate acetate stimulated the production of procollagenase, and synthesis and secretion of procollagenase reached a steady state by 72h, as previously described by Brinckerhoff *et al.* (1979, 1982). Cells pulse-labelled with [^{3}H]leucine for 15min were 'chased' with 1mM-leucine for 0, 0.5, 1, 2 and 4h. At each time point intracellular and extracellular procollagenase was isolated by immunoprecipitation and subjected to SDS/polyacrylamide-gel electrophoresis and fluorography (Fig. 1*a*). Amounts of procollagenase were estimated by counting the radioactivity of the gel slices corresponding to the procollagenase bands (Fig. 1*b*). Procollagenase (M_r 61000 and M_r 57000) pulse-labelled with [^{3}H]leucine for 15min was detectable in culture medium after a 30min chase, and all [^{3}H]leucine-labelled procollagenase was secreted within 60min. No intracellular [^{3}H]-leucine-labelled procollagenase was detected at this point. On the basis of the observation that the rate of secretion of procollagenase was in a steady state, the appearance of procollagenase in the medium was extrapolated to be about 35min after initiation of labelling. Extrapolation of the time required for disappearance of the intracellular pulse-labelled procollagenase after initiation of chase was also 35min (Fig. 1*b*). This was in good agreement with the result obtained by continuous labelling of cells with [^{3}H]leucine, as described in the following experiment.

