Identification and partial characterization of three low-molecular-weight collagenous polypeptides synthesized by chondrocytes cultured within collagen gels in the absence and in the presence of fibronectin

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Culture of chick-embryo sternal-cartilage chondrocytes within three-dimensional collagen gels promotes the synthesis of three low-molecular-weight collagenous polypeptides. The proportions of these novel collagens synthesized and released into the medium are markedly influenced by the presence or the absence of fibronectin in the serum supplement. Chondrocytes cultured on plastic dishes appear to synthesize only small amounts of these low-molecular-weight species. The three species (designated G, H and J) were characterized with respect to the proportion of [^{14}C]proline incorporated into each polypeptide occurring as hydroxy[^{14}C]proline and with respect to their susceptibilities to bacterial collagenase. On the basis of their electrophoretic mobilities under reducing conditions, the G, H and J polypeptides were calculated to have M_r 59 000, 69 000 and 84 000 respectively. Chymotrypsin digestion converted the G collagen into a species containing polypeptides of M_r 45 000, whereas the H and J polypeptides yielded a single band of M_r 53 000. The H and J polypeptides were found to occur as disulphide-linked aggregates, as was the chymotrypsin-digestion product. Peptide 'mapping' has shown that G, H and J polypeptides show no common identity and are distinct from the known interstitial collagens. Native G collagen was digested by human collagenase to discrete products, whereas H and J chains were not cleaved under identical conditions.

For over a decade it has been recognized that collagen type II represents the major collagenous component of cartilagenous tissues (Miller, 1976), although other collagenous polypeptides, designated 1 α -, 2 α - and 3 α -chains, have been extracted specifically from cartilage (Burgeson & Hollister, 1979). In addition, several distinct low-molecular-weight collagenous species have been demonstrated to occur in pepsin digests of cartilagenous tissues (Shimokomaki *et al.*, 1980, 1981; Ayad *et al.*, 1981, 1982; Reese & Mayne, 1981; Reese *et al.*, 1982; Ricard-Blum *et al.*, 1982; von der Mark *et al.*, 1982). The origins of these latter species are very unclear, for their parent molecules have not been described, and their structures and functions in cartilage remain to be determined.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Studies conducted in our laboratory with chick-embryo sternal-cartilage chondrocytes grown in fibronectin-depleted serum have revealed that culture within a three-dimensional gel of collagen type I results in the synthesis of a major collagenous product, termed G collagen (Gibson *et al.*, 1981, 1982). This novel collagen appears to be distinct from the minor collagenous peptides isolated from avian (Reese & Mayne, 1981; Reese *et al.*, 1982; von der Mark *et al.*, 1982) and mammalian (Burgeson & Hollister, 1979; Shimokomaki *et al.*, 1980, 1981; Ayad *et al.*, 1981, 1982; Ricard-Blum *et al.*, 1982) cartilages. In the present paper we report further on the characterization of G collagen. Evidence is also presented indicating that fibronectin in culture medium promotes the synthesis by cultured chondrocytes of two further low-molecular-weight collagens. These proteins, designated H and J collagens, exhibit properties suggesting that they may be the precursors from which disulphide-linked collagenous peptides have been obtained by pepsin

digestion of chick sternal cartilages (Reese & Mayne, 1981; Reese *et al.*, 1982; von der Mark *et al.*, 1982).

Experimental

Materials

Culture medium, foetal calf serum and antibiotics were obtained from Gibco Bio-Cult, Paisley, Scotland, U.K. Pepsin (EC 3.4.23.1) (1:10 000, from pig stomach mucosa), phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Tes, (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonic acid) and Bes {2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid} were obtained from Sigma Chemical Co., Poole, Dorset, U.K. *Staphylococcus aureus* V8 proteinase (batch no. 0850) was obtained from Miles Laboratories, Slough, Berks., U.K., α -chymotrypsin (EC 3.4.21.1) was purchased from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K., and highly purified bacterial collagenase (EC 3.4.24.3) was purchased from Advanced Biofactures, New York, NY, U.S.A.

