Human and sheep growth-plate cartilage type X collagen synthesis and the influence of tissue storage

Gary J. GIBSON,*† Kathy T. FRANCKI,† John J. HOPWOOD* and Bruce K. FOSTER†

*Lysosomal Diseases Research Unit, Department of Chemical Pathology, and †Department of Orthopaedic Surgery, Adelaide Children's Hospital, 72 King William Road, North Adelaide, South Australia 5006, Australia

Direct comparison of type X collagen synthesized by human, sheep and chick growth-plate cartilage has shown that the human type X collagen is similar to the chick in both its molecular mass, containing component α -chains of 59 kDa with helical regions of 45 kDa, and apparent absence of disulphide-stabilized aggregates, whereas the sheep type X collagen has slightly larger α -chains (63 kDa) accounted for by a longer helical region (49 kDa) that contains cystine residues essential for the formation of the high-molecular-mass aggregates found with this species. Type X collagen from all three species showed heterogeneity in primary collagen structure as revealed by *Staphylococcus aureus* V8 proteinase-generated peptide maps. Collagen synthesis by growth-plate cartilage in culture, particularly synthesis of type IX and X collagen, was shown to be very sensitive to prior storage and suggests caution in the interpretation of changes detected when examining collagen synthesis by growth plates in culture.

INTRODUCTION

Growth-plate cartilage, like other cartilages, contains a number of tissue-specific collagens. These include the major chondrocyte gene product, type II collagen, and the less abundant collagen types IX (Shimokomaki *et al.*, 1980), X (Gibson *et al.*, 1982; Schmid & Conrad, 1982a,b) and XI (Burgeson & Hollister, 1979). Type X collagen is distinct among the cartilage collagens in its localization to only growth-plate cartilage, specifically the hypertrophic region of this tissue (Schmid & Linsenmayer, 1985; Gibson *et al.*, 1986). Close association with the sites of initial cartilage calcification and metaphyseal vascular invasion (Schmid & Linsenmayer 1985; Gibson & Flint, 1985) strongly implies its involvement in the processes of endochondral ossification.

Type X collagen, isolated from cultures of chick hypertrophic chondrocytes (Gibson *et al.*, 1981, 1983; Schmid & Conrad, 1982a,b) and from chick-embryo cartilage (Gibson *et al.*, 1984; Kiely *et al.*, 1985), has been extensively characterized and the homopolymeric $\alpha 1(X)$ chain shown to have a molecular mass of 59 kDa composed of *N*- and *C*-terminal non-helical extensions and a helical region of 45 kDa. The chick gene has been sequenced (Ninomiya *et al.*, 1986; Lu Valle *et al.*, 1988) and was shown to be distinct from all other collagen genes, with the exception of the type VIII collagen gene (Yamaguchi *et al.*, 1989). It is composed of only three exons, compared with approximately 50 for the fibrous collagens, one encoding most of the *N*-terminal non-helical extension, the second encoding the rest of the molecule and the third encoding the 5' non-translated region. Recent determination of the cDNA sequence of type VIII collagen has shown a close similarity in primary structure to type X collagen, including near-identity in the location and type of imperfection of the Gly-Xaa-Yaa triplet. These studies suggest that types X and VIII represent part of a family of collagens that may include additional collagens of similar though less well-characterized molecular structure (Majack & Bornstein, 1985; Brown & Balian, 1987; Dixit, 1989).

The relatively few studies of bovine type X collagen synthesis or structure published demonstrate significant differences between this mammalian collagen and chick type X collagen. Analysis of the chick type X collagen gene has shown that there

is a single cysteine residue in the *C*-terminal non-helical region (Yamaguchi *et al.*, 1989); however, this does not appear to form intermolecular disulphide bridges. Bovine type X collagen has been shown to occur as aggregates stabilized by disulphide bonds, which, by their substantial resistance to mild proteolysis, appear to occur between the triple-helical regions of the molecule (Remington *et al.*, 1984; Grant *et al.*, 1985). Conversely, type X collagen synthesized by rabbit growth-plate cartilage was shown to be similar to chick type X collagen in the apparent absence of intermolecular disulphide bonds (Remington *et al.*, 1983).