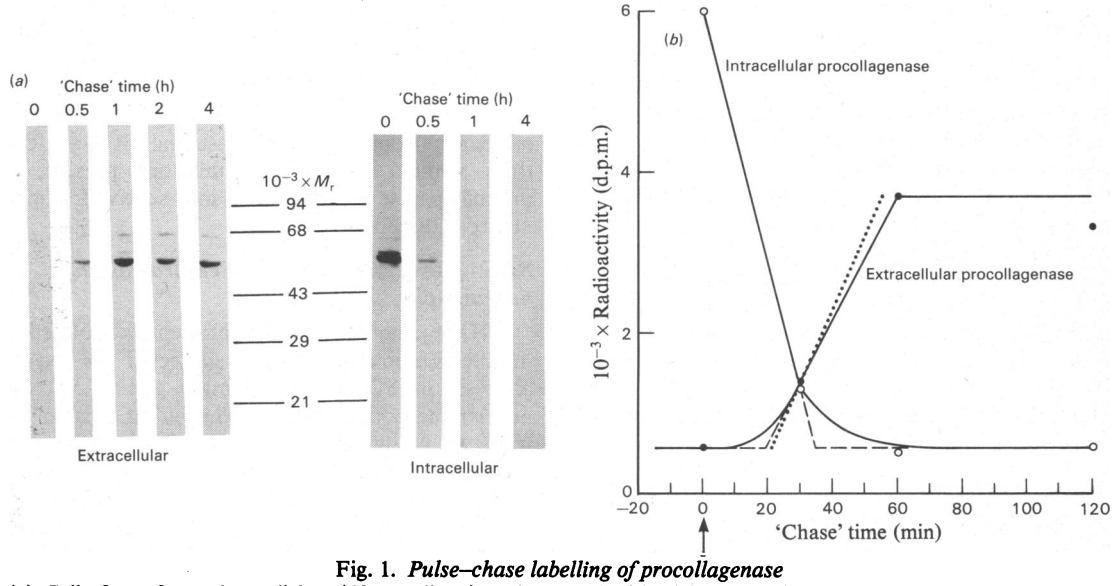

Fig. 1. *Pulse–chase labelling of procollagenase*

(*a*) Cells from five culture dishes (60mm diam.) were treated with phorbol myristate acetate for 72h to stimulate production of procollagenase, and then were incubated with leucine-free Dulbecco's Modified Eagle's Medium for 20min to allow depletion of the intracellular leucine pool. Cells were pulse-labelled with [^{3}H]leucine for 15min and 'chased' with 1mM non-radiolabelled leucine for 0, 0.5, 1, 2 and 4h. Culture medium and cells were harvested at each chase time and subjected to immunoprecipitation, SDS/polyacrylamide-gel electrophoresis and fluorography (*a*). Protein standards co-electrophoresed for determination of M_r were phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 29000) and soya-bean trypsin inhibitor (M_r 21000). In this particular set of cells in culture, an additional immunoreactive protein with M_r 65000 was produced. This protein band was not observed in any other experiments (see Fig. 4). However, since non-immunized sheep serum failed to react to this band, this protein may be a highly glycosylated procollagenase. (*b*) Procollagenase bands located by fluorography were excised, solubilized and counted for radioactivity. The appearance of labelled procollagenase in culture medium was extrapolated to be 35min after the addition of [^{3}H]leucine (15min pulse-labelling time plus 20min 'chase' time) (----). The dotted line (·····) is a hypothetical one that assumes that secretion of procollagenase commenced at 35min and that indicates the complete depletion of [^{3}H]-leucine-pulse-labelled procollagenase within 50min [35min (the time required for non-radiolabelled procollagenase appearance) plus 15min (pulse-labelling time)] after the chase was initiated.

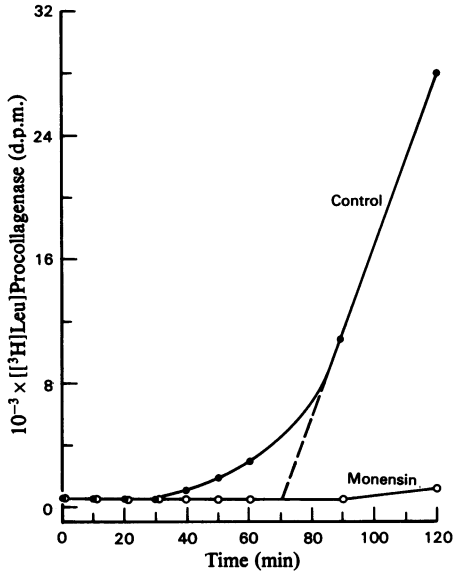

Fig. 2. *Inhibition of procollagenase secretion by monensin*

Phorbol myristate acetate-stimulated cells from two culture dishes (100mm diam.) were treated with 0.5 μM-monensin for 5h and then labelled with [^{3}H]leucine in 7ml of leucine-free Dulbecco's Modified Eagle's Medium. After addition of the labelled leucine, a 0.5ml portion of medium from each dish was removed and combined at (1) 0, (2) 10, (3) 20, (4) 30, (5) 40, (6) 50, (7) 60, (8) 90 and (9) 120min, and was subjected to immunoprecipitation, SDS/polyacrylamide-gel electrophoresis and fluorography. Control experiments were performed identically except that treatment of cells with monensin was omitted. Areas corresponding to procollagenase bands were excised, and proteins were solubilized and counted for radioactivity. The amount of procollagenase secreted was calculated by using the equation described in the Materials and methods section.

Inhibition of procollagenase secretion by monensin

Rabbit fibroblasts stimulated by phorbol myristate acetate were exposed to 0.5 μM-monensin for 5h before the labelling with [^{3}H]leucine. Examination of cell morphology under a phase-contrast microscope showed that monensin treatment caused extensive vacuole formation in the cells, as described by Ledger *et al.* (1980).

Both control and monensin-treated cells were continuously labelled with [^{3}H]leucine. Portions of the culture medium were removed at specified intervals, and labelled procollagenase was identified by immunoprecipitation. In control cells, labelled procollagenase first appeared in the medium 40min after the cells were exposed to [^{3}H]leucine. The time for the complete depletion of the intracellular non-radiolabelled leucine pool used for procollagenase synthesis was estimated to be about 70min (Fig. 2). In this case non-radiolabelled leucine was 'chased' with [^{3}H]leucine. Thus 35min (a half-time of the depletion of intracellular leucine) was the estimated time required for synthesis and secretion of procollagenase by rabbit cells stimulated by phorbol myristate acetate (first appearance of [^{3}H]leucine-labelled procollagenase in the culture medium). This result agreed with that obtained from the previous pulse–chase experiment (Fig. 1).