L-[U-¹⁴C]Proline (270 mCi/mmol) and L-[5-³H]-proline (23 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Highly purified human collagenase was generously given by Dr. D. E. Woolley, University Hospital of South Manchester, Manchester, U.K.

Preparation of chondrocyte cultures and isolation of newly synthesized medium proteins

Chondrocytes were isolated from 18-day chick-embryo sternal cartilages by trypsin/bacterial collagenase digestion and plated on plastic dishes or within 4 ml collagen (type I) gels at a density of 2.5×10^6 cells per 60 mm culture dish (Gibson *et al.*, 1982). The culture medium used was Dulbecco's modified Eagle's medium buffered to pH 7.2 with 10 mM-Hepes, 10 mM-Bes and 10 mM-Tes, and supplemented with penicillin (100 units/ml) and streptomycin (0.1 mg/ml) plus either foetal calf serum (16%, v/v) or fibronectin-depleted foetal calf serum (16%, v/v) prepared as described previously (Gibson *et al.*, 1982).

Cells maintained in culture for 7 days were labelled for 24 h with [¹⁴C]proline (1.5 μ Ci/ml). The cell/matrix layer was then separated from the medium by centrifugation and washed with cold culture medium. The medium and washings were combined, and the following proteinase inhibitors were added to the final concentrations indicated: phenylmethanesulphonyl fluoride (2 mM), *N*-ethylmaleimide (10 mM), 6-aminohexanoic acid (25 mM) and EDTA (25 mM). Labelled proteins, precipitated by the addition of (NH₄)₂SO₄ to 30% saturation, were recovered by centrifugation, resuspended in 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.4 M-NaCl and

the above proteinase inhibitors, and dialysed extensively against this solution. This high-molecular-weight fraction of newly synthesized proteins is referred to below as labelled medium proteins, and was stored frozen at -20°C before further analysis.

In some experiments labelled medium proteins were subjected to mild pepsin digestion (100 μ g of pepsin/ml of 0.5 M-acetic acid for 16 h at 4°C) or chymotrypsin digestion (300 μ g of chymotrypsin/ml of 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.4 M-NaCl for 6 h at 22°C).

Separation of novel low-molecular-weight collagenous polypeptides from medium proteins

Medium proteins precipitated from culture medium with (NH₄)₂SO₄ at 30% saturation were further fractionated under non-denaturing conditions. Labelled medium proteins containing added carrier type I collagen (0.25 mg/ml) in NaCl/Tris buffer were dialysed first against 0.5 M-acetic acid containing pepstatin (0.5 μ g/ml) and then against 0.5 M-acetic acid containing 0.8 M-NaCl and pepstatin (0.5 μ g/ml) at 4°C. Precipitated proteins containing interstitial collagen types I and II, and their precursors, were isolated by centrifugation and redissolved in 0.5 M-acetic acid containing pepstatin. Both the precipitated collagens and supernatant were then dialysed extensively against an acetic acid/pepstatin solution at 4°C. Low-molecular-weight collagens free from contamination with other collagens were obtained in the supernatant fractions and were recovered either by freeze-drying or by precipitation from acidic solution with 2 M-NaCl.

DEAE-cellulose chromatography was also used to purify some of the low-molecular-weight collagens. Labelled medium proteins containing carrier type I collagen (0.25 mg/ml) in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl were applied to a DEAE-cellulose (Whatman DE-52) column (1.5 cm $\times$ 10 cm) equilibrated at 4°C with 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl. The column was washed with the same buffer, and bound proteins were eluted with 0.05 M-Tris/HCl buffer, pH 7.4, containing 1 M-NaCl. Fractions (2 ml) were collected and samples were analysed for radioactivity by liquid-scintillation spectrometry. Peaks of radioactivity were pooled, dialysed against distilled water and freeze-dried.

