Effects of Matrix Macromolecules on Chondrocyte Gene Expression: Synthesis of a Low Molecular Weight Collagen Species by Cells Cultured within Collagen Gels

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ABSTRACT Chick-embryo sternal chondrocytes have been cultured within three-dimensional collagen gels as part of a study concerned with the effects of extracellular matrix macromolecules on chondrocyte gene expression. Data are presented indicating that chondrocytes cultured within such a collagenous environment synthesize significantly more of an hitherto unidentified, low molecular weight collagen species than do cells grown on plastic tissue-culture dishes in the conventional manner. This low molecular weight collagen species contains noncollagenous domains (as indicated by its decreased molecular size after mild pepsin digestion), is distinct from the known collagen types (as judged by CNBr peptide analysis), and forms part of the insoluble collagenous matrix produced by the chondrocytes. Cells growing within the gel tend to form colonies consisting of a linear array of cells reminiscent of the cellular organization in growth cartilage.

Although chondrocytes explanted to plastic tissue-culture dishes initially synthesize cartilage-specific macromolecules, it has proved difficult to maintain the expression of a differentiated phenotype during subsequent culture in vitro. The common observation is that chondrocytes in vitro tend to assume a fibroblastoid morphology with a concomitant switch from the predominant synthesis of type II to type I collagen (26).

A number of culture parameters have been observed to affect chondrocyte behaviour in vitro. For example, Von der Mark et al. (39) have demonstrated that cell density and position within a colony influence the ability of chondrocytes to synthesize a cartilage-specific matrix. Cell-produced matrix macromolecules may play an important role in this regulatory process since the presence of exogenous collagen (23), proteoglycan (17, 18) and fibronectin (32, 40) have all been observed to influence the quantity of cartilage-specific components synthesized by chondrocytes in vitro. Indeed, the differentiation of a number of cell types including epidermal basal cells (29), hepatocytes (34), mammary epithelial cells (41) and fibroblasts (14) has been shown to be affected by culture on a collagen substratum.

The present study was initiated to investigate further the effects of a collagen substratum on the synthesis of different collagen species by chondrocytes in vitro. Type II collagen, which consists of three α1(II) chains, is the predominant collagen species found in hyaline cartilage (27). In recent years, lesser quantities of other collagen species have been identified in cartilage (1, 8, 37). In this communication, data are presented comparing the growth characteristics, morphology, and collagen biosynthesis of chondrocytes cultured within three-dimensional type I collagen matrices and on plastic tissue-culture dishes. We find that cells cultured within the collagen matrix synthesize significantly greater quantities of a low molecular weight collagen species.

MATERIALS AND METHODS

Cell Culture

MEDIUM AND SUBSTRATA: Chick embryo chondrocytes have been cultured on plastic and in collagen gels prepared according to the method of Schor and Court (36). Collagen was prepared from rat-tail tendons by extraction with 0.1 M acetic acid (34) and was dialyzed against distilled water before use. The tissue culture medium used throughout was Dulbecco's Modified Eagle's medium (DME) buffered to pH 7.2 with 10 mM HEPES, 10 mM BES and 10 mM TES, and supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (DME medium) and 16% (vol/vol) fetal calf serum. In the majority of experiments fibronectin was removed from the fetal calf serum before use by gelatin-agarose

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affinity chromatography (12). In this procedure, serum (50 ml) was applied to the affinity column previously equilibrated at 4°C with modified Krebs phosphate buffer (10), and the column was then eluted with DME medium (250 ml). The fibronectin-free serum so recovered from the column was filter-sterilized and stored at 4°C before use. Bound fibronectin was eluted with 0.05 M-Tris-HCl buffer (pH 7.5) containing 6 M-urea. In studies of collagen synthesis by cultured chondrocytes, the DME medium was supplemented with nonessential amino acids 0.1 mM-proline, serine, glutamate, glycine, aspartate, asparagine, and alanine (Flow Laboratories Ltd., Irvine, Scotland), but in studies of the type of collagen synthesis only, 24 h before incubation with isotope the cells were returned to unsupplemented DME medium.

CELLS: Chondrocytes were prepared by enzymic digestion of sternal cartilages dissected from 18-d-old chick embryos. Sterna were dissected free of surrounding perichondrium, and any remaining perichondrial fibroblasts were released by incubation of the tissue with bacterial collagenase [type 1A, Sigma Chemical Co. Ltd., London, England.] (10 mg/ml) and trypsin (3 mg/ml) in DME for 30 min at 37°C. Sternal tissue washed free of contaminating fibroblasts was then chopped and digested with bacterial collagenase (10 mg/ml) and trypsin (3 mg/ml) for 90 min at 37°C. Released chondrocytes were washed three times with culture medium before cells were plated either on plastic Petri dishes (30-mm Diam) at 5×10^5 cells/dish (0.7×10^5 cells/cm^2) or in 1 ml collagen gels at 5×10^5 cells/gel. Cultures in collagen gels were prepared as described by Schor (35) and consisted of 1 ml of gel overlaid with 1 ml of medium. Cell cultures were incubated in 5% CO_2/95% air at 37°C. The medium was changed after 24 h and then at intervals of 48 h with medium supplemented with 25 μg ascorbate/ml.

CELL NUMBER: Cell numbers were determined as described by Schor (35). After selected times of culture, cells grown on plastic substrata were released by incubation with 0.05% (wt/vol) trypsin and 2 mM-EDTA in phosphate-buffered saline (PBS) (2 ml) for 5 min at 37°C. The cell suspension was collected, the dishes were rinsed with culture medium (3 ml), and the washings were combined with the cell suspension and diluted with DME medium to give a final volume of 10 ml. Chondrocytes cultured in collagen gels were recovered by incubation for ~2 h at 37°C in DME medium containing bacterial collagenase (1 mg/ml). Cells were collected by centrifugation, resuspended in 0.05% trypsin and 2 mM EDTA in PBS, and incubated for 5 min at 37°C before adding medium to a final volume of 10 ml. Cells were counted with a Coulter electronic counter (Coulter Electronics, Inc., Hialeah, FL).

