Nature of Collagens Synthesized by Monkey Periodontal-Ligament Fibroblasts *in vitro*

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1. First subcultures of fibroblast-like cells from adult monkey periodontal ligament were incubated in the presence of ^{14}C -labelled amino acids and produced significant amounts of type-I and type-III collagens. 2. The proportion of type-III collagen produced was calculated on the basis of the recovery of procollagens from DEAE-cellulose chromatography to be approx. 20%, and at least 10% when analysed as collagens on CM-cellulose chromatography. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the procollagens, the collagens and their CNBr peptides was used to confirm the identity of the collagen types. 4. In serum-free media extensive conversion of type-I procollagen, but not of type-III procollagen, into collagen was observed, suggesting that a specific type-I procollagen peptidase was produced. 5. The pattern of collagen synthesis was not significantly different from that obtained with fibroblasts derived from skin corium of the same animals.

The periodontal ligament is a highly specialized soft connective tissue that serves to anchor the roots of teeth into bony sockets in the jaw. The cells of the periodontal ligament function in the maintenance and remodelling of the ligament itself, and also to some extent the associated alveolar bone and cementum (Melcher & Eastoe, 1969). The major structural component in the ligament, as in most other connective tissues, is collagen. However, the collagen in periodontal ligament appears to be unique in two important respects, which may be related to one another. First, it has been demonstrated by Butler *et al.* (1975) that approx. 20% of bovine periodontal-ligament collagen is composed of type-III collagen, the remainder being type I. Although type-III collagen is present in significant amounts in most soft connective tissues in foetal life, its contribution in most adult connective tissues is normally very low. Second, the metabolic activity of collagen in rat periodontal ligament has been shown to be unusually high (Sodek, 1976). The half-life for the turnover of the mature collagen has been calculated to be approx. 1 day (Sodek, 1977).

The development in our laboratory of methods for isolating and culturing fibroblast-like cells from monkey periodontal ligament and skin (Marmary *et al.*, 1976) has allowed us to compare the patterns of collagen synthesis by these cells *in vitro* and *in vivo*.

Experimental

Cell culture

Fibroblast-like cells were isolated from skin and

Abbreviation used: SDS, sodium dodecyl sulphate.

periodontal ligament of 2–3-year-old monkeys (*Macaca fascicularis*) by using the procedures and culture conditions described by Marmary *et al.* (1976). Only first-subculture cells grown in 75 cm² flasks were used in this study. The cells were grown to confluency in an α minimal essential medium containing 15% (v/v) heat-inactivated foetal bovine serum (Flow Laboratories, Rockville, MD, U.S.A.). At 5 days after confluency the cells were washed twice with 10 ml of serum-free α minimal essential medium and then cultured in a serum-free α minimal essential medium lacking proline, glycine and lysine, but supplemented with the corresponding U- ^{14}C -labelled L-amino acids (L-[U- ^{14}C]lysine, 318 mCi/mmol, NEC-280; [U- ^{14}C]glycine, 102 mCi/mmol, NEC-276; L-[^{14}C]proline, 254 mCi/mmol, NEC-285; New England Nuclear Corp., Boston, MA, U.S.A.) (2 $\mu\text{Ci/ml}$ each). In addition, β -aminopropionitrile (50 $\mu\text{g/ml}$; Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to prevent collagen cross-linking, and freshly prepared ascorbic acid (100 $\mu\text{g/ml}$; Sigma) to promote collagen hydroxylation. The medium was collected 24 h later and collagenous proteins in the cell layer were extracted by shaking with 10 ml of 0.5 M-acetic acid overnight at 4°C.

Analysis of procollagens by DEAE-cellulose chromatography

Immediately after collection of culture medium, the proteinase inhibitors phenylmethanesulphonyl fluoride and *p*-hydroxymercuribenzoate (both Sigma) were added at concentrations of 50 $\mu\text{g/ml}$ to prevent conversion of procollagen into collagen (Lichtenstein *et al.*, 1975). All subsequent steps were carried out at

4°C. After the addition of 1 mg of rat skin acid-soluble collagen as a carrier, the collagenous proteins were precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 25% saturation, then pelleted by centrifugation at 40000g for 20 min.

