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Independent secretion of proteoglycans and collagens in chick chondrocyte cultures during acute ascorbic acid treatment

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The mechanisms regulating the secretion of proteoglycans and collagens in chondrocytes, in particular those operating at the level of the rough endoplasmic reticulum (RER), are largely unknown. To examine these mechanisms, I studied the effects of acute ascorbate treatment on the secretion of two collagen types (types II and IX) and two proteoglycan types (PG-H and PG-Lb, the major keratan sulphate/chondroitin sulphate proteoglycan and the minor chondroitin sulphate proteoglycan respectively in cartilage) in scorbutic cultures of chick vertebral chondrocytes. I found that the scorbutic chondrocytes synthesized underhydroxylated precursors of types II and IX collagen that were secreted very slowly and accumulated in the RER. When the cultures were treated acutely with ascorbate, both macromolecules underwent hydroxylation within 1–1.5 h of treatment, and began to be secreted at normal high rates starting at about 2 h. Proteoglycan synthesis and secretion, however, remained largely unaffected by ascorbate treatment. Both the half-time of newly synthesized PG-H core protein in the RER and its conversion into completed proteoglycan were unchanged during treatment. Similarly, the overall rates of synthesis and secretion of both PG-H and PG-Lb remained at control levels during treatment. The data indicate that secretion of types II and IX collagen is regulated independently of secretion of PG-H and PG-Lb. This may be mediated by the ability of the RER of the chondrocyte to discriminate between procollagens and proteoglycan core proteins.

INTRODUCTION

The rough endoplasmic reticulum (RER) plays an important regulatory role in protein secretion. For example, different secretory proteins are transported out of the RER at markedly different rates but move through the remaining portion of the secretory pathway at similar rates, suggesting that protein translocation from the RER is a selective, rate-limiting step in protein secretion (Mitchell & Hardingham, 1981; Fitting & Kabat, 1982; Lodish *et al.*, 1983; Fries *et al.*, 1984; Fellini *et al.*, 1984; Campbell & Schwartz, 1988). In addition, abnormal secretory proteins resulting from mutations or pharmacological treatments exit the RER very slowly, if at all, indicating that the RER also has the ability to discriminate between normal and abnormal secretory proteins (Jimenez *et al.*, 1974; Jimenez & Yankowski, 1978; Kreis & Lodish, 1986; Gothing *et al.*, 1986; Sifers *et al.*, 1988).

The mechanisms through which the RER regulates protein secretion are largely unknown. Chondrocytes grown in scorbutic culture conditions represent a useful experimental system with which to analyse these mechanisms (Pacifici & Iozzo, 1988). The scorbutic chondrocytes synthesize underhydroxylated non-helical procollagen molecules that are transported very slowly and accumulate in the RER (Meier & Solursh, 1978; Pacifici *et al.*, 1983; Vertel *et al.*, 1985; Iozzo & Pacifici, 1986). Vitamin C is required for the activity of prolyl hydroxylases, RER enzymes that hydroxylate proline residues in the nascent procollagen chains and allow folding of the chains into a stable triple helix (Peterkofsky & Undenfriend, 1965; Berg & Prockop, 1973; Rosenbloom *et al.*, 1973; Cutroneo *et al.*, 1974; Olsen *et al.*, 1975). We found that upon acute ascorbate treatment the accumulated type II procollagen molecules were hydroxylated within 1–1.5 h of treatment and were then translocated and secreted at their normal high rate (Pacifici & Iozzo, 1988). Clearly, the RER of the chondrocytes shares the ability with that of other cells to discriminate between normal and abnormal secretory proteins, preventing translocation of non-helical procollagen but permitting that of normal procollagen.

To assess further this ability of the chondrocyte RER, I compared in the present study the secretion of type II collagen, type IX collagen (Reese & Mayne, 1981), PG-H and PG-Lb (the major keratan sulphate/chondroitin sulphate proteoglycan and the minor chondroitin sulphate proteoglycan respectively in cartilage; Okayama *et al.*, 1976; Shinomura *et al.*, 1983; Noro *et al.*, 1983) in scorbutic chondrocyte cultures during acute ascorbate treatment. I predicted that secretion of both types II and IX collagen should be enhanced during acute ascorbate treatment, whereas that of PG-H and PG-Lb should proceed unaffected.

EXPERIMENTAL

Collagen analysis

Chondrocytes isolated from 12-day-old chick embryo vertebral cartilage were grown in secondary monolayer cultures under scorbutic conditions for 10 days as described (Pacifici *et al.*, 1983). For pulse–chase experiments, day 10 secondary chondrocyte cultures grown on 60 mm dishes were labelled for 1 h with 40 μCi of [5-^{3}H]proline/ml (Amersham; 31 Ci/mmol) in medium containing 100 μg of β-aminopropionitrile fumarate (β-APN)/ml. After labelling, cultures were rinsed four times (maintaining the temperature at 37 °C) and chased for 0, 0.5, 1, 1.5, 2, 3, 4 and 6 h in medium containing a 100-fold excess concentration of unlabelled proline and β-APN in the absence or presence of 50 μg of freshly prepared L-ascorbic acid/ml (Sigma). At the end of each chase interval, medium and cell layer were separated. The cell layers were extracted with 0.15 M-potassium