Determination of Rates of Collagen Synthesis

The incorporation of [5-^{3}H]proline into peptidyl-hydroxy[^{3}H]proline was used to determine the rate of collagen synthesis by cells maintained in culture for up to 21 d. Since cultures in collagen gels contained a considerable volume of unreplaced medium (~1 ml) within the gel itself, initial adjustment for the corresponding dilution of isotope was made by the addition of an equivalent volume of medium to cultures on plastic. The radioactivity (cpm/ml) of the medium was monitored after 30 min, and any difference between cultures on plastic and in gels was compensated by the addition of unlabeled medium. After incubation for 6 h with [5-^{3}H]proline (10 μCi/ml) at 37°C, the chondrocytes and matrix were scraped from the dishes, separated from the medium by centrifugation, and washed three times with culture medium. Cells plus matrix were homogenized in medium (2 ml), and peptides were precipitated by the addition of trichloroacetic acid to 10% (wt/vol) in the presence of proline (1 mg/ml). The pooled medium and washes were similarly treated, and the precipitates were kept at 4°C overnight. Precipitates were washed (three times) with 5% (wt/vol) trichloroacetic acid containing proline (1 mg/ml), washed twice with ethanol/ether (1:1 by vol), dried in air at 22°C, and then hydrolyzed in 6 M HCl at 110°C for 18 h. Total incorporation of [^{3}H]proline was determined by scintillation counting, and the synthesis of hydroxy[^{3}H]proline was determined by a specific radiochemical assay (22). Control experiments indicated that <0.005% of exogenous [^{3}H]proline bound nonspecifically to the collagen gel.

Isolation of Newly Synthesized Collagens

Cells maintained in culture on plastic or in collagen gels for up to 3 wk in DME medium supplemented with fibronectin-free serum were labeled at selected times for 24 h with [^{3}H]proline (20 μCi/ml). Cells plus matrix were then scraped from the dishes, separated from the medium by centrifugation, and washed three times with the above fibronectin-depleted culture medium (2 ml). To the medium plus washings were added proteinase inhibitors to the following concentrations: phenylmethylsulphonyl fluoride (2 mM), *N* ethylmaleimide (10 mM), EDTA (25 mM) and 6 amino hexanoic acid (25 mM). Medium was cooled at 4°C and radiolabeled proteins precipitated by the addition of $(NH_4)_2SO_4$ to 30% saturation were recovered by centrifugation at 30,000 *g* for 30 min at 4°C, then redissolved in 0.1 M Tris/HCl buffer (pH 7.4) containing 0.4 M NaCl and proteinase inhibitors before extensive dialysis against this solution.

The matrix-containing fraction was digested with pepsin (1 mg/ml) in 0.5 M acetic acid (1.0 ml) for 16 h at 4°C. The incubation mixture was centrifuged at 30,000 *g* for 30 min at 4°C and the collagens present in the supernatant were precipitated by the addition of NaCl to 2.0 M. The precipitate was dissolved in 0.5 M acetic acid and dialyzed extensively against acetic acid before analysis.

In some experiments the radiolabeled proteins precipitated from culture medium by $(NH_4)_2SO_4$ were also digested with pepsin (100 μg/ml) in 0.5 M acetic acid for 16 h at 4°C. After incubation, pepsin was inactivated by addition of NaOH solution to 0.5 M, and samples were dialyzed against 0.1% (wt/vol) SDS in 0.02 M Tris/HCl buffer (pH 7.4) before electrophoretic analysis.

Electrophoretic and Chromatographic Analyses

Radiolabeled proteins were resolved by PAGE according to the method of Laemmli (25) using a separating gel of 8% and a stacking gel of 3%. All gels were run under reducing conditions. The procedure of Bonner and Laskey (7) was used to impregnate the gel with 2,5-diphenyloxazole, and gels dried on to filter paper under vacuum were exposed to preflashed x-ray film (Kodak Royal X-Omat XH-1) at −70°C (24).

Radiolabeled proteins were also analyzed under reducing conditions by gel-filtration chromatography on 6% (Bio-Gel A-5m; Bio-Rad Laboratories, Richmond, CA) agarose column (90 × 1 cm) as described by Heathcote et al. (20). Fractions (~1 ml) were collected and a portion (0.1 ml) of each was taken for measurement of radioactivity by scintillation spectrophotometry. The remainder of the fraction was hydrolyzed in 6 M HCl for 18 h at 105°C and assayed for 4-hydroxy[^{3}H]proline (22). In some experiments selected peaks were rechromatographed on the same agarose column, and isolated proteins were analyzed for 4-hydroxy[^{3}H]proline by ion-exchange chromatography using a JEOL JLC-6AH amino-acid analyzer. Isolated peaks from gel-filtration columns were also digested for 4 h at 30°C with CNBr in 70% formic acid (10 mg/ml) under N_2. Distilled water (10 vol) was then added and the samples were lyophilized and analyzed by electrophoresis on 15% polyacrylamide gels and subsequent fluorography.

RESULTS

Growth of Chondrocytes on Plastic Dishes and within Three-dimensional Collagen Gels

Chondrocytes were plated on plastic dishes at 5×10^5 cells/dish and within 1 ml collagen gels at 5×10^5 cells/gel. All cultures were incubated at 37°C and cell number was determined at various times thereafter as described in Materials and Methods. The results of a typical growth experiment are shown in Fig. 1 *a* for cells maintained in medium supplemented with 16% whole fetal calf serum. Cells reach a higher saturation density on plastic (11×10^6 cells/dish) compared to cells cultured within the collagen gel matrix (2.5×10^6 cells/dish). Similar results have been obtained at initial cell densities both lower (1×10^5 cells/dish) and higher (2×10^6 cells/dish) than shown in Fig. 1 *a*.