Electrophoretic and molecular-weight analyses

The nature of the labelled polypeptides synthesized by cultured chondrocytes was examined by discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Samples were denatured by heating at 100°C for 2 min in sample-preparation buffer containing 2% (w/v) SDS, and either 5% (v/v) mercaptoethanol or 0.1 M-iodoacetamide. Electrophoresis was performed on slab gels, which con-

sisted of a separating gel (15 cm × 15 cm × 0.3 cm) and a stacking gel (15 cm × 1 cm × 0.3 cm). Electrophoresis was performed in a water-cooled apparatus at 28 mA/slab gel. Gels were fixed in 10% (v/v) acetic acid/25% (v/v) methanol and then impregnated with 2,5-diphenyloxazole for fluorography, dried and exposed to pre-flashed Kodak Royal X-Omat X-ray film XH-1 (Bonner & Laskey, 1974; Laskey & Mills, 1975).

The molecular weights of newly synthesized collagenous polypeptides and their enzymic digestion products were determined by SDS/polyacrylamide-gel electrophoresis by comparison with radio-labelled $\alpha 1(I)$ -chains and peptides derived therefrom of known molecular weights. Radiolabelled type I collagen was prepared from 17-day chick-embryo leg tendons that had been incubated in modified Krebs medium (Dehm & Prockop, 1971) for 1 h with [3H]proline and 'chased' for 4 h in the presence of unlabelled proline (100 μ g/ml). The 3H -labelled collagen was extracted into 0.1 M-acetic acid, and $\alpha 1(I)$ -chains were purified by CM-cellulose chromatography (Piez *et al.*, 1963). Native type I 3H -labelled collagen was incubated with human collagenase to yield the digestion products fragment TC_A (M_r 73 500) and fragment TC_B (M_r 24 500), and the labelled $\alpha 1(I)$ -chain was incubated with CNBr (Gibson *et al.*, 1982) to provide the following peptides: $\alpha 1$ -CB5,8,3 (M_r 42 000), $\alpha 1$ -CB8 (M_r 25 000), $\alpha 1$ -CB7 (M_r 24 000), $\alpha 1$ -CB6AB (M_r 17 500) and $\alpha 1$ -CB3 (M_r 13 500).

Peptide 'mapping'

In order to compare the primary structures of the low-molecular-weight collagens and other medium proteins, they were subjected to SDS/polyacrylamide-disc-gel electrophoresis combined with V8 proteinase digestion and subsequent peptide 'mapping' in a second dimension by using a modification of the techniques of Cleveland *et al.* (1977) and Lizardi (1979). Labelled medium proteins were denatured under reducing conditions and separated in an 8% polyacrylamide gel with a 3% stacking gel. A slab gel consisting of a 15% polyacrylamide separating gel and 3% stacking gel was prepared in bevelled plates similar to those described by O'Farrell (1975). The disc gel containing electrophoresed labelled medium proteins was placed horizontally on this slab gel and fixed in place with a solution of 1% agarose (10 ml) containing 2.5 mg of V8 proteinase together with Bromophenol Blue tracking dye. Electrophoresis was performed at 20 mA/gel until the dye front reached the separating gel. The power was switched off for 60 min to allow enzyme digestion to occur, and then the resulting peptides were separated by continuing the electrophoretic separation until the dye front reached the bottom of

the gel. The peptides were detected by fluorography as described above.

Results

Effect of fibronectin on the synthesis of [^{14}C]-proline-labelled polypeptides by cultured chondrocytes

Chondrocytes were cultured on plastic dishes or within collagen gels in the presence or in the absence of fibronectin as described in the Experimental section. After 1 week in culture, the cells were incubated for 24 h with [^{14}C]proline, and the newly synthesized proteins released into the medium were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography (Fig. 1). High-molecular-weight collagenous polypeptides migrating in positions corresponding to pro- α -, p- α - and $\alpha 1(II)$ -chains were synthesized under all the culture conditions employed. The appearance of a radio-labelled band migrating in the position of $\alpha 2(I)$ -chains in medium from cells grown on plastic in the presence of fibronectin (Fig. 1, track 3) is consistent with previous reports indicating a switch from type-II to type-I collagen synthesis by chondrocytes cultured under these conditions (West *et al.*, 1979; Gauss & Muller, 1981). In addition, three components of lower molecular weight were also detected, but their synthesis was clearly influenced by the cellular environment. The previously reported G collagen (Gibson *et al.*, 1981, 1982) was synthesized in large amounts by chondrocytes grown in collagen gels with fibronectin-depleted serum (Fig. 1, track 2), and was also seen after growth in collagen gels with normal serum. However, only trace amounts of G collagen were detected in cultures grown on plastic in the absence of fibronectin, and with fibronectin present little or none was seen.