No detectable [^{3}H]leucine-labelled procollagenase was found in the culture medium of monensin-treated cells for periods up to 90min. A small amount of labelled procollagenase was observed after 120min (Fig. 2). Both control and monensin-treated cells were harvested after 120min labelling, and the intracellular procollagenase was isolated by immunoprecipitation and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography (results not shown). Larger amounts of procollagenase were accumulated in the cells treated with monensin compared with control cells. Radioactivities detected in the corresponding procollagenase bands from control cells and monensin-treated cells were 32.8×10^3 d.p.m.

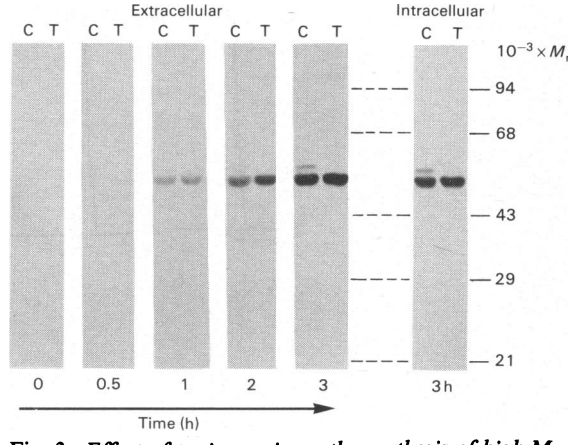

Fig. 3. *Effect of tunicamycin on the synthesis of high-M_r procollagenase*

Phorbol myristate acetate-stimulated cells from two culture dishes (100mm diam.) were treated with tunicamycin (1 μg/ml) for 4h and then labelled with [^{3}H]leucine in 7ml of leucine-free Dulbecco's Modified Eagle's Medium. Culture medium (0.5ml) was removed after 0, 0.5, 1, 2 and 3h of labelling. Duplicate samples were combined and subjected to immunoprecipitation. Cells were harvested after 3h labelling, and cell lysates were subjected to immunoprecipitation. Samples were run on SDS/polyacrylamide-gel electrophoresis and fluorographed. Protein standards for estimation of M_r were as in Fig. 1. C, Control cells; T, tunicamycin-treated cells.

and 63.2×10^3 d.p.m. respectively. Fluorography of total cell lysates of monensin-treated cells showed that protein synthesis in cells treated with monensin was not grossly impaired. The total intracellular collagenase activity that accumulated in the monensin-treated cells during the 8h incubation was 26 ± 6 units, whereas that detected in the control cells was 5.4 ± 1.2 units. However, the activatable collagenase secreted from the monensin-treated cells during the last 3h of treatment was negligible, whereas control cells secreted 157 ± 25 units of collagenase. [One unit of collagenase degrades 1 μg of collagen/h at 37°C after activation by 4-aminophenylmercuric acetate (Nagase *et al.*, 1982).]

Effect of tunicamycin

Phorbol myristate acetate-stimulated rabbit fibroblasts were treated with tunicamycin (1 μg/ml) for 4h or 24h before the labelling with [^{3}H]leucine. After the treatment with tunicamycin, cells were examined for morphological changes and for possible cytotoxicity. At the concentration of tunicamycin used for the experiment, no marked morphological changes of the cells or cytotoxic effects were noted.