The addition of fibronectin to chondrocytes growing on plastic tissue-culture dishes has been previously observed to promote the transition to a fibroblastoid cell morphology and synthesis of type I collagen (32, 40). In view of this effect of exogenous fibronectin on chondrocyte behavior, cells were grown in medium supplemented with fetal calf serum depleted of fibronectin. The results of a typical growth experiment in fibronectin-depleted medium are shown in Fig. 1 *b*, which indicate that under these conditions cells proliferate at approximately the same rate both in plastic dishes and within the collagen gels. All subsequent experiments were performed using medium supplemented with fibronectin-depleted fetal calf serum.

Morphology of Cells Growing on Plastic Dishes and within the Collagen Gels

The morphology of chondrocytes cultured on plastic tissue-culture dishes (Fig. 2 *a*) was similar to that previously described by Oakes et al. (30). The chondrocytes were observed to

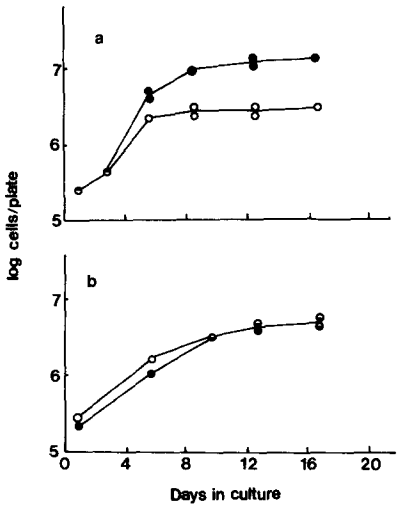

FIGURE 1 Growth curves of chondrocytes cultured on plastic dishes and in collagen gels in the presence and absence of fibronectin. (*a*) Cells maintained in DME medium plus 16% whole fetal calf serum. (*b*) Cells maintained in DME medium plus 16% fetal calf serum depleted of fibronectin. (○), in collagen gel; (●), on plastic.

maintain a characteristic round cell morphology when cultured at high density, with cells displaying a fibroblastoid morphology appearing after ~1 wk in areas of lower cell density.

Cells cultured within the collagen gel matrix undergo a transition to a fibroblastoid morphology after ~1–2 d in culture (Fig. 2*b*), followed by a reversion to a round cell morphology after 5–6 d (Fig. 2*c*) which is then maintained for the duration of the culture period (up to 3 wk). As can also be seen in Fig. 2*c*, cells cultured within the collagen gel matrix form characteristic colonies consisting of a linear array of cells resembling beads on a string. When stained with toluidine blue, cells growing both on plastic and within the collagen gel (Fig. 2*d*) are seen to be invested with a metachromatic matrix.

Rate of Collagen Synthesis

Chondrocytes were plated on plastic dishes and within collagen gels and incubated for 3 wk at 37°C. The rates of collagen biosynthesis and total protein synthesis were determined during this incubation period. No significant differences were detected in the rates of collagen synthesis between cultures on plastic and those within collagen gels (Fig. 3). Similar results were obtained when total protein synthesis by cells cultured on plastic and within collagen gels was examined (not shown). The rates of both protein and collagen syntheses per cell reached maxima after ~6 d of culture but readily detectable quantities of collagen were synthesized at all times examined. The proportion of the total non-diffusible radioactivity present as peptidyl-4-hydroxy[^{3}H]proline was fairly constant at about 12% in both culture systems, although in longer-term cultures (3 wk) this figure was reduced to ~5%, suggesting a decrease in the proportion of collagenous proteins synthesized at this time.

Analyses of the distribution of ^{3}H-collagen between the culture medium and cells plus matrix revealed marked differences between the two culture systems. Of the total hydroxy-[^{3}H]proline synthesized during a 6-h labeling period by cells grown on plastic, between 60 and 80% was released into the medium at the times examined whereas cells grown in the collagen matrix released only 25 to 40% into the culture medium. It is interesting to note that, during culture of chondrocytes in collagen gels, the gel volume decreased by 50% during the first week in culture (as estimated by isotope dilution) and then decreased only slightly with further culture. This shrinkage of the collagen gels coincided with the adoption of a fibroblastoid cell morphology (see Fig. 2*b*) and was caused by the included chondrocytes, since control gels incubated without cells showed negligible volume change.

Collagen Types Detected in the Culture Medium

Cultures were labeled for 24 h with [^{3}H]proline after various times of culture, and ^{3}H-proteins released into the medium were examined by PAGE and fluorography. When cells were labeled after only one day in culture, the major high molecular weight proteins precipitated from culture medium at 30% $(NH_4)_2SO_4$ saturation were found to migrate as two bands, one in the region of pro-α collagen polypeptides, the other slightly preceding this band (Fig. 4, tracks *1* and *2*). The nature of these two bands is under investigation but preliminary studies indicate that they are both sensitive to digestion by highly purified bacterial collagenase, and treatment with chymotrypsin yields two bands of slightly lower molecular weight, one of which migrates in the position of α1(I) standard and the other fractionally more slowly. The high molecular weight species migrating just slower than the standard of β_{12} collagen chains (Fig. 4, tracks *1* and *2*) is not digested by collagenase and is probably fibronectin which is known to be synthesized by chondrocytes in the first days of culture but is not synthesized once the cells have established a surrounding cartilage-type matrix (11). At this time point no differences were observed between the products secreted by chondrocytes cultured in collagen gels and those secreted by chondrocytes grown on plastic.