Abbreviations used: RER, rough endoplasmic reticulum; β-APN, β-aminopropionitrile; PG-H and PG-Lb, the major keratan sulphate/chondroitin sulphate proteoglycan and the minor chondroitin sulphate proteoglycan respectively in cartilage (nomenclature according to Shinomura *et al.*, 1983); proα1(II), pCα1(II), pNα1(II) and α1(II), precursor and mature forms of type II collagen; α1(IX), α2(IX) and α3(IX), the subunits of type IX collagen.

phosphate buffer (pH 7.6) for 30 min at 4 °C to solubilize the secreted pericellular collagenous proteins (Schmid & Conrad, 1982). Extracts were combined with their respective chase medium samples and collagenous proteins were precipitated by 30% ammonium sulphate (Uitto, 1977). Precipitated material and the extracted cell layers were solubilized in sample buffer (Laemmli, 1970), and aliquots containing identical portions of medium or cell layer samples were analysed by SDS/PAGE on 6% polyacrylamide slab gels. The resulting fluorograms were scanned with a video digitizer interfaced with an IBM microcomputer (Haselgrove *et al.*, 1985).

Pulse–chase analysis of PG-H synthesis

Day 10 secondary chondrocyte cultures were processed for pulse–chase experiments as above. Cultures were labelled for 10 min in serine-free medium containing 35 μCi of [3-^{3}H]serine/ml (Amersham; 37 Ci/mmol). Cultures were rapidly rinsed at 37 °C with medium containing a 100-fold excess of unlabelled serine, a step requiring about 2 min to perform. They were chased in medium containing unlabelled serine and β-APN (used in the above experiments of procollagen secretion and included here as a control) in the absence or presence of ascorbate for 0, 5, 10, 15, 20, 30 and 45 min. At the end of each chase period, cell layers were quickly cooled to 4 °C, solubilized in Laemmli sample buffer and analysed by SDS/PAGE and fluorography as above. Alternatively, cell layers were processed for quantitative immunoprecipitation using a rabbit polyclonal antiserum to PG-H core protein (Pacifici *et al.*, 1983) and analysed by SDS/PAGE and fluorography. In similar experiments, the day 10 cultures were first pretreated with ascorbate and β-APN for 3 h and then used for pulse–chase experiments. Cultures treated with β-APN alone served as a control.

To determine the rates of conversion of core protein into completed PG-H, cultures were incubated for 3 h with or without ascorbate, pulsed for 10 min with [^{3}H]serine and chased in serum-free medium as above. At each time point, the combined medium and cell layer were mixed with 1 vol. of 8 M-guanidine hydrochloride containing proteinase inhibitors; PG-H was isolated by centrifugation on dissociative CsCl gradients (Fellini *et al.*, 1984) followed by agarose gel electrophoresis as described below.

Proteoglycan synthesis and analysis

Day 10 cultures were treated with ascorbate and β-APN for 0.5, 1, 2, 3, 4, 5 and 6 h. Cultures treated with β-APN alone served as a control. Cultures were labelled with 50 μCi of [^{35}S]sulphate/ml (Amersham; 1.3 Ci/μmol) during the last 30 min of treatment, and incorporation into medium and cell layer macromolecules was determined after Sephadex G-50 column chromatography in 4 M-guanidine hydrochloride containing proteinase inhibitors and 0.5% Triton (Yanagishita & Hascall, 1983). Radioactivity in the void volume material was determined by liquid-scintillation counting.

For rate-zonal centrifugation analysis, portions of the void volume material were fractionated by dissociative sucrose gradient centrifugation (Okayama *et al.*, 1976). For agarose gel electrophoresis, the top 11 fractions of sucrose gradients that contain PG-Lb and type IX collagen were pooled and chromatographed on Sephadex G-25 PD-10 columns equilibrated with 8 M-urea, 50 mM-NaCl and 0.5% Triton X-100 in 50 mM-Tris/HCl, pH 7.0 (urea buffer) (Yanagishita & Hascall, 1983). Void volume material was applied to DEAE-Sephacel columns equilibrated with urea buffer and eluted with a linear 0.1–0.8 M-NaCl gradient. Fractions containing PG-Lb and type IX collagen (eluting at about 0.4–0.5 M-NaCl) were pooled, mixed with carrier chondroitin sulphate, dialysed in the presence of 0.1 mM-EDTA/0.5 mM-benzamidine/0.1 mM-phenylmethanesulphonyl fluoride/0.5 mM-*N*-ethylmaleimide, and freeze-dried. Samples were then processed for agarose gel electrophoresis (see below).