The other low-molecular-weight components, designated H and J polypeptides, appeared to be synthesized simultaneously, and in approximately equal proportions. The conditions most favourable for their synthesis were growth in collagen gels with normal serum (Fig. 1, track 4), but easily detectable amounts of these components were also present after growth on plastic in the presence or in the absence of fibronectin. Only trace amounts of H and J collagenous polypeptides were detected after growth in collagen gels with fibronectin-depleted serum. The total amounts of H and J species synthesized under these conditions appears to vary markedly with the time period of culture (G. J. Gibson, unpublished work).

Isolation of G, H and J chains and demonstration of their collagenous nature

The three low-molecular-weight species G, H and

J were separated from the high-molecular-weight [^{14}C]proline-labelled medium proteins by differential salt precipitation. Medium proteins from cultures grown on plastic for 1 week in the absence of fibronectin were dissolved in 0.5 M-acetic acid and the solution was made 0.8 M in NaCl. Under these

Fig. 1. Fluorogram of [^{14}C]proline-labelled medium proteins synthesized by cells cultured in the presence and in the absence of fibronectin on plastic dishes and in collagen gels for 1 week

Cells were labelled for 24 h with [^{14}C]proline, and the medium proteins precipitable at 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ were analysed by electrophoresis on an 8%-polyacrylamide gel. Samples from cultures grown on plastic are in tracks 1 and 3, and samples from cultures grown in collagen gels are in tracks 2 and 4. The cultures used in tracks 1 and 2 were maintained in medium supplemented with fibronectin-depleted serum, whereas the cultures in tracks 3 and 4 were maintained in medium supplemented with complete foetal calf serum. Track 5 contains a standard of [^3H]acetylated rat tail tendon collagen, in which the $\alpha 2(\text{I})$ -chain is poorly labelled. The short-chain collagenous polypeptides G, H and J are arrowed, and the migration positions of standard $\alpha 1(\text{II})$ - and $\alpha 2(\text{I})$ -chains are indicated.

conditions high-molecular-weight species were precipitated, and a preparation containing H and J species was recovered from the supernatant fraction (Fig. 2). Similar treatment of medium proteins from cultures grown in collagen gels for 1 week in the presence (results not shown) or in the absence (Fig. 2) of fibronectin yielded a fraction rich in G, H and J chains. The recovery of the G collagen species was occasionally found to be poor, and this may be explained by its tendency to form high-molecular-weight aggregates that are precipitated at 0.8 M-

Fig. 2. Fluorogram of low-molecular-weight [^{14}C]proline-labelled collagens electrophoresed under reducing and non-reducing conditions

Cells were cultured for 1 week on plastic and in collagen gels in medium containing fibronectin-depleted serum before labelling for 24 h with [^{14}C]proline. Medium proteins were fractionated by differential salt precipitation, and the low-molecular-weight species present in the 0.8 M-NaCl supernatant were analysed on 8% polyacrylamide gels. The samples in track 1 (reduced) and track 2 (unreduced) from cultures grown on plastic were rich in H and J species, with only a trace of α -chains. The samples in track 3 (reduced) and track 4 (unreduced) from cultures grown in collagen gels contain G, H and J chains, with a trace of a polypeptide migrating in a α -chain position.

Fig. 3. Ion-exchange chromatography of [^{14}C]proline-labelled collagenous polypeptides

Medium proteins were subjected to ion-exchange chromatography on DEAE-cellulose as described in the text. (a) Elution profile of radioactivity. The arrow indicates the point at which buffer containing 1 M-NaCl was applied to recover polypeptides bound to the column. (b) Electrophoretic and fluorographic analysis of (i) sample applied to the column, (ii) unbound fraction eluted in peak I, and (iii) bound fraction eluted in peak II. Species of interest are arrowed.