Analyses of proteins released into the medium after longer culture periods demonstrated that the prominent [^{3}H]proline-labeled species were the α chains and p-α chains, and it was consistently found that cultures grown in gels had a greater proportion of the newly synthesized medium macromolecules present as the collagen precursor polypeptides rather than α-chains. Such an observation suggests a slower conversion of procollagen to collagen in these cultures in gels. Furthermore, cells grown on plastic dishes for 2 and 3 wk were observed to synthesize some type I collagen as indicated by the presence of a band migrating in the position of α2(I) chains (Fig. 4, track *7* and Fig. 6). This species was seldom detected in cultures grown in gels and, when present, was always much more faint than the equivalent band from cultures grown on plastic.

As can be seen in Fig. 4, the most outstanding difference between the cells grown on the different substrata for 1 to 3 wk was the presence of a fast-migrating species (band G) in the medium of cultures grown in gels. This band was prominent after one week of culture and became the predominant labeled species in the medium when cells were incubated with [^{3}H]-proline after 3 wk of culture (Fig. 4, tracks *4*, *6*, and *8*). Cells grown on plastic synthesized detectable but markedly lower amounts of this component.

Chromatography of these medium proteins on 6% agarose (Fig. 5) coupled with analysis of fractions for 4-hydroxy[^{3}H]-proline revealed two major peaks of collagenous polypeptides. Peak *1* was found by gel electrophoresis to contain proα_1, pα and α-chains whereas peak *2* of hydroxy[^{3}H]proline-rich peptides from cultures in collagen gels (Fig. 5*b*) contained only band G. When this low molecular weight peak was rechromatographed on 6% agarose and reanalyzed for hydroxy[^{3}H]-

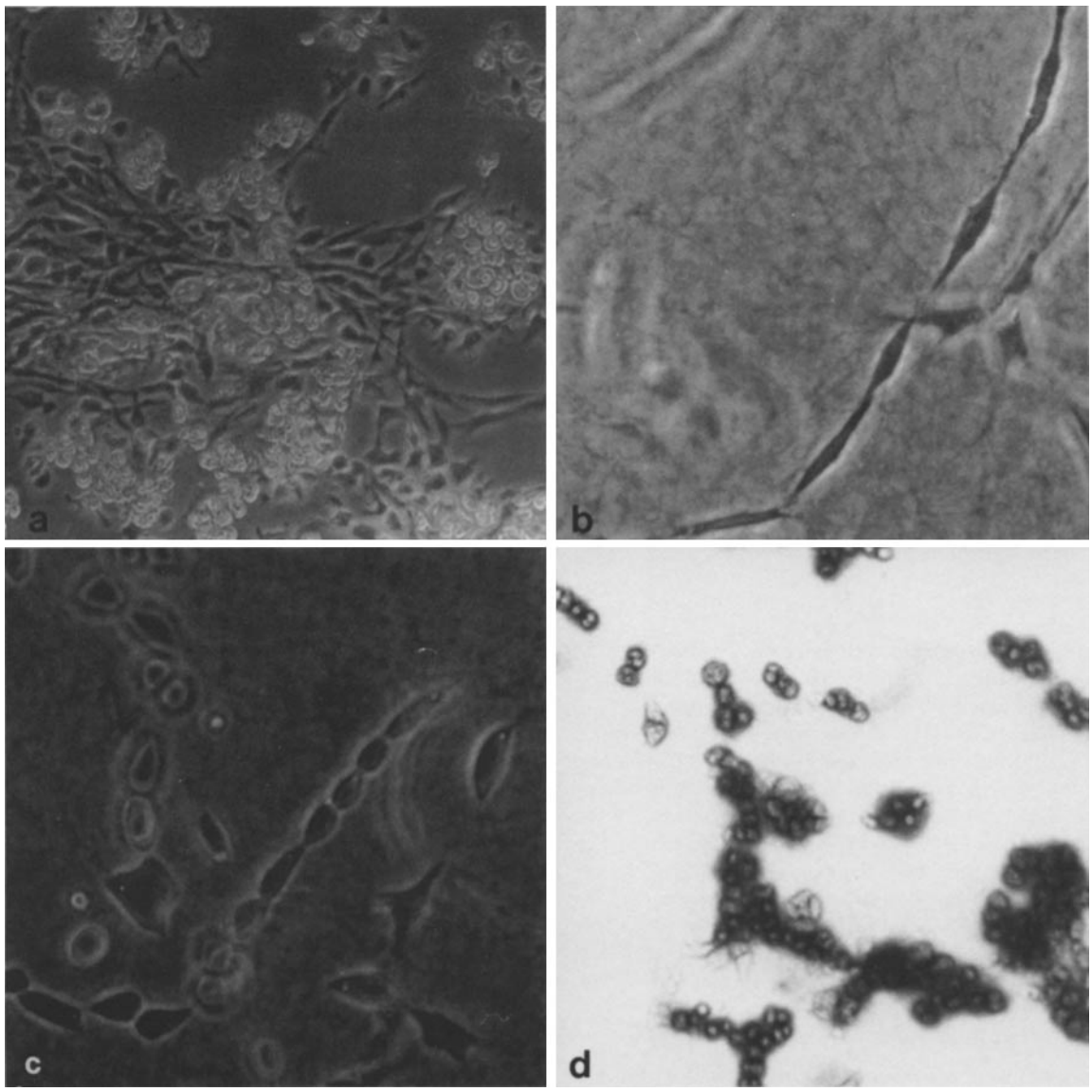

FIGURE 2 The morphology of chondrocytes growing on plastic dishes and within collagen gels. Chondrocytes were plated onto plastic dishes and within collagen gels at an initial density of 5×10^5 cells/culture. (*a*) Cells growing on plastic dishes after 8 d of incubation. Compact colonies of round cells are observed interspersed among cells displaying a fibroblastoid morphology. × 300. (*b*) Cells cultured within the collagen gel after 3 d of incubation. Majority of cells adopt a spindle-shaped (fibroblastoid) morphology. Colonies consist of a linear array of such cells. × 500. (*c*) Cells cultured within the collagen gel after 8 d of incubation. Majority of chondrocytes revert to round cell morphology, with typical colonies consisting of a linear array of cells. × 500. (*d*) Cells cultured within collagen gel after 10 d of incubation. Preparation fixed with formalin and stained with toluidine blue. Majority of cells invested with prominent metachromatic matrix. × 300.

proline, 42% of the incorporated label occurred as 4-hydroxy[^{3}H]proline whereas rechromatographed peak *1* (Fig. 5*b*) exhibited 46% hydroxylation (Table 1).