Agarose gel electrophoresis of PG-H, PG-Lb and type IX collagen

During the course of this study, it became apparent that a procedure was needed for the simultaneous analysis of samples containing various amounts of PG-H, PG-Lb and type IX collagen. Accordingly, [^{35}S]sulphate-labelled PG-H, PG-Lb and type IX collagen, isolated as described (Okayama *et al.*, 1976; Shinomura *et al.*, 1983; Noro *et al.*, 1983), were used to establish conditions for their quick separation by agarose gel electrophoresis. We modified existing methods (Heinegard *et al.*, 1985; Thornton *et al.*, 1986) as follows. Agarose (type II; Sigma) was dissolved at a final concentration of 1% by boiling in 0.1 M-Tris/HCl (pH 6.8)/2.5 mM-Na_2SO_4/0.25% SDS. The solution was allowed to cool down to about 60 °C and was used to cast 2.5 mm thick slab gels. Gels were stored overnight at 4 °C before use. Gels were pre-electrophoresed at 75 mA for 2 h at 4 °C. Samples were dissolved in 40 mM-Tris/HCl (pH 6.8)/1 mM-Na_2SO_4/0.1% SDS and preincubated at 37 °C for 2 h (Heinegard *et al.*, 1985). Samples were then loaded on to the pre-electrophoresed gels and electrophoresis was continued for 3 h. Gels were fixed with 50% methanol/10% acetic acid, processed for fluorography when indicated, and exposed to Kodak XAR-5 films at −70 °C.

As shown in Fig. 1, this electrophoretic procedure gave excellent separation of PG-H, type IX collagen and PG-Lb (lanes 1, 5 and 9 respectively). Portions were digested with repurified

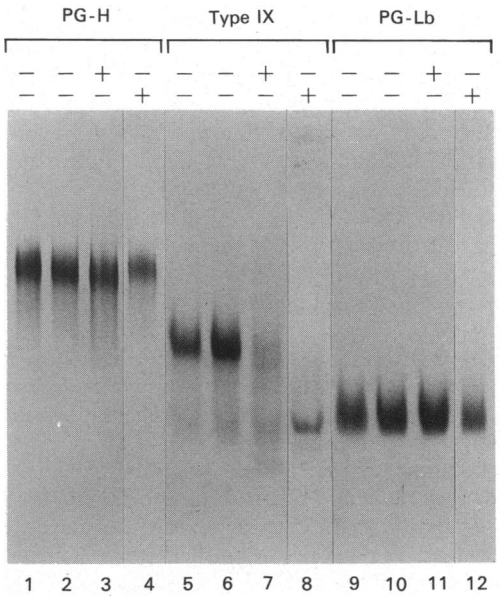

Fig. 1. Fluorograme of an agarose gel to separate PG-H, type IX collagen and PG-Lb

Before electrophoresis, portions were digested with collagenase (Coll) or reduced by β-mercaptoethanol (β-ME) treatment, as indicated. Lanes 1–4, untreated, mock-digested, collagenase-digested and reduced PG-H respectively; lanes 5–8, untreated, mock-digested, collagenase-digested and reduced type IX collagen respectively; lanes 9–12, untreated, mock-digested, collagenase-digested and reduced PG-Lb respectively. As expected, only type IX collagen was sensitive to collagenase digestion (lane 7) and reduction (lane 8). The band in lane 8 probably represents the α2 subunit of type IX collagen that carries a glycosaminoglycan chain (Vaughan *et al.*, 1985).

bacterial collagenase as described (Pacifici & Iozzo, 1988); mock-digested portions served as a control. As expected, PG-H and PG-Lb were not sensitive to collagenase digestion (lanes 3 and 11 respectively) whereas type IX collagen was (lane 7). Similarly, treatment with 5% β-mercaptoethanol for 1 h at 70 °C showed that type IX collagen was sensitive to reduction (Vaughan *et al.*, 1985) as indicated by increased mobility (lane 8), whereas PG-H and PG-Lb were essentially insensitive to reduction (lanes 4 and 12 respectively).

RESULTS

Collagen secretion

To study the effects of acute ascorbate treatment on secretion of types II and IX collagen, scorbutic chondrocyte cultures were pulsed for 1 h with [^{3}H]proline and chased in the absence or presence of ascorbate for 0.5, 1, 1.5, 2, 3, 4 and 6 h. Electrophoretic analysis of medium plus pericellular material during the chase revealed that type II collagen secretion increased slightly as early as 1–1.5 h after the start of ascorbate treatment, as compared with the slow secretion rate in control cells (Fig. 2, lanes 1–7; see quantifications in Fig. 3*a*). The electrophoretic mobility of type II collagen subunits secreted by the treated chondrocytes had decreased, indicating that the macromolecule had undergone hydroxylation by 1.5 h of treatment (lane 7). By 2 h of treatment the rate of type II collagen secretion increased rapidly and markedly (Fig. 2, lanes 8–15; Fig. 3*a*), with kinetics identical to those observed previously (Kao *et al.*, 1983; Pacifici & Iozzo, 1988).

Type IX collagen secretion was similarly affected by the acute ascorbate treatment. The secretion rate of this macromolecule remained low during the initial 1–1.5 h of treatment as it was in control cells (Fig. 2, lanes 1–7). The low abundance of this macromolecule at these early chase time points made quantification of the data difficult (Fig. 3*b*). Starting at 2 h of treatment, however, the secretion rate of type IX collagen in the treated cells increased markedly, and remained at this high level with further treatment (Fig. 2, lanes 8–15; Fig. 3*b*).

When the cell layer samples were analysed by similar procedures, only a minor decrease in intracellular collagen precursors had occurred by 6 h of chase (results not shown), confirming that longer periods are needed to deplete the large RER-associated pool of accumulated procollagens (Meier & Solursh, 1978; Pacifici & Iozzo, 1988).