Part of the stimulus for the studies described here was a proposed investigation of human genetic disorders of unknown aetiology involving growth-plate defects, e.g. the chondrodysplasias, and an investigation of the resultant effects on collagen synthesis of a group of genetic disorders of defined aetiology, the mucopolysaccharidoses, which also affect growth-plate function. The synthesis and characteristics of human type X collagen, however, have not been previously described. The present paper describes the synthesis of type X collagen by sheep and human growth-plate cartilage in direct comparison with radiolabelled chick type X collagen, and an examination of the effect of tissue storage time and temperature on the spectrum of collagen types detected.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium, foetal-calf serum and antibiotics were obtained from Gibco, Glen Waverley, Vic., Australia. Pepsin (1:60000 from pig stomach mucosa), phenylmethanesulphonyl fluoride, *N*-ethylmaleimide and 6-aminohexanoic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. *Staphylococcus aureus* V8 proteinase was obtained from Miles Scientific, Naperville, IL, U.S.A. Highly purified collagenase (CLSPA; 437 units/mg) was purchased from Worthington Diagnostic, Freehold, NJ, U.S.A. α -Chymotrypsin (bovine pancreas) was purchased from Calbiochem Corp. Australia, Alexandria, N.S.W., Australia. L-[4-³H]Proline and Hyperfilm-MP were purchased from

† To whom reprint requests should be sent, at present address: Bone and Joint Center, Henry Ford Hospital, 2799 West Grand Blvd., Detroit, MI 48202, U.S.A.

Amersham Australia Pty. Ltd., North Ryde, N.S.W., Australia. ^3H -labelled chick collagen types II and X were prepared from culture medium of chick sternal chondrocytes as described previously (Gibson *et al.*, 1983).

Tissue culture

Growth-plate tissue was dissected from either proximal tibia and distal femur or rib of foetal lambs (115–122 days gestation). Growth-plate tissue was dissected from human (5 weeks to 12 months of age) rib specimens obtained at autopsy after an interval of 8–23 h *post mortem*. By slicing dissected growth-plates with adjacent epiphyseal cartilage into approx. 2 mm sagittal sections in some experiments, dissection of the tissue slices into regions of hypertrophic growth-plate, combined proliferative and resting growth-plate, articular cartilage and also cartilage surrounding secondary centres of ossification was facilitated. Sagittal slices enabled dissection along the often undulating contours of the growth-plate. Dissected growth-plate cartilage or growth-plate zones were finely sliced and incubated with 40 μCi of L- ^3H proline in Dulbecco's modified Eagle's medium containing 15 mM-Hepes, 10% (v/v) foetal-calf serum and ascorbic acid (0.5 mg/ml) in 5% CO_2 in air at 37 °C for 24 h.

Isolation of newly synthesized collagen

Radiolabelled collagens were isolated from either culture medium or tissue slices. Proteinase inhibitors phenylmethanesulphonyl fluoride (2 mM), *N*-ethylmaleimide (10 mM), EDTA (25 mM) and 6-aminohexanoic acid (25 mM) were added to the medium of cartilage cultures to achieve the concentrations shown and radiolabelled collagens were precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation. The pellet was recovered by centrifugation (40 000 *g* for 30 min), redissolved in 0.4 M-NaCl/0.1 M-Tris/HCl buffer, pH 7.4, containing the proteinase inhibitors, then dialysed against the same buffer. In some experiments, samples of radiolabelled proteins from the culture medium were digested with chymotrypsin (300 $\mu\text{g}/\text{ml}$) for 16 h at 20 °C.

The radiolabelled collagens of the cartilage matrix were extracted by digestion with pepsin (1 mg/ml) in 0.5 M-acetic acid/0.5 M-NaCl overnight at 4 °C, and the pellet, after centrifugation, was re-extracted with pepsin, as above. Pooled extracts were dialysed against 0.5 M-acetic acid overnight at 4 °C and collagens were separated by salt fractionation. Type II collagen was precipitated by dialysis against a solution of 0.8 M-NaCl/0.5 M-acetic acid, and the minor collagens by dialysis against a solution of 2.0 M-NaCl/0.5 M-acetic acid. Collagens were redissolved and dialysed against 0.5 M-acetic acid. Samples of radiolabelled proteins extracted from the matrix or precipitated from culture medium were digested with bacterial collagenase (7 units/ml) in 0.15 M-NaCl/5.0 mM- CaCl_2 /50 mM-Tris/HCl buffer, pH 7.4, containing *N*-ethylmaleimide (10 mM) and phenylmethanesulphonyl fluoride (0.2 mM) for 6 h at 37 °C.

Electrophoretic analysis

Radiolabelled proteins after denaturation, usually in the presence of dithioerythritol (130 mM), were resolved by SDS/PAGE according to the method of Laemmli (1970) with a separating gel of 7.5% (w/v) and a stacking gel of 4% (w/v). The gel was impregnated with 2,5-diphenyloxazole, according to the procedure of Bonner & Laskey (1974), dried on to filter paper and exposed to pre-flashed X-ray film (Amersham Hyperfilm-MP) for approx. 7 days at –70 °C (Laskey & Mills, 1975). Molecular masses were determined by comparison with the

peptide $\alpha 1(\text{II})$ CB11 (25 kDa), generated by CNBr digestion of type II collagen, $\alpha 1(\text{II})$ chains (98 kDa) and pepsin-digested chick type X collagen (45 kDa).