The pellet was dissolved in 2 ml of 0.5 M-acetic acid and dialysed against the same solvent. The dialysed material was made 8 M in urea and dialysed against two 300 ml changes of 30 mM-Tris/HCl buffer (pH 7.4)/2 M-urea before DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.) chromatography (Lichtenstein *et al.*, 1975). Chromatography was carried out on a 1.5 cm × 30 cm column and the collagenous proteins were eluted with a linear gradient of 0–200 mM-NaCl in the Tris/HCl/urea buffer over a total volume of 300 ml. A flow rate of 7 ml/h was used to elute the radioactive proteins, which were collected in 5 ml fractions. Radioactivity in the fractions was measured by dissolving 0.2 ml samples in 7.0 ml of Aquasol scintillation 'cocktail' (New England Nuclear) and counting the material in a Nuclear-Chicago Isocap 300 (Searle Instrumentation, Toronto, Ont., Canada). Fractions associated with peaks of radioactivity were pooled and desalted by dialysis against 0.5 M-acetic acid. Samples containing 10000 c.p.m. were freeze-dried for subsequent identification procedures.

Analysis of collagens by salt fractionation

A mixture of type-I and type-III collagens (0.5 mg each) prepared from newborn-rat skin by the method of Chung & Miller (1974) was added to medium and cell extracts to act as a carrier for the labelled collagen. Medium samples and cell extracts were dialysed exhaustively against 0.5 M-acetic acid and then digested with pepsin (100 µg/ml) for 5 h at 15°C to convert the precursor collagens into collagen (Layman *et al.*, 1971). The digests were freeze-dried and an additional 4.5 mg of rat skin type-I collagen was added. The collagens were separated by the salt-fractionation procedure described by Chung & Miller (1974). The material was dispersed in 20 ml of 50 mM-Tris/HCl buffer, pH 7.4, containing 5.0 M-NaCl and the insoluble material pelleted at 40000g for 1 h. The pellet was re-extracted with a second 20 ml of the buffer and the supernatants were combined. Similarly, 20 ml extractions were carried out on the insoluble pellets with 2.5 M- and 1.5 M-NaCl solutions in the same buffer. Supernatants from each extraction procedure were filtered through a 0.2 µm Millipore membrane and the clear solution was dialysed against 0.5 M-acetic acid. The final pellet, insoluble in 1.5 M-NaCl, was dispersed in 0.5 M-acetic acid and dialysed exhaustively against the same solvent. After dialysis each sample was divided into portions and freeze-dried before analysis by CM-cellulose chromatography.

Analysis of collagen by CM-cellulose chromatography

Samples containing mixtures of labelled collagens were analysed on CM-cellulose (CM-52, Whatman) by using the method originally described by Piez *et al.* (1963) for separating collagen α -chains. Freeze-dried samples were dissolved in 2–3 ml of starting buffer (40 mM-sodium acetate/4 M-urea, adjusted to pH 4.8 with acetic acid) containing 0.2 µg of pepstatin (a gift from Dr. H. Umezawa, Institute of Microbiological Chemistry, Tokyo, Japan) and dialysed against two 200 ml changes of the same buffer. The sample was incubated at 56°C for 30 min before application to a column (1.0 cm × 15 cm) of the CM-cellulose equilibrated with the starting buffer at 42°C. The denatured collagen α -chains were eluted from the column with a linear gradient of 0–160 mM-NaCl in starting buffer over a total volume of 200 ml. A flow rate of 7.0 ml/h was used, and 2.5 ml fractions were collected. Samples (0.2 ml) from each fraction were analysed for radioactivity as described above. Radioactive peaks were pooled, dialysed and freeze-dried for further analysis on SDS/polyacrylamide-gel electrophoresis.