Thus, after an initial lag of about 1.5 h, probably needed for hydroxylation to occur, secretion of both types II and IX collagen is markedly stimulated by acute ascorbate treatment.

PG-H core protein translocation and secretion

To determine whether the above changes in collagen secretion during ascorbate treatment were accompanied by similar changes in PG-H secretion, pulse–chase experiments were performed. In a first set of experiments, we determined the kinetics of intracellular translocation and processing of PG-H core protein during the first hour of ascorbate treatment (i.e. before the rates

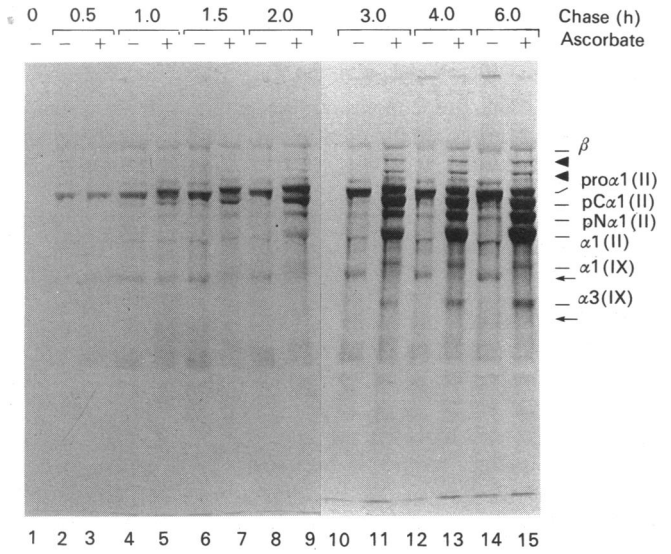

Fig. 2. Fluorogram of an SDS/polyacrylamide gel to analyse secreted collagenous proteins

Chondrocyte cultures pulsed for 1 h with [^{3}H]proline were chased in the absence (−) or presence (+) of ascorbate, and the combined medium and pericellular collagenous proteins secreted at each chase time point were analysed by electrophoresis and fluorography. Arrows indicate the electrophoretic positions of the underhydroxylated α1(IX) and α3(IX) subunits. The underhydroxylated α3(IX) subunit produced a broad band that was difficult to reproduce photographically but was readily measurable in the original fluorogram. The α2(IX) subunit of type IX collagen co-migrated with the abundant type II collagen subunits and was thus not resolved by the electrophoretic system used. Arrowheads mark the positions of type XI collagen subunits; note that secretion of this collagen type is also stimulated markedly by ascorbate treatment.

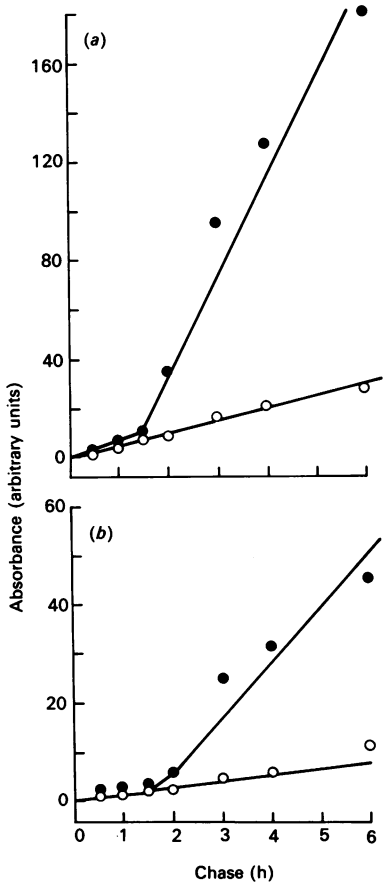

Fig. 3. Kinetics of collagen secretion

Lanes 1–15 in the fluorograms shown in Fig. 2 and other fluorograms from different exposures were scanned by video digitization. Absorbance values of types II and IX collagen subunits were summed and the resulting data were plotted in (*a*) and (*b*) respectively. Note that the kinetics of secretion of these macromolecules are first-order processes in both control (○) and ascorbate-treated (●) cells.