NaCl from acidic solution (G. J. Gibson & M. E. Grant, unpublished work). When these samples containing the low-molecular-weight species were analysed under non-reducing conditions, the H and J chains were found to occur as a doublet of disulphide-linked aggregates, which just migrated into the 6.5%-polyacrylamide gel. In contrast, the G collagen exhibited the same electrophoretic mobility under both reducing and non-reducing conditions.

It was also possible to separate the low-molecular-weight species H and J from the higher-molecular-weight species by ion-exchange chromatography. When labelled medium proteins were applied in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl to a DEAE-cellulose column, the type II collagen and its precursors were not retained, whereas the H and J species were bound and could be eluted with buffer containing 1.0 M-NaCl (Fig. 3). In these samples small amounts of G collagen were initially present, but these were not detectable in the fractions recovered during ion-exchange chromatography.

The [^{14}C]proline-labelled peak containing only H and J chains, as judged by electrophoretic and fluorographic analyses, was assayed for hydroxy-[^{14}C]proline (Juva & Prockop, 1966). Two separate preparations were found to contain 34% and 36.5% respectively of the ^{14}C as 4-hydroxy[^{14}C]proline. In subsequent studies, where H and J chains were separated on cylinder SDS/polyacrylamide gels and

the hydroxy[^{14}C]proline content was determined in 1 mm slices of the gel, it was demonstrated that both components were rich in hydroxy[^{14}C]proline and may be considered as polypeptides containing collagenous sequences. Thus in band H 42.7% of the radioactivity was present as hydroxy[^{14}C]proline and in band J 33.9% was so present. These values can be compared with the value of 42% hydroxylation of proline residues in G collagen, as determined previously (Gibson *et al.*, 1982).

Further evidence for the collagenous nature of H and J chains was obtained in experiments demonstrating their susceptibility to digestion by highly purified bacterial collagenase (Fig. 4). All three low-molecular-weight collagenous polypeptides were digested to small peptides.

Determination of the molecular weights of the G, H and J collagenous polypeptides and their chymotrypsin-digestion products

The molecular weights of the G, H and J collagenous polypeptides were determined by SDS/polyacrylamide-gel electrophoresis, with the use of the peptides of type I collagen produced by mammalian collagenase digestion or CNBr treatment as standards. On the basis of their electrophoretic mobilities, G, H and J collagenous polypeptides were calculated to have M_r approx. 59 000, 69 000 and 84 000. As shown in Fig. 5, chymotryptic digestion caused the conversion of type II

Fig. 4. Effect of bacterial collagenase on medium proteins isolated from cultures grown in collagen gels. $[^{14}\text{C}]$ Proline-labelled medium proteins isolated from cells grown in collagen gels in serum-containing medium were prepared as described in the text. A sample was dialysed against 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.4 M-NaCl, 10 mM- CaCl_2 , 2 mM-phenylmethanesulphonyl fluoride and 10 mM-N-ethylmaleimide. To half the sample was added 250 units of highly purified bacterial collagenase, and the enzyme digest and control were incubated for 24 h at 36°C. The reaction was stopped by the addition of EDTA to 10 mM, and samples were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. The fluorogram was scanned with an LKB laser densitometer at 632.8 nm. —, Control sample; ----, digested sample.

collagen precursors into collagen molecules that migrate as α -chains after denaturation. Similarly chymotrypsin digestion caused the increased migration of the low-molecular-weight collagens. In previous studies we have reported that the M_r of the isolated G collagen was decreased to 45 000 by pepsin digestion (Gibson *et al.*, 1982), and a species of identical electrophoretic mobility is obtained after chymotrypsin treatment. Chymotrypsin digestion of the isolated H and J collagenous polypeptides gave under reducing conditions a single band of M_r 53 000 (Fig. 5). Also noteworthy is the fact that the unreduced chymotrypsin-derived product of H and J chains migrated as a somewhat diffuse band with a mobility slightly faster than that of the undigested unreduced sample (Fig. 2) but still slower than that of β -chains of type I collagen (Fig. 5).