Peptide mapping

To compare the primary structure of type X collagens from human, sheep and chicken sources they were subjected to SDS/PAGE combined with V8-proteinase digestion and subsequent peptide mapping in a second dimension with the use of a modification of the techniques of Cleveland *et al.* (1977), Lizardi (1979) and Tijssen & Kurstak (1983). Proteins were denatured under reducing conditions and separated by electrophoresis in an SDS/7.5% polyacrylamide gel with a 4% stacking gel. Tracks cut from the first gel were placed horizontally on a second polyacrylamide gel (15% main gel; 4% stacker) polymerized with 0.004% (w/v) riboflavin rather than ammonium persulphate as catalyst and fixed in place with extra stacking gel. Sample buffer [5% (w/v) sucrose/1% (w/v) SDS/10 mM-Tris/HCl buffer, pH 7] containing V8 proteinase (10 μg or equivalent to 500 ng/ mm^2 of gel surface) was overlaid and electrophoresed until tracker dye reached approx. 2 mm above the resolving gel. The current was then decreased to 1 mA for 30 min to allow digestion of the collagens. The current was then returned to 40 mA and electrophoresis continued until the dye front reached the bottom of the gel. Peptides were detected by fluorography.

Effect of storage time and temperature on collagen synthesis

Ribs numbered (cephalic to caudal) 1 to 9 left side and 1 to 9 right side were dissected from a foetal lamb (kept at room temperature) 3.5 h after slaughter of the ewe. Costochondral junction, together with adjoining costal cartilage and approx. 1 cm of proximal bone, was stored in sterile phosphate-buffered saline (0.14 M-NaCl/6 mM-sodium phosphate buffer, pH 7.4) containing penicillin and gentamicin. Costochondral junctions from the left side were stored at 4 °C, and those from the right side at 22 °C. After an additional storage interval (ribs 2, 5 and 7, 0 h; ribs 1, 4 h; ribs 4, 21.5 h; ribs 6, 27.5 h; ribs 8, 46.5 h; ribs 9, 100.5 h) growth-plates were dissected from both contralateral ribs and cultured for 24 h in the presence of ^3H proline as described above. Radiolabelled proteins were isolated from culture medium and cartilage matrix as described above and subjected to SDS/PAGE and fluorography. Fluorograms were scanned with a Schoeffel densitometer with peak integrator to determine the relative incorporation of radiolabel into the collagen types.

RESULTS

Location of type X collagen synthesis in sheep and human growth-plates

The incorporation of ^3H proline into collagens by growth-plate in short-term tissue culture was sufficient to enable examination of the collagen types synthesized by individual human or sheep growth-plates. Most of the studies of collagen synthesis in human growth-plate reported here are from single human costochondral growth-plates, whereas the studies of sheep growth-plate are from pooled costochondral, tibial or femoral growth-plates.

Human and sheep growth-plates showed a similar pattern of collagen synthesis. The majority of radioactivity incorporated into proteins extracted from the tissue by pepsin digestion were precipitated from acetic acid solutions with 0.8 M-NaCl. This fraction contained a single species on SDS/PAGE, with mobility

Fig. 1. SDS/PAGE of [³H]proline-labelled proteins isolated from the matrix and culture medium of regions of growth-plate cartilage

Human (a) and sheep (b) articular and growth-plate cartilage was dissected into regions and incubated for 24 h with [³H]proline. Proteins were isolated from the tissue by pepsin extraction and fractional precipitation at 2.0 M-NaCl (matrix) or from culture medium by precipitation with (NH₄)₂SO₄ (medium), and denatured in the presence of 130 mM-dithioerythritol before electrophoresis. The cartilage regions examined were articular cartilage peripheral to the growth plate (lane 1), the combined resting and proliferative regions (lane 2), the hypertrophic region of the growth-plate (lane 3) and the cartilage surrounding the secondary centre of calcification (lane 5). Lane 4 is radiolabelled proteins extracted from chick chondrocyte cultures after pepsin digestion. The migration positions of $\alpha 1$ chains ($\alpha 1$), type X collagen chains (X) and pepsin-digested type X collagen chains (Xp) are indicated.

Fig. 2. Effect of disulphide reduction and chymotrypsin digestion on the electrophoretic mobility of collagens synthesized by human and sheep growth-plate cartilage

Radiolabelled proteins isolated from the culture medium of human or sheep growth-plate cartilage cultures were subjected to SDS/PAGE after denaturation in the presence (+) or absence (–) of 130 mM-dithioerythritol. Some samples were digested with chymotrypsin (+) (300 μ g/ml) for 16 h at 22 °C before denaturation and electrophoresis. Std, radiolabelled chick chondrocyte proteins extracted by pepsin digestion. $\alpha 1$, X and Xp indicate migration positions of $\alpha 1$ collagen chains, type X collagen chains and pepsin-digested chick type X collagen chains respectively. The migration positions of collagen molecular-mass markers ($\alpha 1$ chains, 98 kDa; pepsin-digested type X collagen, 45 kDa; $\alpha 1$ (II) CB11, 25 kDa) are shown.