Pepsin Digestion of Medium Proteins

The results of mild pepsin digestion on the electrophoretic behavior of the medium proteins is shown in the fluorogram presented in Fig. 6. As expected, pepsin digestion results in the conversion of collagen precursor polypeptides to α chains. A more surprising finding is the increased migration of the G band after pepsin digestion to give a lower molelcular weight band designated Gp. The apparent decrease in size of the G collagen polypeptide with pepsin digestion was confirmed by gel chromatography on 6% agarose. A representative chromatogram is presented in Fig. 7, which demonstrates that pepsin digestion of the medium proteins from 2-wk cultures gave three well-separated peaks. PAGE showed that first peak (peak *1*) to contain α chains and peak *2* to contain collagen chains with the same mobility as the Gp band. The third peak (peak *3*) migrates on 8% polyacrylamide gels at the dye front, and its nature has not been further investigated. Hydroxyproline analysis showed the α chain species (peak *1*) from plastic and collagen gel cultures to contain 47 and 49% hydroxylation and the Gp peak (peak *2*), from collagen gel cultures, to contain 52% hydroxylation of proline (Table I). Hydroxyproline analysis suggests that peak *2* from cultures on plastic is contaminated with noncollagenous material, and this peak contains only trace amounts of the Gp component as judged by gel electrophoresis and fluorography (Fig. 6, track *2* in which the

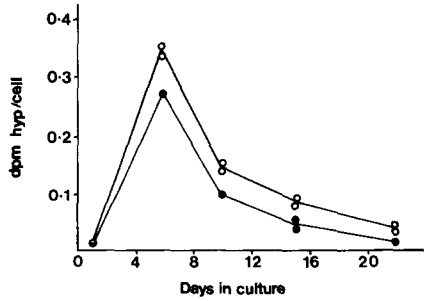

FIGURE 3 Rates of collagen synthesis by cells cultured on plastic dishes and in collagen gels. Cells cultured for up to 3 wk were incubated for 6 h with [5-^{3}H]proline, and the 4-hydroxy[^{3}H]proline incorporated into TCA-precipitable material (medium plus cell layer) was taken as an index of collagen synthesis. (○) in collagen gel; (●) on plastic.

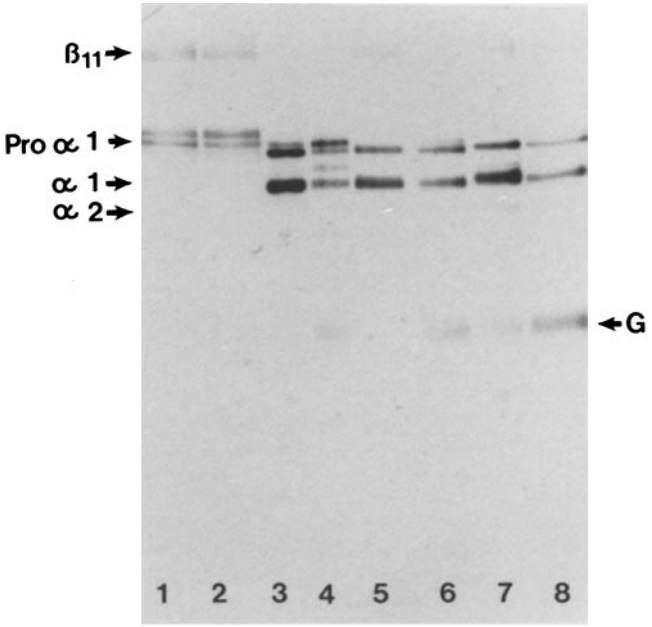

FIGURE 4 Fluorogram of [^{3}H]proline-labeled medium proteins synthesized by cells cultured on plastic and in collagen gels. Cells were labeled for 24 h with [5-^{3}H]proline, and the medium proteins precipitable at 30% $(NH_4)_2SO_4$ saturation were analyzed on an 8% polyacrylamide gel. Samples from cultures grown on plastic for 1, 7, 14, and 21 d are in tracks *2*, *3*, *5*, and 7. Samples from cultures grown in collagen gels for the same periods of time are in tracks *2*, *4*, *6*, and 8, respectively. The migration positions of proα1(II) and of [^{3}H]-acetylated type I collagen standards are indicated. Species of interest (see text) is labeled G.

Gp band is barely visible but was detected on the original fluorogram).