Analysis of collagenous proteins by SDS/polyacrylamide-gel electrophoresis and radioautography

SDS/polyacrylamide-gel electrophoresis was carried out by using the discontinuous buffer system on 7.5% polyacrylamide slabs with 20 cm separating gels (Laemmli, 1970). Freeze-dried samples, generally containing 10000 c.p.m. of radioactivity, were dissolved in 100 µl of sample buffer (25 mM-Tris/glycine, containing 2 M-urea and 0.2% SDS at pH 8.0) and heated at 56°C for 30 min before application to sample slots cast in 5% polyacrylamide-sample gel. Samples were run into the separating gels at 100 V. The voltage was then increased to 150 V and electrophoresis continued until Bromophenol Blue tracking dye had travelled approx. 14 cm (6–7 h). The gels were soaked in 100 ml of 10% (v/v) acetic acid, containing 1% (v/v) glycerol, for 20 min, then vacuum-dried by using a slab-gel-drier apparatus [model 224; Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont., Canada]. Some samples were reduced by the addition of 2-mercaptoethanol (1%, v/v) to the sample buffer before denaturation. 2-Mercaptoethanol was also added to the electrophoresis buffers for these analyses at 0.1% (v/v).

The dried gels were exposed to 20.3 cm × 25.4 cm (8 in × 10 in) Kodak SB-54 X-ray film for 1–4 weeks. Densitometer profiles were obtained by scanning radioautogram tracks at 550 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.). Radioactivity associated with each radioautographic band was estimated by measuring areas under each scan peak.

CNBr digestion of radioactive collagens

Fractions corresponding to the different peaks from the CM-cellulose column chromatography were pooled and dialysed against 0.5 M-acetic acid. Samples containing 10000 c.p.m. were freeze-dried, then redissolved in 250 µl of 0.5 M-acetic acid, and formic acid (90%, v/v) was added to 70% (v/v) concentration. Solid CNBr was added to a concentration of approx. 0.5% (w/v). The reaction was carried out with constant stirring at room temperature (25°C) for 4 h. The samples were diluted 5-fold with water, then freeze-dried before analysis by SDS/polyacrylamide-gel electrophoresis.

Amino acid analysis

The ratio of radioactive hydroxyproline to radioactive proline in labelled collagen samples was determined after separating the imino acids on a Beckman 121M amino acid analyser by using a system described previously (Sodek, 1977). Samples were hydrolysed in 6M-HCl for 24 h at 110°C under vacuum. The imino acids eluted from the analyser column were collected in a fraction collector, and the total radioactivity of each imino acid was measured as described by Sodek (1977).

Results

Characterization of the procollagens synthesized by periodontal-ligament fibroblasts

The incorporation of radioactive proline into collagenous and non-collagenous proteins synthesized by monkey periodontal-ligament fibroblasts during the 24 h culture period is shown in Table 1. This analysis showed that the (NH₄)₂SO₄ precipitation was effective in removing all collagenous protein from solution and that most of the collagen (90%) was recovered in the medium. Approx. 45% of the protein-bound radioactive proline in the medium was calculated to be incor-

porated into collagen, whereas collagen was found to be a minor component of the cell-layer proteins.

The collagenous proteins in the medium were separated into three major peaks by DEAE-cellulose chromatography (Fig. 1). The first peak generally

Fig. 1. DEAE-cellulose separation of [¹⁴C]collagen from periodontal-ligament-fibroblast medium produced in the absence of foetal bovine serum

Monkey periodontal-ligament fibroblasts were cultured for 24 h in medium containing ¹⁴C-labelled amino acids but lacking foetal bovine serum. The radioactive collagens secreted into the medium were precipitated with 25%-satd. (NH₄)₂SO₄ and separated by DEAE-cellulose chromatography, with 300 ml of 30 mM-Tris/HCl buffer, pH 7.4, containing 2 M-urea and a salt gradient (----) of 0–200 mM-NaCl. Each 5 ml fraction was assayed for ¹⁴C radioactivity (—).

Table 1. Incorporation of [¹⁴C]proline into collagen and non-collagenous proteins during 24 h culture with monkey periodontal-ligament fibroblasts

The incorporation of [¹⁴C]proline into collagen and other proteins by monkey periodontal-ligament fibroblasts after a 24 h culture was determined by amino acid analysis as described in the text. The total [¹⁴C]proline radioactivity associated with collagen was calculated by multiplying the [¹⁴C]hydroxyproline radioactivity by a factor of 1.3, and the percentage of label in collagenous protein was determined from this value. The average cell number per flask was approx. 5.5 × 10⁶.