and CNBr peptide map characteristic of type II collagen (results not shown). The minor cartilage collagens were isolated by a subsequent 2.0 M-NaCl precipitation of tissue extracts and their behaviour on SDS/PAGE, together with medium proteins precipitated with (NH₄)₂SO₄ but without further fractionation, is shown in Fig. 1. Radiolabelled proteins isolated from the medium of human hypertrophic growth-plate cultures contained a distinct low-molecular-mass protein (labelled 'X') not present in the medium of cultures of the proliferative region of the growth plates (Fig. 1a). A similar pattern of radiolabelled proteins was seen in the culture medium of sheep hypertrophic growth-plate cultures (results not shown). Pepsin extracts of the hypertrophic zone of human growth-plate cartilage contained a prominent radiolabelled protein that migrated on SDS/PAGE with the pepsin-digested chick type X collagen (Xp) (Fig. 1a). Incorporation of trace amounts of radiolabel into polypeptides migrating with chick type X collagen standard by non-hypertrophic regions of the growth-plate was occasionally observed and was attributed to imperfect dissection of the hypertrophic from proliferating and resting zone.

Pepsin extraction of sheep growth-plate revealed a similar pattern of collagen synthesis to the human, except that the low-molecular-mass radiolabelled protein synthesized by hypertrophic zones migrated slightly more slowly than the pepsin-digested chick type X collagen (Fig. 1b). Cartilage surrounding the secondary centre of ossification showed the same pattern of collagen synthesis as the hypertrophic zone of the growth-plate, including synthesis of the characteristic low-molecular-mass protein (Fig. 1b). Because of their similarity in tissue location and molecular size to chick type X collagen, these proteins have been labelled human and sheep type X collagen.

Molecular characteristics of human and sheep type X collagens

The type X collagen species from human, chick and lamb showed small though distinctly different migration on SDS/PAGE. By comparison with collagen molecular-mass

Fig. 3. Effect of bacterial collagenase on radiolabelled proteins isolated from cultures of sheep growth-plate cartilage

Radiolabelled protein isolated from the culture medium (lanes 3 and 4) or extracted from the matrix by pepsin digestion (lanes 1 and 2) were digested with purified bacterial collagenase in the presence of proteinase inhibitors and subjected to SDS/PAGE after denaturation. Migrations of $\alpha 1$ chains ($\alpha 1$) and pepsin-digested type X collagen chains (Xp) from chick chondrocyte cultures (lane 5) are shown.

Fig. 4. Peptide maps of radiolabelled protein extracted from sheep and human growth-plate cartilage and chick chondrocyte cultures

Radiolabelled proteins extracted from sheep and human growth-plate cartilage or chick chondrocytes by pepsin digestion were denatured in the presence of SDS and dithioerythritol and separated by electrophoresis on a 7.5% polyacrylamide gel (a). Excised lanes were overlaid on a 15% polyacrylamide gel and subjected to digestion with V8 proteinase. Resulting peptides were separated by electrophoresis and detected by fluorography (b). The migration positions of the type X collagen chains and the peptides derived therefrom are indicated.

standards, the molecular masses of type X collagen isolated from culture medium of human and sheep growth-plate cartilage were calculated to be 59 kDa and 63 kDa respectively when electrophoresed under reducing conditions (Fig. 2, lanes 9 and 4). After mild chymotrypsin digestion, the molecular masses of the type X collagen from human and sheep growth-plates were decreased to 45 kDa and 49 kDa respectively (Fig. 2, lanes 7 and 2). The migration of the type X collagen species extracted from

the tissues with pepsin was identical with that of the species generated from the corresponding medium protein by mild chymotrypsin digestion (cf. Fig. 1).

When electrophoresed without disulphide reduction, migrations of the human and sheep type X collagens were distinctly different. The sheep type X collagen that had been precipitated from culture medium migrated as a high-molecular-mass aggregate, just penetrating the polyacrylamide gel (Fig. 2, lane 5). The sheep type X collagen extracted from the matrix with pepsin (results not shown) or precipitated from the culture medium and subjected to mild chymotrypsin digestion also migrated as high-molecular mass aggregates not distinctly different from those of the intact type X collagen (Fig. 2, lane 3). Type X collagen from human growth-plate, like that from the chick, was unaffected by disulphide reduction and migrated as the 59 kDa protein or 45 kDa protein after chymotrypsin digestion, irrespective of the presence of reducing agents (Fig. 2, lanes 9, 10 and 7, 8). The high-molecular-mass species observed on SDS/PAGE of non-reduced human collagen samples was attributed to aggregated type IX collagen (Fig. 2, lanes 8 and 10).

The collagenous nature of all the radiolabelled proteins precipitated from pepsin extracts of the matrix and all but two of the major radiolabelled proteins precipitated from the culture medium was demonstrated by their susceptibility to bacterial collagenase (Fig. 3). The bacterial-collagenase-sensitivity of sheep and human proteins synthesized by cartilage in tissue culture were the same (only the sheep proteins have been shown in Fig. 3).