CNBr Peptide Analysis of the Low Molecular Weight Collagen

The possibility of a direct relationship between the G collagen polypeptide and one of the known collagens synthesized by cultured chondrocytes was investigated by CNBr peptide analysis. The G collagen species was isolated by column chromatography of the medium proteins from cells cultured within collagen gels for 3 wk and purified by rechromatography on the same column. CNBr digestion followed by gel electrophoresis and fluorographic analysis demonstrated that the G collagen was digested to very low molecular weight peptides (Fig. 8). No high molecular weight fragments were detected and the peptides obtained exhibited electrophoretic mobilities distinct from those of CNBr peptides derived from standard collagen α chains. These observations appear to exclude the possibility

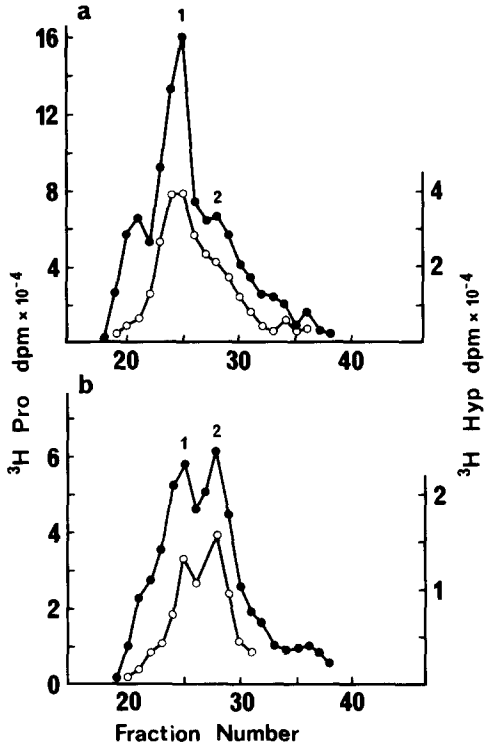

FIGURE 5 Gel-filtration chromatography on SDS/agarose (Bio-Gel A-5m) of [^{3}H]proline-labeled medium proteins. Cells cultured for 14 d on (*a*) plastic dishes and (*b*) in collagen gels were incubated with [5-^{3}H]-proline for 24 h, and proteins precipitated at 30% $(NH_4)_2SO_4$ saturation were applied to a column (90 × 1 cm) eluted with 0.1% SDS in 0.02 M-Tris/HCl buffer (pH 7.4). Fractions collected were analyzed for total ^{3}H-radioactivity (●) and 4-hydroxy[^{3}H]proline (○). Peaks of interest are labeled *1* and *2* (see text).

TABLE I

Hydroxylation of Proline Residues in Collagenous Proteins Isolated by Gel Chromatography

Culture conditions	Fraction analyzed*	$\frac{\text{4-hydroxy[}^3\text{H]proline (cpm)}}{\text{4-hydroxy[}^3\text{H]proline + [}^3\text{H]proline (cpm)}} \times 100$
		%
Collagen gels	α	45.9
	α pepsin	48.9
	G	41.8
	G pepsin	51.6
Plastic dishes	α	42.8
	α pepsin	46.8

* Peaks *1* and *2* from gel filtration (Figs. 5 and 7) were rechromatographed and shown by gel electrophoresis to comprise the purified species indicated, except that the α chain fractions contained pro-α and p-α chains also. Samples were hydrolyzed in 6 M HCl at 110°C for 18 h, and their 4-hydroxy[^{3}H]proline and [^{3}H]proline contents were determined by ion-exchange chromatography using a JEOL JLC-6AH amino acid analyzer.

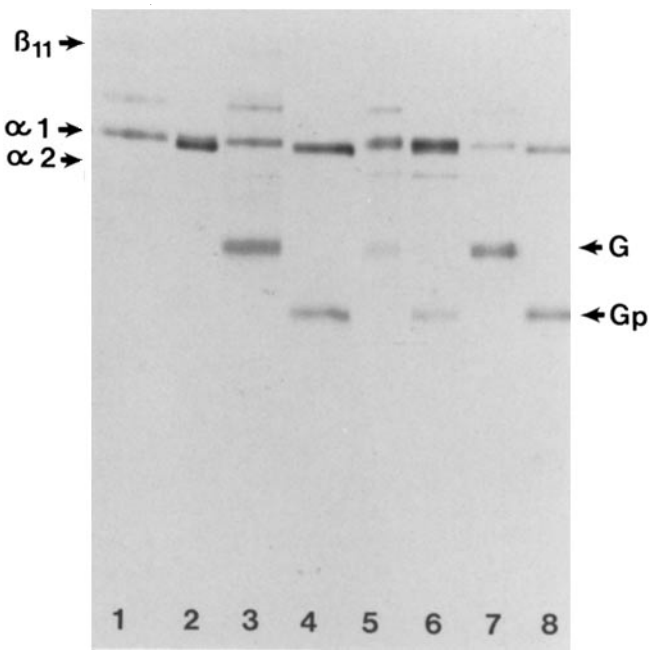

FIGURE 6 Fluorogram of [^{3}H]proline-labeled medium proteins and their pepsin-digestion products. Cells were incubated for 24 h with [5-^{3}H]proline and medium proteins precipitable at 30% $(NH_4)_2SO_4$ saturation isolated. Aliquots were incubated with pepsin (100 μg/ml) at 4°C for 16 h and both the pepsin digestion products and untreated controls were analyzed on 8% polyacrylamide gels. Samples from cultures grown for 14 and 21 d on plastic dishes are shown in tracks *1* and *5* and their respective pepsin-digested products in tracks *2* and *6*. Samples from cultures grown for 14 and 21 d in collagen gels are shown in tracks *3* and *7* and their pepsin digestion products in tracks *4* and *8*. Migration positions of [^{3}H]acetylated type I collagen standards are indicated. The short chain collagenous polypeptides (*G*) and their pepsin-derived products (*Gp*) are arrowed.