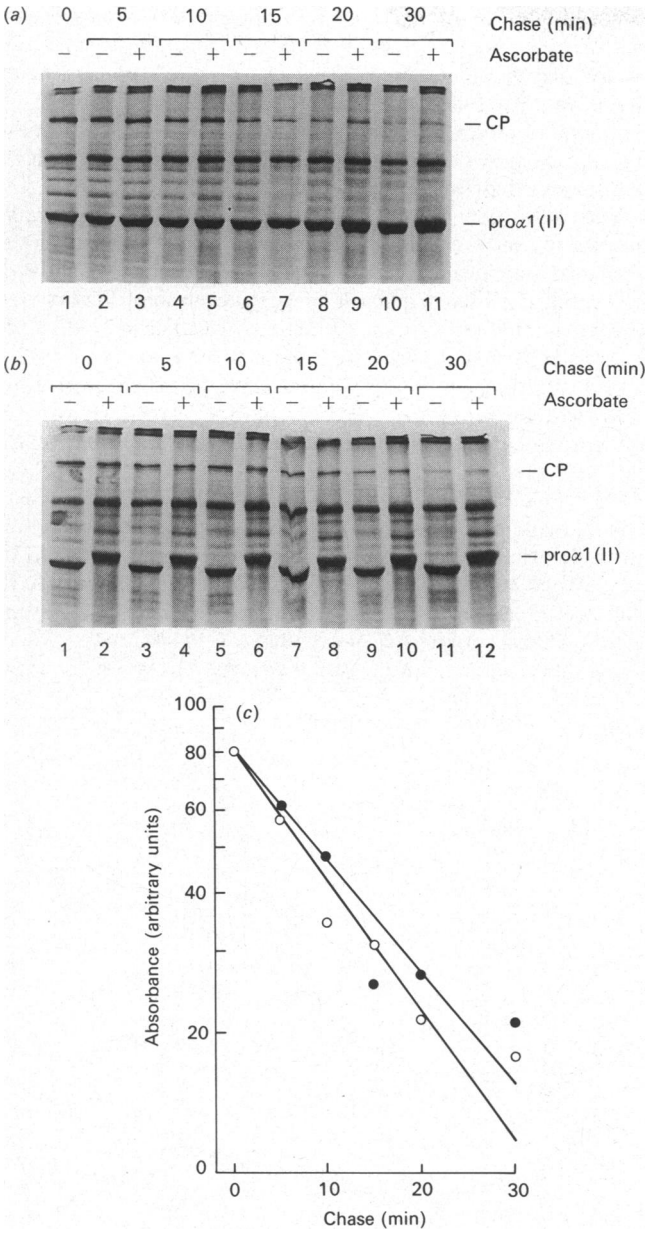

Fig. 4. Analysis of PG-H core protein processing

(*a*) Cultures pulsed for 10 min with [^{3}H]serine were chased in the absence (−) or presence (+) of ascorbate for the indicated periods. Cell homogenates were then analysed by SDS/PAGE and fluorography. (*b*) Cultures were first preincubated in the absence or presence of ascorbate for 3 h and were then pulse–chased as in (*a*). (*c*) The core protein (CP) band in each lane was quantified by video digitization and the absorbance values were plotted as a function of chase time. The estimated half-life of core protein processing was about 12 min in both control (○) and ascorbate-treated (●) cultures.

of types II and IX collagen secretion had changed during ascorbate treatment; see Fig. 3). Scorbutic chondrocyte cultures pulsed for 10 min with [^{3}H]serine were chased in the absence or presence of ascorbate for 5, 10, 15, 20, 30 and 45 min. The cell layers were processed for SDS/PAGE and fluorography. The prominent PG-H core protein band present at the end of the 10 min pulse was rapidly lost during chase (Fig. 4*a*, lanes 1–11). Throughout the chase period examined, the core protein exhibited sensitivity to endoglycosidase H digestion (results not shown), indicating that it was localized in the RER (Kornfeld & Kornfeld, 1980). Fluorograms were scanned with a video digitizer and absorbances of the core protein band were plotted as a function of chase time (Fig. 4*c*). A first-order fit of these data revealed that the core protein had a short intracellular half-life of about 12 min (Campbell & Schwartz, 1988) and that its half-life was not significantly affected by ascorbate treatment. Comparable data were obtained when PG-H core protein was analysed by immunoprecipitation followed by SDS/PAGE (results not shown).

In a second set of experiments, cultures were preincubated with ascorbate for 3 h, at which time the secretion rates of types II and IX collagen had increased to their maximum (Fig. 3). Companion untreated cultures served as a control. Cultures were then pulsed with [^{3}H]serine for 10 min, and chased and analysed as described above. The resulting fluorograms (Fig. 4*b*) and video digitization revealed that the intracellular half-life of PG-H core protein was again similar in both control and treated cells.

We next determined the rates of maturation of PG-H core protein into completed proteoglycan during ascorbate treatment. Cultures preincubated without or with ascorbate for 3 h were pulsed with [^{3}H]serine for 10 min and chased for up to 45 min as above. At each chase time point, PG-H was isolated from the combined medium and cell layer by CsCl gradient centrifugation and analysed by electrophoresis. Video digitization of the resulting fluorograms showed that newly synthesized core protein was rapidly converted into completed PG-H with an estimated half-life of about 10 min, and that this rate of conversion remained essentially unchanged during ascorbate treatment (Fig. 5).

Together, the above data indicate that the kinetics of translocation and processing of PG-H core protein and of its conversion into proteoglycan are not significantly altered during acute ascorbate treatment.

Proteoglycan synthesis and secretion

To substantiate this conclusion, we determined the rates of proteoglycan synthesis and secretion during ascorbate treatment. Chondrocyte cultures incubated in the absence or presence of

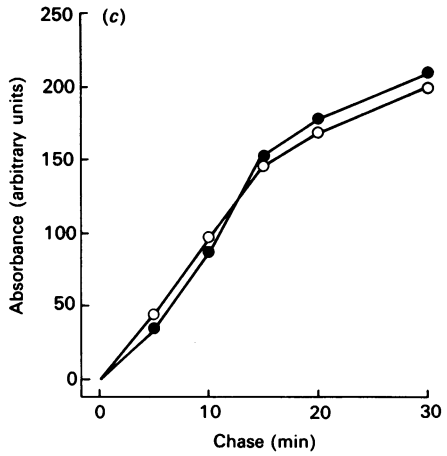

Fig. 5. Kinetics of conversion of PG-H core protein into proteoglycan

Cultures preincubated for 3 h without or with ascorbate were pulsed for 10 min with [^{3}H]serine and chased for up to 45 min. PG-H was isolated from the combined cell layer and medium and separated by sequential CsCl gradient centrifugation and electrophoresis. The PG-H band in the resulting fluorograms was quantified and the absorbance values were plotted as a function of chase time. Note that the rates of conversion of core protein into PG-H are similar in control (○) and ascorbate-treated (●) chondrocytes.