After [^{3}H]leucine was added, portions of the culture medium were removed at 0, 0.5, 1, 2 and 3h to examine secretion of labelled procollagenase. Results very similar to those shown for control cells in Fig. 2 were obtained. [^{3}H]Leucine-labelled procollagenase was detected in the culture medium after 30min. Time for the complete depletion of the intracellular non-radiolabelled leucine pool was extrapolated to be 72min for both control and tunicamycin-treated cells. The total amounts of procollagenase synthesized and secreted from control cells (C) and tunicamycin-treated cells (T) were not markedly different (Fig. 3). The amounts of collagenase activity found in culture medium from tunicamycin-treated cells and control cells were similar: 220 ± 27 units for control and 173 ± 44 units for tunicamycin-treated cells. However, the synthesis of high-M_r procollagenase (M_r 61000) was completely blocked by the treatment of cells with tunicamycin. Identical results were obtained with 4h and 24h tunicamycin treatment of the cells. This

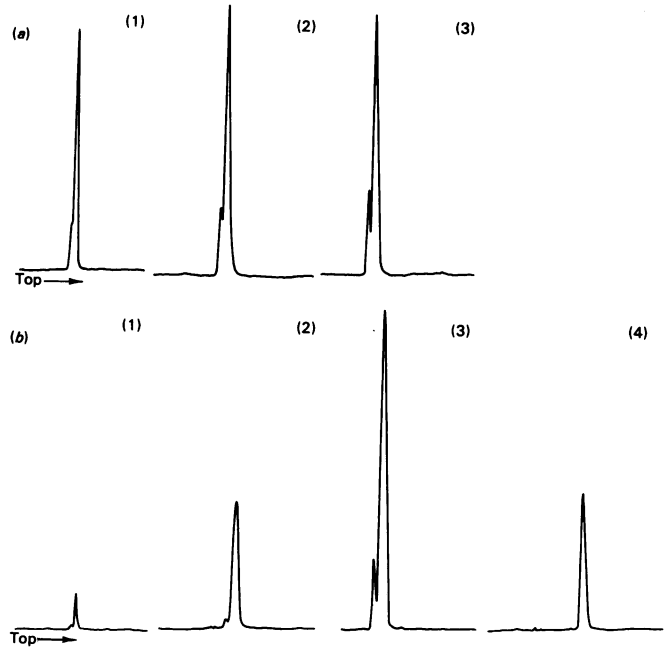

Fig. 4. *Ratios of glycosylated to unglycosylated procollagenase in cell lysate and in culture medium*
Intracellular and extracellular procollagenase after labelling with [^{3}H]leucine for various times was isolated by immunoprecipitation and subjected to SDS/polyacrylamide-gel electrophoresis. The gels were impregnated with 2,5-diphenyloxazole, dried and exposed to X-ray film in order to obtain fluorograms, which were scanned within a linear range at 800nm with a Gilford spectrophotometer attached to a Gilford 2410-S Linear Transport. (*a*) Intracellular procollagenase after labelling with [^{3}H]leucine for (1) 15min, (2) 2h and (3) 3h. (*b*) Extracellular procollagenase after labelling with [^{3}H]leucine for (1) 30min, (2) 2h, (3) 3h and (4) 2h from cells treated with tunicamycin (1 μg/ml).

indicates that the 61000-M_r species of procollagenase is a glycoprotein with asparagine-linked oligosaccharides formed via lipid–saccharide intermediates.

Ratio of glycosylated to unglycosylated procollagenase

Fluorograms of intracellular and extracellular procollagenase were scanned to determined the ratio of glycosylated to unglycosylated forms. Representative patterns for intracellular and extracellular procollagenase are shown in Fig. 4. In cell lysates $18.2 \pm 0.8\%$ ($n = 4$) of the total procollagenase was glycosylated, whereas only $7.0 \pm 1.6\%$ ($n = 4$) of secreted procollagenase was found as glycoprotein.