Fig. 5. Effect of chymotrypsin on the migration of H and J chains electrophoresed under reducing and non-reducing conditions.

Medium proteins from cells cultured on plastic in fibronectin-depleted serum and labelled with $[^{14}\text{C}]$ -proline were separated by SDS/polyacrylamide-gel electrophoresis and analysed by fluorography. (a) Medium proteins. (b) Medium proteins digested with chymotrypsin. The migration positions of standards of $\beta 1,1(\text{I})$ -chains, $\alpha 1(\text{II})$ -chains, H and J chains are indicated. —, Reduced sample; ----, unreduced sample.

Peptide 'mapping' of the $[^{14}\text{C}]$ proline-labelled medium proteins

In order to investigate the relationship, if any, between the collagenous polypeptides released into culture medium by the chondrocytes cultured within collagen gels, the medium proteins were subjected to digestion with *Staphylococcus aureus* V8 proteinase. Electrophoretic separation of the polypeptides in one dimension was followed by enzymic digestion and subsequent electrophoresis in a second dimension. Fluorographic analysis of the peptide 'maps' revealed a considerable similarity in the peptides derived from the α -chains and higher-molecular-

Fig. 6. Peptide 'mapping' of [^{14}C]proline-labelled medium proteins

Medium proteins obtained from a culture of cells grown in collagen gels in the presence of fibronectin-containing medium were denatured in SDS in the presence of mercaptoethanol and separated by electrophoresis in an 8% polyacrylamide gel (upper frame). The disc gel containing electrophoresed proteins was embedded in a 15%-polyacrylamide slab gel and subjected to digestion with *S. aureus* V8 proteinase as described in the text. The resulting peptides were separated by electrophoresis and detected by fluorography. The migration positions of the collagenous polypeptides of interest and peptides derived therefrom are indicated.

weight polypeptides, supporting the suggestion that these polypeptides are predominantly the collagen type II precursor chains, namely pro- α -chains and p- α -chains. In contrast, the V8-proteinase-digest peptides of the low-molecular-weight G, H and J collagenous polypeptides show no common identity (Fig. 6). A similar conclusion is indicated in preliminary studies of chymotryptic peptides of these collagenous polypeptides (results not shown).

Influence of mammalian collagenase on chondrocyte-derived collagens

Further evidence that species G, H and J are unrelated to the well-characterized interstitial col-

lagens was obtained in studies of their susceptibilities to purified human collagenase. The G collagen was initially cleaved to products of M_r approx. 51 000, 32 000 and 9000 (Fig. 7a, track 2). With longer periods of incubation the intensity of the band at M_r 32 000 (designated band GpA; Fig. 7a, tracks 3 and 4) increased, and the mobility of the low-molecular-weight product also increased slightly to yield a component designated GpB. The intermediate degradation product of M_r 51 000 was occasionally seen in some preparations of the G collagen recovered from culture medium, and faint traces are detectable in the controls (Fig. 7a, tracks 1 and 5). When the pepsin-derived Gp collagen was similarly treated with human collagenase, it too was degraded to species GpA and GpB, of M_r 32 000 and 8000 respectively (Fig. 7b). In the 4 h digest there was evidence for two species of sizes intermediate between Gp and GpA, suggesting that cleavage was occurring at more than one site.

The susceptibilities of the collagenous polypeptides H and J were examined under conditions identical with those that had been used to cleave G collagen, Gp collagen and also collagen types I and II. However, in these experiments no evidence for cleavage of either H or J chains was obtained (results not shown).

Discussion

Although fibronectin is reported to be absent from normal cartilage (Dessau *et al.*, 1978; Stenman & Vaheri, 1978), it does appear to be synthesized by chondrocytes in the early stages of culture (Dessau *et al.*, 1978), and addition of fibronectin to culture medium has marked effects on the phenotypic expression of chondrocytes in culture (Pennypacker *et al.*, 1979; West *et al.*, 1979). In the present paper we have demonstrated that the absence or the presence of fibronectin in culture medium has a strong influence on the synthesis by chick-embryo sternal-cartilage chondrocytes of three novel low-molecular-weight collagenous molecules.