To compare chick, human and sheep type X collagens further, they were subjected to V8-proteinase peptide mapping. The 2 M-NaCl precipitates from the three sources of growth-plate cartilage were fractionated by SDS/PAGE. The three gel strips were excised and subjected to V8-proteinase digestion and then peptide mapping on a single 15% (w/v) polyacrylamide gel. The type X collagens showed no significant interspecies similarity in peptide pattern (Fig. 4). This procedure was repeated a number of times with different enzyme concentrations and also with chymotrypsin rather than V8 proteinase (results not shown). In no case was a significant similarity between sheep, chick or human type X collagen observed.

Effect of tissue storage on growth-plate collagen synthesis

To examine the effect of tissue storage on the synthesis of collagen by cultured growth-plate cartilage, growth-plates were excised from foetal-sheep costochondral junction after various intervals of storage at either 4 °C or 22 °C, incubated in tissue culture with radiolabelled proline for 24 h, and collagens were isolated from culture medium and tissue as described above. The synthesis of type X collagen appeared to be particularly sensitive to storage of the rib before dissection and culture of growth-plate cartilage (Fig. 5). After more than 25 h storage at 22 °C the incorporation of [3 H]proline into type X collagen in both culture medium and matrix was decreased to trace levels. Storage at 4 °C increased the persistence of type X collagen synthesis for storage intervals of up to 49 h, although the pattern of collagen types synthesized was changed by this interval of storage. The loss of type X collagen synthesis was seen in both the culture medium and cartilage-matrix extracts.

The radiolabelled proteins from the culture medium migrating in the presence of reducing agents as two bands between the $\alpha 1$ and type X collagen chains (shown by the asterisks in Fig. 5) were concluded to be type IX collagen chains because of similarity in molecular size (86 and 69 kDa) to the chick type IX collagen chains (Gibson *et al.*, 1983; Bruckner *et al.*, 1983), similar susceptibility to mild chymotrypsin digestion to the chick type IX collagen yielding products of 57 kDa (Fig. 2, lane 2, Fig. 5;

Fig. 5. Effect of tissue storage time and temperature on the synthesis of growth-plate collagens

Sheep growth-plate cartilage was dissected from ribs stored for various intervals at either 4 °C or 22 °C for up to 104 h. The storage interval described includes the 3.5 h between slaughter of the ewe and the dissection along with subsequent storage of foetal ribs in phosphate-buffered saline. Radiolabelled proteins were isolated from culture medium or matrix after incubation of dissected cartilage with [3 H]proline for 24 h. Proteins isolated from culture medium (a), samples of these proteins after mild chymotrypsin digestion (b) and the 2.0 M-NaCl precipitate of proteins extracted from the matrix by pepsin digestion (c) were denatured in the presence of dithioerythritol and subjected to SDS/PAGE and fluorography. $\alpha 1$, X, Xp and * indicate the migration positions of $\alpha 1$ chains, sheep type X collagen chains, collagen chains from pepsin- or chymotrypsin-digested sheep type X collagen and type IX collagen chains and peptides derived from type IX collagen respectively.

Gibson *et al.*, 1983) and ability, like the chick collagen (Gibson *et al.*, 1983; Bruckner *et al.*, 1983), to form disulphide-stabilized aggregates (Fig. 2, lanes 3 and 5). The 57 kDa polypeptides extracted from the cartilage matrix together with polypeptides of 41, 34 and 32 kDa (shown by asterisks in Fig. 5) are assumed, because of their similarity to polypeptides derived from bovine and rat type IX collagen (Ricard-Blum *et al.*, 1985; Furuto *et al.*, 1989), to be degradation products of type IX collagen.

Synthesis of type IX collagen was also sensitive to storage, but, in contrast with type X collagen, increased as a relative proportion of total collagen synthesis. The type IX peptide of 57 kDa appeared more prominently in pepsin extracts after 32 h storage of the rib at both 4 °C and 22 °C (Fig. 5).

The radiolabelled proteins extracted after digestion with pepsin or obtained by chymotrypsin digestion of radiolabelled culture medium proteins that migrated as three bands with a mobility slightly slower than the $\alpha 1$ band on SDS/PAGE are characteristic of type XI collagen (Burgeson & Hollister, 1979) and demonstrated little change with storage time or temperature.