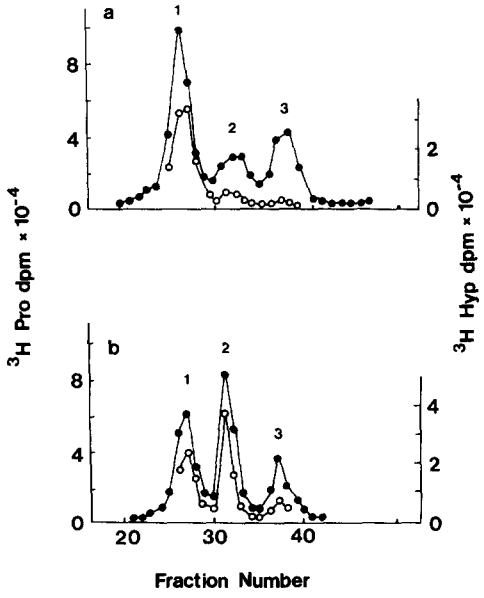

FIGURE 7 Gel-filtration chromatography on SDS/Agarose of [^{3}H]-proline-labeled medium proteins after pepsin digestion. Medium proteins isolated from cells cultured for 14 d on (*a*) plastic dishes, and (*b*) in collagen gels were isolated and analyzed as described in Fig. 5, after digestion with pepsin (100 μg/ml) for 16 h at 4°C. (●) total radioactivity, (○) 4-hydroxy[^{3}H]-proline.

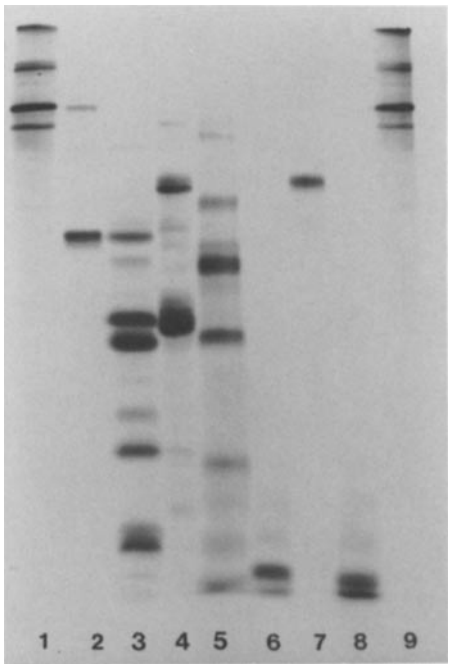

FIGURE 8 Analysis of CNBr peptides derived from the G collagen by SDS PAGE and fluorography. Tracks *1* and *9*: [^{3}H]Acetylated rat-tail tendon collagen. Track *2*: Pepsin-treated G collagen together with trace amounts of pepsin-treated α1(II) chains. Track *3*: CNBr peptides of authentic α1(I) chains that were prepared by incubating 18-d embryonic-chick tendons with [^{3}H]proline and purifying the type I collagen by CM-cellulose chromatography under denaturing conditions. Track *4*: CNBr peptides of authentic α2(I) chains obtained during the above preparation of α1(I) chains. Track *5*: CNBr digest of α1(II) chains preparation obtained when 18-d embryonic-chick sternal cartilage was labeled with [^{3}H]proline. Tracks *6* and *8*: Medium proteins from cells cultured for 14 d in collagen gels were separated by gel filtration chromatography and the peak fraction of the G collagen (Peak *2*, Fig. 5 *b*) was rechromatographed on the same column. The purified G collagen was digested with CNBr as described in the text. Track *7*: Purified G collagen before CNBr digestion.

that the G collagen species is a partial degradation product of a known collagen molecule.

Collagen Types Deposited in the Cell Matrix

The study of the isolated matrix collagens confirmed the distribution of hydroxy[^{3}H]proline found after 6 h synthesis, described above, and showed that ~60% of the newly synthesized collagen is laid down as part of the insoluble matrix in cultures in collagen gels, compared with 10 to 20% with cultures on plastic. When the collagen gel matrix containing labeled polypeptides was subjected to pepsin digestion, the collagenous components were brought into solution and shown by gel electrophoresis and fluorography to contain a predominant species comigrating with the Gp band isolated from medium protein together with a fainter band migrating in the position of α1 chains. The presence of the Gp species was not at first appreciated as it was found to be soluble in 0.8 M NaCl, 0.5 M acetic acid, conditions initially used to precipitate proteins during the isolation of the pepsin-extractable collagens.

DISCUSSION

In this study, chick sternal chondrocytes have been cultured both on plastic tissue-culture dishes and within a three-dimen-

sional gel matrix of type I collagen. After 1 wk in culture, chondrocytes in both culture systems exhibited a typical round cell morphology and abundant toluidine blue-staining matrix. Cells cultured within the collagen gel matrix exhibited a beaded arrangement reminiscent of the cellular organization in growth cartilage (38). Histotypical structures have also been reported to be produced when mammary epithelial cells are cultured within a three-dimensional collagenous matrix (41). Human skin fibroblasts cultured within a collagen gel matrix form a random array of elongated, spindle-shaped cells quite distinct from the linear array adopted by the chondrocytes (35).

We observe that chondrocytes cultured within the collagen gel matrix assume a fibroblastoid morphology for the first few days in culture and that there is an ~50% reduction in gel volume during this time. Bell et al. (2) have observed that skin fibroblasts contract a collagen gel when cultured in this manner, thus indicating that the "fibroblastoid" chondrocytes share this functional property with skin fibroblasts.

Cell proliferation was very similar in both collagen gels and plastic dishes when fibronectin-depleted serum was used in the culture medium. The presence of fibronectin appeared to stimulate cell division on plastic but not in collagen gels (Fig. 1), possibly because a large proportion of the fibronectin was bound to the collagen framework of the gel. Stimulation of cell division by fibronectin has been observed with other cell types in culture (31).