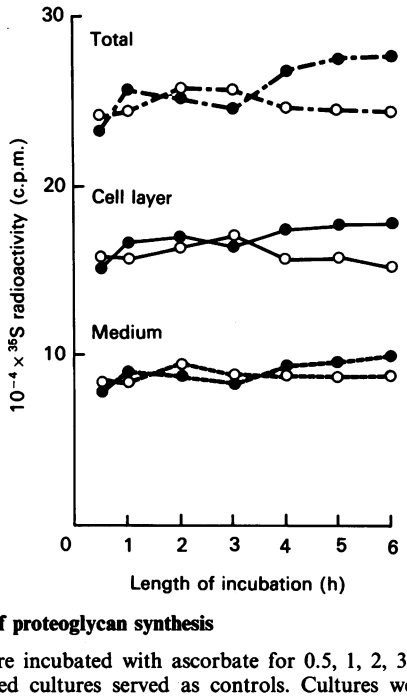

Fig. 6. Rates of proteoglycan synthesis

Cultures were incubated with ascorbate for 0.5, 1, 2, 3, 4, 5 and 6 h; untreated cultures served as controls. Cultures were pulsed with [^{35}S]sulphate during the last 30 min of incubation and radioactivity incorporated into medium-released and cell-layer-associated macromolecules was determined after column chromatography by liquid-scintillation counting. ○, Control cultures; ●, ascorbate-treated cultures.

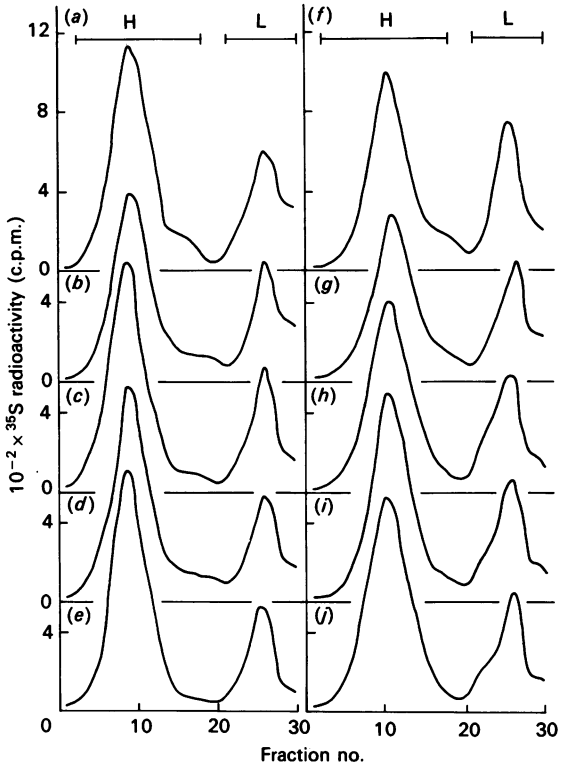

Fig. 7. Analysis of medium-released macromolecules by sucrose gradient centrifugation

Portions of the samples in Fig. 6 were centrifuged on dissociative linear sucrose gradients to separate PG-H (peak H) from PG-Lb and type IX collagen (peak L). *a–e*, 1, 2, 3, 4 and 6 h control samples respectively; *f–j*, 1, 2, 3, 4 and 6 h ascorbate-treated samples respectively.

ascorbate for 0.5, 1, 2, 3, 4, 5 and 6 h were pulsed with [^{35}S]sulphate during the last 30 min of incubation. At each time point examined about one-third of the incorporated radioactivity was found in medium-released macromolecules, and the remaining two-thirds was in cell-layer-associated macromolecules (Fig. 6). Incorporation into medium-released macromolecules remained unchanged throughout ascorbate treatment compared with control values (Fig. 6). Incorporation into cell-layer-associated macromolecules also remained unchanged for the first 2–3 h of treatment but increased slightly thereafter, reaching a new steady-state rate during the last 3 h of treatment (Fig. 6).

Samples of the [^{35}S]sulphate-labelled medium and cell layer samples were fractionated on dissociative sucrose gradients to separate PG-H migrating at the bottom of the gradient (peak H) from the small PG-Lb and type IX collagen that remain together at the top (peak L; see Okayama *et al.*, 1976; Shinomura *et al.*, 1983; Noro *et al.*, 1983). In control medium samples, PG-H accounted for about 70–75% of the total recovered radioactivity (Figs. 7*a–7e*; see also Fig. 9*a*), whereas peak L represented the remaining 25–30% at each time point (Figs. 7*a–e*; see also Fig. 9*b*). No significant change was appreciable in the relative amounts of peak H and L in ascorbate medium samples (Figs. 7*f–7j*; see also Figs. 9*a* and 9*b*).