Discussion

Two species of procollagenase (M_r 57000 and M_r 61000) are synthesized by phorbol myristate acetate-stimulated rabbit synovial cells and secreted continuously, without any apparent changes in M_r value, into the culture medium. Both forms have been shown to be inactive zymogens unless activated by limited proteolysis or treatment with organic mercurial compounds (Nagase *et al.*, 1981; Vater *et al.*, 1981). The findings reported in the present paper indicate that intracellular procollagenase was observed as early as 15 min after labelling the cells with [^{3}H]leucine. The time required for both synthesis and secretion of procollagenase was estimated to be about 35 min by the pulse–chase experiment, and this value was supported by two separate continuous-labelling experiments. Present observations with stimulated rabbit synovial fibroblasts are in good agreement with the results reported by Valle & Bauer (1979) for procollagenase biosynthesis in cultures of normal human skin fibroblasts. These authors demonstrated that the times of appearance for intracellular and extracellular procollagenase were 15 min and 30 min respectively after labelling was started.

Rapid appearance of procollagenase in the culture medium suggests that the intracellular transport of the proenzyme after synthesis on polyribosomes may follow a pathway common to other secretory proteins. The univalent ionophore monensin was employed to test this possibility. Monensin has been shown to block secretion of proteins from various cells (Tartakoff & Vassalli, 1977, 1978; Uchida *et al.*, 1979; Smilowitz, 1979; Strous & Lodish, 1980). Ultrastructural studies suggested that monensin caused the disappearance of normal Golgi elements and the formation of numerous vesicles in the Golgi zone (Tartakoff & Vassalli, 1978; Ledger *et al.*, 1980). However, the actual stage at which monensin blocks intracellular transport is unclear. On the basis of radioautographic studies and cell fractionation along with ultrastructural studies, Tartakoff & Vassalli (1978) have proposed that the effect of monensin is exerted as secretory proteins are released from the Golgi apparatus. Possible effects on the transport of secretory proteins from the rough endoplasmic reticulum to the Golgi apparatus have been also suggested (Ledger *et al.*, 1980; Strous & Lodish, 1980; Uchida *et al.*, 1980). Our results obtained for procollagenase secretion from cells treated with monensin were very similar to those reported for procollagen secretion by Uchida *et al.* (1980). They reported that secretion of [^{3}H]proline-labelled procollagen commenced about 35 min after labelling the cells, whereas the effect of monensin on secretion was markedly delayed (about 105 min). This suggests that procollagenase and procollagen may share a common secretory route from their sites of synthesis.

We suggested previously that high-M_r procollagenase (M_r 61000) was a product of post-translational glycosylation, because only a single pre-procollagenase with M_r 59000 was produced in translation *in vitro* without microsomal fraction and because the processed 61000-M_r procollagenase bound to concanavalin A–Sepharose (Nagase *et al.*, 1981). In the present investigation we have obtained further evidence of glycosylation by showing complete inhibition of the synthesis of high-M_r procollagenase in cells treated with tunicamycin without a concurrent effect on protein biosynthesis. The result has indicated that the 61000-M_r species is a glycoprotein with asparagine-linked oligosaccharides. Appearance of the glycosylated form after 15 min pulse-labelling indicates that glycosylation of the core protein takes place immediately after or during the synthesis of procollagenase in the microsomal fraction. The ratio of glycosylated to unglycosylated procollagenase in the cell lysates was approximately 1:4, and did not increase even after a chase with non-radiolabelled leucine. In contrast, only about 7% of procollagenase in culture medium was found to be glycosylated. In our previous studies (Nagase *et al.*, 1981) we reported that glycosylated procollagenase represented 20% of that found in culture medium. In those studies the cells were induced to synthesize procollagenase with crystals of monosodium urate monohydrate rather than with phorbol myristate acetate as described in the present work. This discrepancy may be due to different stimuli and different cell populations used. Even in the monensin-treated cells, in which the exit of procollagenase was blocked, not all of the procollagenase was glycosylated. This indicates that the block of secretion induced by monensin occurred after the post-translational glycosylation took place.

The significance of glycosylation of procollagenase is not clear. The cause of the difference in ratios of glycosylated to unglycosylated forms in cell

lysates and in culture medium remains to be resolved. The difference may reflect the possibility that a portion of glycosylated procollagenase is attached to cellular organelles rather than destined for secretion, or that there are different kinetics of secretion for the glycosylated and unglycosylated forms.

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