Summation of radioactivity in the culture medium (after

chymotrypsin digestion) and the 0.8 M-NaCl and 2.0 M-NaCl precipitates of pepsin extracts of the cartilage matrix provided an estimate of the total collagen synthesis by rib growth-plate. Less than 10 % of the radiolabelled collagen, estimated from radioactivity in the final residue, remained unextracted and has not been included in the calculation of total collagen synthesis. Six rib growth-plates were analysed after the minimum storage interval of 3.5 h and were shown to incorporate between 1.0×10^6 and 1.85×10^6 c.p.m. into collagen. Growth-plates from contralateral ribs showed similar rates of collagen synthesis (contralateral ribs are indicated by the same symbol, 3.5 h time point, Fig. 6), whereas the rates of collagen synthesis from more caudal or cephalic ribs were less similar (Fig. 6a). Collagen synthesis by rib growth-plate showed a steady decline with storage time such that relatively little collagen synthesis was observed after 104 h. However, the rate of collagen synthesis was consistently higher in those ribs stored at 4 °C compared with those stored at 22 °C.

Scanning densitometry of fluorograms of SDS/polyacrylamide gels (Fig. 5) of chymotrypsin-digested culture medium proteins

Fig. 6. Effect of tissue storage time and temperature on the collagen types synthesized by growth-plate cartilage

The effect of tissue storage time and temperature on collagen synthesis was examined by SDS/PAGE of radiolabelled proteins isolated from the culture medium and cartilage matrix as shown in Fig. 5. Estimation of the total collagen synthesis (a) was obtained by summation of radioactivity in chymotrypsin-resistant culture medium proteins and pepsin-extracted proteins (both 0.8 M- and 2.0 M-NaCl-precipitated fractions). The proportions of total radioactivity incorporated into collagen types X (b), IX (c) and II together with XI (d) were determined by scanning densitometry of chymotrypsin-digested proteins isolated from the culture medium and the 2.0 M-NaCl precipitate of pepsin-extracted collagens after SDS/PAGE and fluorography. Collagen synthesis by growth-plates from six ribs was examined 3.5 h after slaughter ($\nabla \nabla \diamond \diamond \cdot \cdot$); syntheses by growth-plates from contralateral ribs are indicated by the same symbol. The lines shown begin at the means derived from the six determinations. Collagen synthesis was examined after subsequent storage of pairs of contralateral ribs at either 4 °C ($\square$) or 22 °C ($\triangle$).

and 2.0 M-NaCl precipitates of pepsin extracts of the cartilage matrix enabled the determination of the relative proportion of radioactivity incorporated into collagen types X, IX, XI and II (Fig. 6). Synthesis of type X collagen showed a significant increase followed by a rapid decline with storage time. Storage at 22 °C caused an earlier minor stimulation of type X collagen synthesis and an earlier decline compared with storage at 4 °C. The stimulation of type X collagen synthesis at 4 °C was also much greater than observed at 22 °C (Fig. 6b).

The relative proportion of radioactivity incorporated into type IX collagen peptides increased with storage time (Fig. 6c). However, there was no significant influence of storage temperature and the rapid decline seen in the relative proportion of type X collagen synthesis was not seen with type IX collagen synthesis. The increase in type IX collagen synthesis as a proportion of total collagen synthesis from less than 10% with minimal storage to greater than 25% after 32 h was maintained for the longer storage times even though total collagen synthesis (Fig. 6a) declined markedly over this time.

The synthesis of type II collagen was similar to type XI collagen. However, the incorporation of labelled proline into these two collagens was pooled as their separation was not always complete. The synthesis of types XI and II as a proportion of total collagen synthesis remained fairly constant and was not significantly influenced by storage temperature (Fig. 6d).

DISCUSSION

By direct comparison of type X collagen synthesized by human, sheep and chick growth-plate chondrocytes, we have demonstrated interspecies heterogeneity of type X collagens. The chicken type X collagen has been well characterized (Gibson *et al.*, 1981, 1983, 1984; Kielty *et al.*, 1985; Schmid & Conrad, 1982a,b) and some studies of the bovine type X collagen (Remington *et al.*, 1984; Grant *et al.*, 1985) and one report of rabbit type X collagen (Remington *et al.*, 1983) have been published. This is the first report of the characterization of human and sheep type X collagens. The identity of the bacterial-collagenase-sensitive proteins as type X collagen was concluded from the localization of their synthesis to regions of hypertrophic growth cartilage (cartilage surrounding secondary ossification centres contains hypertrophic chondrocytes and can be considered a type of growth cartilage) and a similarity in the size of the helical and non-helical regions of the molecule to previously reported type X collagens. The human type X collagen was surprisingly shown to be more similar to the chick type X collagen in both molecular size and apparent absence of disulphide aggregation than to the sheep type X collagen. Like the chick collagen, the human collagen is composed of α -chains of 59 kDa, concluded from studies of chymotrypsin-susceptibility to be composed of a helical region of 45 kDa with non-helical extensions. Like the chick collagen (Gibson *et al.*, 1983) and the reported structure of rabbit type X collagen (Remington *et al.*, 1983), human type X collagen does not appear to form disulphide-stabilized aggregates. The sheep collagen, however, is slightly larger, consisting of α -chains of 63 kDa containing a helical region of 49 kDa. A similar molecular size of 63 kDa with a helical region of 46 kDa has been reported for the bovine collagen (Grant *et al.*, 1985). Similarly, the bovine collagen has, like the sheep collagen described here, been shown to form disulphide-stabilized aggregates (Remington *et al.*, 1984; Grant