In the cell layer samples PG-H was also the most abundant macromolecule, accounting however for 80–85% of the recovered radioactivity (Figs. 8*a–8e*); the remainder of the

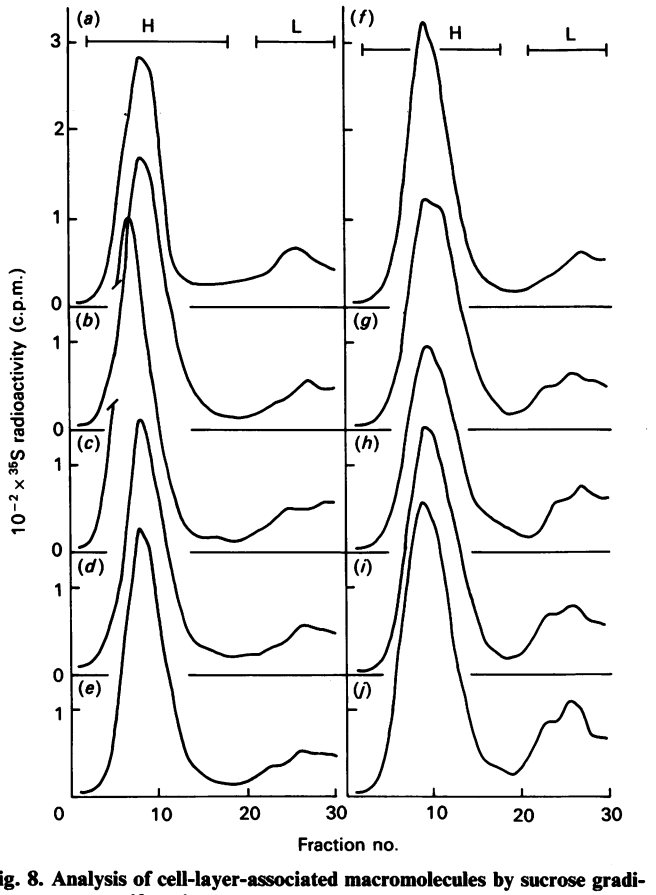

Fig. 8. Analysis of cell-layer-associated macromolecules by sucrose gradient centrifugation

Portions of samples in Fig. 6 were centrifuged on linear sucrose gradients. (*a*)–(*e*) 1, 2, 3, 4 and 6 h control samples respectively; (*f*)–(*j*) 1, 2, 3, 4 and 6 h ascorbate-treated samples respectively. Note in (*h*)–(*j*) the increased proportion of peak L and its bimodal appearance, probably reflecting the presence of both PG-Lb and type IX collagen (see Fig. 10).

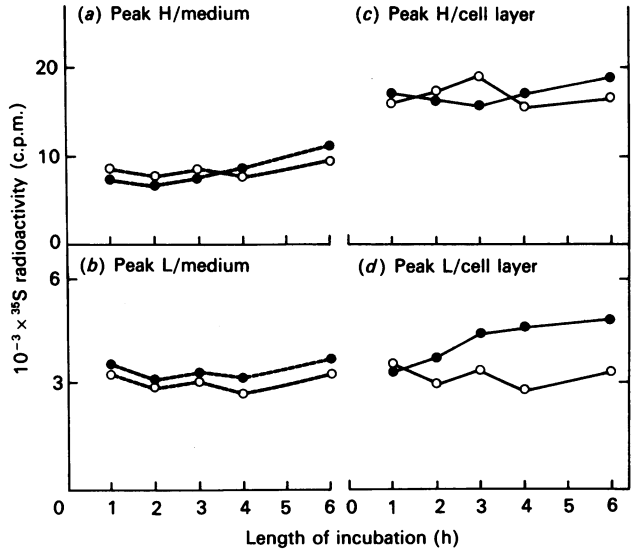

Fig. 9. Quantification of peaks H and L

Radioactivity recovered at each time point under peaks H and L after sucrose gradient centrifugation (Figs. 7 and 8) was plotted as a function of incubation time: ○, control cultures; ●, ascorbate-treated cultures.

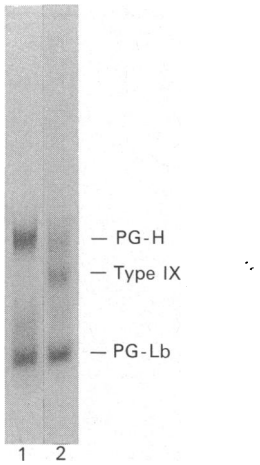

Fig. 10. Electrophoretic analysis of peak L samples

Macromolecules present in peak L from control and ascorbate-treated samples in Figs. 7 and 8 were separated by agarose gel electrophoresis followed by autoradiography and video digitization. The 6 h control and ascorbate-treated cell layer samples are shown to exemplify the results obtained (lanes 1 and 2 respectively). Note the conspicuous amount of type IX collagen in the ascorbate-treated sample (lane 2) relative to control sample (lane 1), the similar levels of PG-Lb in both samples, and the presence of some contaminating PG-H.

radioactivity was in a broad peak L at the top of the gradient. Whereas the relative amounts of peaks H and L did not change during the first 2 h of ascorbate treatment (Figs. 8*f*–8*g*, 9*c* and 9*d*), the proportion of peak L increased significantly with further treatment, from 10–15% to about 25% of the total recovered radioactivity (Figs. 8*h*–8*j* and 9*d*). At these late time points, peak L also had a distinct bimodal appearance.