Culture of chondrocytes in collagen gels promotes the synthesis of low-molecular-weight collagenous polypeptides. The smallest of these species, G collagen, has been previously detected in cultures maintained in fibronectin-depleted serum (Gibson *et al.*, 1981, 1982). Trace amounts of the other two low-molecular-weight species, H and J chains, were subsequently detected in such cultures. However, the inclusion of fibronectin in the medium markedly increased the amounts of H and J chains synthesized (Fig. 1). No detailed characterization of the higher-molecular-weight collagens has been undertaken, but preliminary studies suggest that some type I collagen, type I trimer and 1 α -, 2 α - and 3 α -collagens are synthesized after 1 week of culture

Fig. 7. Effect of human collagenase on G collagen and its pepsin-derived product Gp collagen

Native G collagen was prepared and purified from medium of cells cultured for 3 weeks in collagen gels in fibronectin-depleted medium (Gibson *et al.*, 1982). The pepsin-derived product, Gp collagen, was prepared by using the conditions described in the text. Radiolabelled type I and type II collagens were prepared by incubating 17-day chick-embryo leg tendons and sternal cartilages respectively with [14 C]proline in modified Krebs medium (Dehm & Prockop, 1971) containing β -aminopropionitrile (50 μ g/ml) followed by extraction into 0.1 M-acetic acid. The G, Gp, type I and type II collagens were digested with human collagenase for up to 24 h at 25°C in 0.1 M-Tris/HCl buffer, pH 8.0, containing 10 mM- CaCl_2 . The products were separated by electrophoresis on an SDS/15%-polyacrylamide slab gel and detected by fluorography. (a) Samples in tracks are: 1, G collagen; 2, 3 and 4, G collagen digested with human collagenase for 4 h, 18 h and 24 h respectively; 5, G collagen control digest for 24 h without enzyme; 6, partial human collagenase digest of type I collagen; 7, Gp collagen and a trace of $\alpha 1(\text{II})$ -chains. (b) Samples in tracks are: 1, G collagen; 2, Gp collagen; 3, 4 and 5, Gp collagen digested with human collagenase for 4 h, 18 h and 24 h respectively; 6, Gp collagen control digest for 24 h without enzyme; 7, type II collagen; 8, 9 and 10, type II collagen digested with human collagenase for 4 h, 18 h and 24 h respectively; 11, type II collagen control digest for 24 h without enzyme. Species and digestion products of interest (see the text) are arrowed.

on the plastic substrate in fibronectin-containing medium (G. J. Gibson, unpublished work).

The three low-molecular-weight species, G, H and J, all appear to contain collagenous and non-collagenous domains, as judged by their decreased molecular size after mild peptic or chymotryptic digestion. In the chondrocyte cultures, no evidence has been obtained for the G, H or J collagenous polypeptides undergoing a procollagen-into-collagen conversion analogous to that for cartilage type II procollagen. The triple-helical region of these collagens, seen as proteinase-resistant peptides on gel-electrophoretic analysis, is much smaller than that of the interstitial collagens. Consequently one might predict that for such molecules to be assembled and secreted at 37°C they might possess proline- and hydroxyproline-rich collagenous sequences similar to those that provide a very stable

helical segment in the N-propeptides of type I procollagen (Engel *et al.*, 1977). Some support for this prediction is seen in the high percentage of [^3H]proline occurring as hydroxyl[^3H]proline in the pepsin-derived Gp polypeptide (Gibson *et al.*, 1982). An additional factor in the stabilization of the H and J chains may be the involvement of interchain disulphide bonds, some of which occur within the chymotrypsin-resistant peptides (Fig. 5). The absence of disulphide bonds between the polypeptides of the G collagen is a noteworthy observation that raises questions about the mode of assembly of the G-collagen helix. In the case of interstitial procollagens interchain disulphide-bond formation appears to be a factor determining helix formation (Grant & Jackson, 1976). However, although the G collagen has the characteristics of a procollagen-like molecule with proteinase-sensitive extension pep-

tides, no evidence has been obtained for a higher-molecular-weight disulphide-linked precursor of this species.