The storage interval described includes the 3.5 h between slaughter of the ewe and the dissection along with subsequent storage of foetal ribs in phosphate-buffered saline.

et al., 1985). Since aggregates of the sheep and bovine collagens are not dissociated by chymotrypsin digestion, at least some of the stabilizing interchain disulphide bonds must occur between helical regions or very close to the helical region of the molecules, and, since the sheep type X collagen contains a helical region approx. 3 kDa longer than those type X collagens (i.e. human and chick) that do not form disulphide-stabilized aggregates, it may be concluded that this extra helical sequence contains the cysteine residues forming interchain disulphide bonds. It has been suggested that the unusual chick type X collagen gene, containing only three exons, one coding for more than 90 % of the amino acid residues, may result in greater interspecies heterogeneity in amino acid sequence (Ninomiya *et al.*, 1986). This proposal was supported by the observation that chick, human and sheep type X collagens generated distinct peptide maps on digestion with V8 proteinase.

The close and specific association of human type X collagen with the site of endochondral ossification, as demonstrated here and inferred from studies of other species (Schmid & Linsenmayer, 1985, 1990; Gibson & Flint 1985; Grant *et al.*, 1985; Gibson *et al.*, 1986; Poole & Pidoux, 1989; Kirsch & von der Mark, 1990), suggests strongly that disruption of type X collagen structure would specifically affect skeletal growth. Furthermore, since the majority of genetic diseases resulting in skeletal growth retardation or deformity are of unknown aetiology, an examination of type X collagen synthesis in growth-plates of growth-affected children has been proposed. In a preliminary to these studies, we have shown that the temperature and duration of storage of tissue samples before growth-plate dissection and culture has a major effect on the spectrum of collagen types, particularly types IX and X, synthesized. The increased proportion of type X collagen synthesized after short-term storage may be explained if the number of chondrocytes synthesizing type X collagen increased as a result of differentiation *in vitro*. Differentiation *in vitro* appears to occur where non-hypertrophic chondrocytes hypertrophy and initiate and maintain high levels of type X collagen synthesis in culture (Schmid & Conrad, 1982a,b; Gibson & Flint, 1985; Castagnola *et al.*, 1987). The increased proportion of type X collagen synthesized may also reflect a response to altered cellular environment. Several factors have been shown to stimulate type X collagen synthesis by chondrocytes in culture, including culture medium and substrate (Gibson *et al.*, 1982; Bates *et al.*, 1987; Solursh *et al.*, 1986; Bruckner *et al.*, 1989), β -glycerophosphate and levamisole (Thomas *et al.*, 1990). However, any factor influencing collagen synthesis in the studies reported here must either remain associated with the tissues or induce relatively long-term changes in the chondrocytes, as collagen synthesis was studied in culture medium after dissection of the stored growth-plate. The loss of type X collagen synthesis after longer storage may reflect a loss of those cells synthesizing type X collagen. The region of hypertrophic chondrocytes, particularly in mammals (Kirsch & von der Mark, 1990), appears to be only a few cells deep and is adjacent to the metaphyseal blood supply. These 'exposed' cells may be the first chondrocytes lost during storage. Furthermore, hypertrophic chondrocytes and the synthesis of type X collagen have previously been shown to be vulnerable to loss during cell culture (Gibson *et al.*, 1982). Storage at 22 °C rather than 4 °C hastens the apparent loss of hypertrophic chondrocytes and may also thus prevent the greater stimulation of type X collagen synthesis seen after brief storage at 4 °C. The increased proportion of type IX collagen synthesized may also reflect a response to changes in the cellular environment. Chondrocytes have previously been shown to respond to dissociation from the cartilage by production of a high proportion (relative to total collagen synthesis) of type IX collagen during

the first few days of culture (Gibson *et al.*, 1984). As all chondrocytes within cartilage appear to synthesize type IX collagen, loss of peripheral hypertrophic chondrocytes with prolonged storage would not be expected to affect significantly type IX collagen synthesis as a proportion of total collagen synthesis. Histological assessment of the viability of hypertrophic chondrocytes after storage was precluded by loss of fragile chondrocytes during routine histological processing and will require use of improved tissue preparation and staining procedures.

Regardless of the cause, it is clear that the spectrum of collagen types synthesized (particularly type X collagen) by growth-plate cartilage is acutely sensitive to tissue storage. A major misinterpretation of the association between type X collagen synthesis and the cause of growth deformities could result from storage of tissue samples from growth-affected individuals before commencement of studies of collagen synthesis.