To clarify whether the increased proportion of peak L during late ascorbate treatment reflected increased levels of PG-Lb or type IX collagen, the top 11 fractions containing peak L from the above sucrose gradients (Figs. 7 and 8) were pooled, and material was analysed by agarose gel electrophoresis. Both control and ascorbate-treated cell layer samples contained similar amounts of PG-Lb at each time point (Fig. 10). However, the ascorbate-treated samples also contained appreciable amounts of type IX collagen starting at around 3 h of treatment (Fig. 10). Similar data were obtained with the medium samples; however, the amount of type IX collagen present was very low (results not shown).

Thus the rates of synthesis and secretion of PG-H and PG-Lb are essentially unaffected by acute ascorbate treatment (see Leboy *et al.*, 1989); similarly, the distribution of these macromolecules between medium and cell layer compartments is also unaffected (Kim & Conrad, 1980, 1982). The ascorbate treatment, however, leads to increased secretion of type IX collagen, the bulk of which remains associated with the cell layer. Both sets of data corroborate well the data on collagen secretion described above.

DISCUSSION

The results of the present study clearly show that, as predicted, translocation and secretion of both types II and IX collagen are readily enhanced by acute exposure of cultured scorbutic chondrocytes to ascorbate. Secretion of PG-H and PG-Lb, however, remains largely unaffected by the treatment. The differential effects of ascorbate treatment on secretion of these four macromolecules strengthens the conclusion that the chondrocyte RER must possess mechanisms able to discriminate between different secretory proteins and, in particular, between procollagens and proteoglycan core porteins.

These data are best interpreted based on the simple assumption that, whereas ascorbate has a specific role in collagen synthesis and processing, it has no known direct role in proteoglycan synthesis. Thus, in the scorbutic culture condition, both types II and IX collagen precursors are synthesized as underhydroxylated molecules (Fig. 2). Both molecules must thus retain an abnormal, non-helical configuration, preventing them from leaving the RER at normal rates and leading to their accumulation in this organelle. Upon treatment with exogenous ascorbate, the molecules are hydroxylated in the RER (Fig. 2), acquire a normal helical configuration and begin to be translocated and secreted at high rates starting at about 2 h of treatment (Fig. 3). On the other hand, the newly synthesized core porteins of PG-H and PG-Lb must acquire a normal configuration regardless of whether the chondrocytes are scorbutic. Their normal configuration must allow them to be translocated from the RER, processed into proteoglycans in the Golgi complex and secreted at normal rates both before and during ascorbate treatment (Figs. 6 and 9).

Other examples have been described of abnormal secretory proteins that are retained in the RER. In some cases the abnormality induced by exogenous treatments or mutations affects the tertiary structure of the protein; in others, it affects the quaternary structure, as in the collagen types studied here. Little, however, is known of the mechanisms which allow the RER to discriminate between normal and abnormal secretory proteins. In particular it is not clear whether the abnormal proteins remain associated with RER resident proteins or fail to be packaged into transport vesicles and reach the Golgi (Rothman, 1989). With regard to the former possibility, prolyl hydroxylases were proposed to have such a role in procollagen secretion (Jimenez & Yankowski, 1978). These RER (Cutroneo *et al.*, 1974; Olsen *et al.*, 1975), ascorbate-dependent (Peterkofsky & Undenfriend, 1965) enzymes have high binding affinity for their underhydroxylated non-helical procollagen substrate, but lose this affinity upon hydroxylation (Juva & Prockop, 1969). Thus in

scorbutic cells prolyl hydroxylases would remain bound to non-helical procollagen molecules, preventing them from leaving the RER. Upon ascorbate treatment, the enzymes would hydroxylate and lose their binding affinity for the procollagen molecules; the newly hydroxylated, helical, procollagen molecules would then be able to be transported out of the RER. The discovery (Pihlajaniemi *et al.*, 1987) that prolyl 4-hydroxylase is closely related to protein disulphide isomerase (another RER-resident protein involved in protein folding; see Rothman, 1989) provides renewed interest and support for this hypothesis.

We have previously shown that increased type II procollagen secretion in chondrocyte cultures during acute ascorbate treatment is accompanied by a striking remodelling of the RER and a redistribution of the procollagen within it (Pacifici & Iozzo, 1988). Although these morphological events may have some role in increased procollagen secretion, they clearly have no effects on either proteoglycan synthesis or secretion as shown here. Vertel and co-workers recently showed that PG-H core protein does not co-localize with type II procollagen within the chondrocyte RER (Vertel *et al.*, 1989). Their data suggest that these two proteins are not randomly distributed in the RER and that different mechanisms regulate their localization within, and translocation from, the RER. Their data and conclusions correlate well with the differential translocation and secretion of procollagens and proteoglycans during ascorbate treatment described here. By being to a large extent physically segregated from procollagen molecules within the RER, proteoglycan core proteins may continue to be translocated and secreted at normal rates despite changes in RER morphology, procollagen distribution and procollagen translocation rates occuring during acute ascorbate treatment.

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