The G collagen polypeptides can be clearly distinguished from H and J chains and from the various low-molecular-weight pepsin-derived collagenous peptides from cartilaginous tissues (Shimomaki *et al.*, 1980, 1981; Ayad *et al.*, 1981, 1982; Reese & Mayne, 1981; Reese *et al.*, 1982; Ricard-Blum *et al.*, 1982; von der Mark *et al.*, 1982) by its non-disulphide linked nature, by its susceptibility to CNBr and by its molecular weight. In addition, peptide 'mapping' has shown that the polypeptides G, H and J are all distinct species and are unrelated to higher-molecular-weight components in the culture medium. H and J polypeptides are found to be co-purified by salt precipitation procedures and ion-exchange chromatography, and they exhibit disulphide aggregation behaviour. Their presence in approximately equal amounts in culture medium, as judged by radiolabelled-proline labelling, and the detection of a high-molecular-weight doublet on SDS/polyacrylamide-gel electrophoresis under non-reducing conditions argue against the occurrence of H and J chains in a single molecular species. However, chymotryptic digestion of preparations containing H and J chains yields polypeptides of M_r approx. 53 000 that remain disulphide-linked and on electrophoresis under non-reducing conditions migrate as a single band of high molecular weight. Peptides having similar properties to the chymotryptic products of H and J chains have been described in peptic digests of chicken hyaline cartilage (Reese & Mayne, 1981; Reese *et al.*, 1982; von der Mark *et al.*, 1982). Particularly noteworthy is their lack of susceptibility to digestion with mammalian collagenase (Liotta *et al.*, 1982), which accords well with the resistance of H and J chains to this enzyme.

The susceptibility of the native G collagen to mammalian collagenase digestion has provided an opportunity to propose a working model for its structure. The products of digestion of G collagen (Fig. 7a) indicate that more than one site is being cleaved, but it is possible that non-helical regions are being digested by non-specific proteolytic activity of the collagenase preparations during the longer incubation periods. These studies demonstrate that mammalian collagenase cleaves the helical region of the G collagen in a manner analogous to that of the cartilage type II collagen (Harris & McCroskery, 1974), yielding fragments of approximately three-quarters and one-quarter length. If the assumption is made that the site of cleavage by mammalian collagenase is towards the C-terminus, as for interstitial collagens, then the molecule can be envisaged as having a helical segment of M_r approx. 40 000–45 000 with a large proteinase-sensitive

peptide (M_r approx. 13 000–19 000) at the N-terminus and a short non-helical extension at the C-terminus. Rigorous determinations of molecular weight other than those based on electrophoretic mobility will be necessary to establish the exact size of the various domains.

Further studies will also be needed to assess the function and location of these novel collagens in cartilage. Preliminary experiments have demonstrated that chick-embryo sternal cartilage is capable of the synthesis of G, H and J chains in organ culture (C. M. Kielty, S. L. Schor & M. E. Grant, unpublished work). Evidence is also accumulating to suggest that these collagens may have quite separate roles, for the G collagenous polypeptide is synthesized only by chondrocytes isolated from the presumptive calcification region of 18-day chick-embryo sterna (G. J. Gibson & M. Flint, unpublished work). In contrast, cells isolated from other regions of the sterna do not synthesize the G collagenous polypeptide but do secrete large amounts of the H and J collagenous polypeptides. The influence of extracellular-matrix macromolecules in the attachment and proliferation of chondrocytes at different stages of development will be an important aspect of future work seeking to understand the regulatory role of the matrix on chondrocyte gene expression.

Note added in proof (received 24 February 1983)

Since the completion of this work Schmid & Conrad (1982a,b) have reported on a low-molecular-weight collagen secreted by cultured chondrocytes derived from the tibiotarsus of 12-day chick embryos. This collagen comprising polypeptides of M_r 59 000 exhibits all the properties of G collagen described in the present paper and our earlier publications (Gibson *et al.*, 1981, 1982).

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