We thank Dr. A. Bourne, Department of Histopathology, Adelaide Children's Hospital, for the provision of autopsy material and the South Australian Meat Corporation for the sheep tissue samples. The secretarial assistance of Peta Knapman is gratefully acknowledged. This work was supported by grants from the Adelaide Children's Hospital and the National Health and Medical Research Council of Australia.

REFERENCES

- Bates, G. P., Schor, S. L. & Grant, M. E. (1987) *In Vitro Cell Dev. Biol.* **23**, 374–386
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Brown, C. C. & Balian, G. (1987) *J. Cell Biol.* **105**, 1007–1012
- Bruckner, P., Mayne, R. & Tuderman, L. (1983) *Eur. J. Biochem.* **136**, 333–339
- Bruckner, P., Horler, I., Mandler, M., Houze, Y., Winterhalter, K. H., Eich-Bender, S. G. & Spycher, M. A. (1989) *J. Cell Biol.* **109**, 2537–2545
- Burgeson, R. E. & Hollister, D. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1124–1131
- Castagnola, P., Torella, G. & Cancedda, R. (1987) *Dev. Biol.* **123**, 332–337
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Dixit, S. N. (1989) *Eur. J. Biochem.* **186**, 411–414
- Furuto, D., Bhowan, A. S. & Miller, E. J. (1989) *Matrix* **9**, 353–365
- Gibson, G. J. & Flint, M. H. (1985) *J. Cell Biol.* **101**, 277–284
- Gibson, G. J., Schor, S. L. & Grant, M. E. (1981) *Biochem. Soc. Trans.* **9**, 550–551
- Gibson, G. J., Schor, S. L. & Grant, M. E. (1982) *J. Cell Biol.* **93**, 767–774
- Gibson, G. J., Kielty, C. M., Garner, C., Schor, S. L. & Grant, M. E. (1983) *Biochem. J.* **211**, 417–426
- Gibson, G. J., Beaumont, B. W. & Flint, M. H. (1984) *J. Cell Biol.* **99**, 208–216
- Gibson, G. J., Berman, C. H. & Flint, M. H. (1986) *Collagen Relat. Res.* **6**, 163–184
- Grant, W. T., Sussman, M. D. & Balian, G. (1985) *J. Biol. Chem.* **260**, 3798–3803
- Kielty, C. M., Kwan, A. P. L., Holmes, D. F., Schor, S. L. & Grant, M. E. (1985) *Biochem. J.* **27**, 545–554
- Kirsch, T. & von der Mark, K. (1990) *Biochem. J.* **265**, 453–459
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Lizardi, P. M. (1979) *Cell* **18**, 581–589
- Lu Valle, P., Ninomiya, Y., Rosenblum, N. D. & Olsen, B. R. (1988) *J. Biol. Chem.* **263**, 18378–18385
- Majack, R. A. & Bornstein, P. (1985) *J. Cell Biol.* **100**, 613–619
- Ninomiya, Y., Gordon, M. van der Rest, M., Schmid, T., Linsenmayer, T. & Olsen, B. R. (1986) *J. Biol. Chem.* **261**, 5041–5050
- Poole, A. R. & Pidoux, I. (1989) *J. Cell Biol.* **109**, 2547–2554
- Remington, M. C., Bashey, R. I., Brighton, C. T. & Jimenez, S. A. (1983) *Collagen Relat. Res.* **3**, 271–278
- Remington, M. C., Bashey, R. I., Brighton, C. T. & Jimenez, S. A. (1984) *Biochem. J.* **224**, 227–233

- Ricard-Blum, S., Tiollier, J., Garrone, R. & Herbage, D. (1985) *J. Cell. Biochem.* **27**, 347–358
- Schmid, T. M. & Conrad, H. E. (1982*a*) *J. Biol. Chem.* **257**, 12444–12450
- Schmid, T. M. & Conrad, H. E. (1982*b*) *J. Biol. Chem.* **257**, 12451–12457
- Schmid, T. M. & Linsenmayer, T. F. (1985) *J. Cell Biol.* **100**, 598–605
- Schmid, T. M. & Linsenmayer, T. F. (1990) *Dev. Biol.* **138**, 53–62
- Shimokomaki, M., Duanne, V. C. & Bailey, A. J. (1980) *FEBS Lett.* **121**, 51–54
- Solursh, M., Jensen, K. L., Reiter, R. S., Schmid, T. M. & Linsenmayer, T. F. (1986) *Dev. Biol.* **117**, 90–101
- Thomas, J. T., Boot-Hanford, R. P. & Grant, M. E. (1990) *J. Cell Sci.* **95**, 639–648
- Tijssen, P. & Kurstak, E. (1983) *Anal. Biochem.* **128**, 26–35
- Yamaguchi, N., Benya, P. D., van der Rest, M. & Ninomiya, Y. (1989) *J. Biol. Chem.* **264**, 16022–16029

Received 31 October 1990/15 January 1991; accepted 28 January 1991