The rates of total protein and collagen syntheses were very similar in both culture systems though there is a much greater proportion of newly synthesized protein in the matrix-cell layer in cultures in collagen gels. This may be a result of precipitation of newly synthesized collagens on the fibers of the collagen gel. It does not appear that the gel matrix simply poses a barrier to diffusion of the newly synthesized proteins as extensive extraction of the homogenized matrix with 1.0 M NaCl or pepsin released only a small proportion of these proteins. Examination of the newly synthesized medium collagens by gel electrophoresis suggested that there is a higher proportion of procollagen to collagen, indicating less efficient conversion of procollagen to collagen in cultures in collagen gels.

The synthesis of type I collagen, as detected by the presence of bands comigrating with α2(I) chains on PAGE (Figs. 4 and 6) and confirmed by CNBr peptide analysis (not shown), occurred earlier in culture and constituted a higher proportion of total collagen synthesis in cultures on plastic than in cultures of collagen gels. The switch to type I collagen synthesis in both culture systems, however, was much less pronounced than has been previously observed (3, 15, 28). This may be due to the relatively high initial plating densities used (lower plating densities have been reported to result in a more rapid switch to type I collagen synthesis [28]), the use of an efficiently buffered medium (30) or the use of fibronectin-depleted serum (32, 40).

However, the major difference between the two culture systems was the surprising observation that chondrocytes grown within a three-dimensional matrix of type I collagen are induced to synthesize a short-chain collagenous protein (Fig. 4, band *G*). Trace amounts of this species were detected in the medium of cells grown on plastic (Fig. 4) but the short-chain collagen constituted a very significant fraction, frequently being the major constituent, of the newly synthesized [^{3}H]-proline-labeled species in the medium from chondrocytes cultured in collagen gels. Its partial resistance to mild pepsin digestion suggests that the protein, as isolated, has both a collagenous and noncollagenous domain. This concept was supported by hydroxyproline analysis of the isolated proteins, which showed an increased proline hydroxylation after pepsin digestion, similar to that found with the digestion of procollagen to collagen. Furthermore it appears that this short-chain species forms part of the insoluble collagenous matrix in the gel cultures for a ^{3}H-labeled polypeptide whose migration on polyacrylamide gels is identical to that of the pepsin digestion product of the short-chain collagen was isolated by pepsin digestion of the matrix-cell layer of 3-wk cultures in collagen gels.

Initial considerations of the properties of the short-chain collagen suggested that it was unlikely to be a product of proteolytic attack on newly synthesized type II collagen. Although a stimulation of mammalian fibroblast collagenase production in response to added collagen has been reported in tissue cultures (6), the products of mammalian collagenase digestion (fragments TCA and TCB) have different molecular sizes than the short-chain collagen or the pepsin digestion product. Using the TCA and TCB peptides produced by mammalian collagenase digestion of type I collagen as reference standards, initial estimates of the molecular weights of the short-chain collagen and its pepsin digestion product were calculated to be 59,000 and 45,000, respectively, on SDS PAGE (16). Similarly, these collagenous proteins are unlikely to be mammalian collagenase digestion products of type II procollagen, mammalian collagenase digestion of which gives peptides of 88,000 and 61,000 mol wt (19). With subsequent mild pepsin digestion these should be reduced to TCA or TCB fragments the same as those produced by mammalian collagenase digestion of collagen, i.e. 75,000 and 25,000 mol wt, respectively.

It has been reported that the bacterial collagenase used in the isolation of chondrocytes from cartilage can be taken up and stored by the chondrocytes (9). If the short-chain collagens observed were fragments produced by the action of this enzyme, it seems unlikely that the proportion of short-chain collagen to α chains would increase as a function of time in culture, because we would expect the bacterial collagenase taken up in this way to be continuously depleted. It has also been reported that up to 30% of collagen synthesized by lung fibroblasts in vitro is rapidly degraded, although in this case the final degradation products appears to be small peptides (4, 5). In addition, if the short-chain collagen was a fragment of normal type II collagen or procollagen produced by proteolytic digestion it would not be expected to maintain a stable triple helix under the culture conditions (37°C). By comparison, the TCA and TCB fragments of type I collagen produced by mammalian collagenase digestion have melting temperatures of 32°C and 29°C, respectively (33).

On the basis of the above evidence it appears that the short-chain collagen recovered from collagen gel cultures represents a distinct collagenous species whose triple helical structure is rendered more stable than similar-sized fragments of normal type II collagen by characteristics as yet unknown. Definitive evidence that this low molecular weight species represents a new gene product and is not a partial degradation product of a known collagen molecule was obtained by CNBr peptide analysis (Fig. 8). Other short-chain collagens have recently been isolated from pepsin digests of bovine nasal cartilage (1), porcine articular and bronchial cartilage (37), and human intervertebral disc (1). These peptides also exhibit solubility properties similar to those of the G collagen described here but are distinguished from it by their lower molecular weights

(33,000) and their occurrence as disulphide-bonded aggregates in the unreduced state (16). Other short-chain collagens have also been isolated from pepsin digests of bovine (21) and human (13) placenta; these too are very soluble and disulphide-linked but have molecular weights (40,000) nearer those of the short-chain collagen synthesized by chrondrocytes in collagen gels.

Type I collagen is not a constituent of the macromolecular matrix normally surrounding chondrocytes in vivo. The results presented in this communication, however, indicate that a type I collagenous environment does affect the biosynthetic activity of chrondrocytes cultured in vitro. Previous studies have indicated that several types of collagen (including type I) stimulate the production of a metachromatic matrix by somites explanted in vitro, although type II collagen was most effective (23). It is also noteworthy that in preliminary studies in which we have cultured chondrocytes within collagen gel matrices in a medium containing fibronectin, a markedly different profile of collagenous polypeptides is obtained including two more short-chain collagens that are distinct from the G collagen species reported here (Gibson, Schor, and Grant. Unpublished observations.). The mechanism by which extracellular matrix macromolecules influence cellular biosynthetic activity is not understood but is the subject of current investigation.

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