Culture fraction	25%-satd.-(NH ₄) ₂ SO ₄ fraction	10 ⁻⁶ × [¹⁴ C]Hydroxyproline (d.p.m./flask)	10 ⁻⁶ × [¹⁴ C]Proline (d.p.m./flask)	Percentage of total protein label in collagen
Medium	Precipitate	0.47	1.19	45.0
	Supernatant	0	0.73	
Cell extract	Precipitate	0.056	0.56	7.8
	Supernatant	0	1.03	

accounted for approx. 50% of the total radioactivity, the remainder being more or less equally distributed between the second and third peaks. From the elution positions of these peaks, peak 1 was expected to contain collagen α -chains, peak 2, type-I procollagens, and peak 3, type-III procollagens. More definite identification was made from analysis of each peak before and after pepsin digestion by using an SDS/polyacrylamide-gel electrophoresis radioautography technique (Fig. 2). The first peak, before and after pepsin digestion, consisted of essentially two high-molecular-weight proteins with the electrophoretic characteristics of collagen α_1 - and α_2 -chains. The ratio of the areas under the corresponding scan peaks gave values of 2.92:1 before and 2.68:1 after pepsin digestion, indicating that this material was composed predominantly of type-I collagen. The second DEAE-cellulose peak was resolved into several bands, which had migration characteristics of the procollagens previously described for type-I collagen (Lichtenstein *et al.*, 1975). Both intact procollagens and p-collagens [p-collagen is used to describe the intermediate formed when either the *N*-terminal or the *C*-terminal extension peptide has

been removed from the procollagen molecule, and p_c-collagen refers to the intermediate that has lost the *N*-terminus only (see Uitto & Lichtenstein, 1976)] for α_1 - and α_2 -chains could be identified. In this system α_2 -p-collagen appeared to migrate between the α_1 - and α_2 -chains, whereas α_2 -procollagen comigrated with α_1 -p-collagen of type-I collagen. This pattern is similar to the one obtained from human skin fibroblast type-I procollagen (Lichtenstein *et al.*, 1975), except that the resolution of the components was improved by using the slab-gel radioautographic technique. After pepsin digestion, the densitometric ratio between the α -chains was 1.93:1, which is close to the 2:1 ratio expected for type-I collagen. The third peak was resolved into two bands with mobilities corresponding to type-III procollagen and p-collagen. The identity of the material in this peak was confirmed after pepsin digestion, which produced a single component that migrated in the position corresponding to α_1 -chains and gave a densitometric ratio of 7.2:1 compared with a minor band migrating in the position of α_2 -chains. In addition, under non-reducing conditions, the α_1 -chains were observed to run in the position

Fig. 2. Densitometric tracings of radioautographic band patterns of DEAE-cellulose peaks before and after pepsin digestion. Radioactive collagens secreted into the medium of monkey periodontal-ligament fibroblasts during the 24h culture were separated on a DEAE-cellulose column. The fractions of the three resulting radioactive peaks (Fig. 1) were pooled, dialysed and freeze-dried in batches (10000 c.p.m.). Samples, before and after pepsin digestion, were run on SDS/7.5% polyacrylamide slab gels under reducing conditions. The slab gels were vacuum-dried and exposed to X-ray films, which were then developed and scanned at 550nm. The resulting densitometric tracings are shown above. Direction of electrophoresis is from the negative to the positive electrode. (a) Peak 1; (b) peak 2; (c) peak 3; (d) peak-1 pepsin digest; (e) peak-2 pepsin digest; (f) peak-3 pepsin digest.

Fig. 3. DEAE-cellulose separation of [^{14}C]procollagens from periodontal-ligament-fibroblast medium produced in the presence of 10% (v/v) foetal bovine serum

Monkey periodontal-ligament fibroblasts were cultured for 24 h in medium containing ^{14}C -labelled amino acids and supplemented with 10% (v/v) foetal bovine serum. The radioactive collagens secreted into the medium were harvested and separated by DEAE-cellulose chromatography. The elution buffer was 30 mM-Tris/HCl, pH 7.4, containing 2 M-urea and the salt gradient (----) was 0–200 mM-NaCl over a total volume of 300 ml of the same buffer. Each 5 ml fraction was analysed for ^{14}C radioactivity (—).

expected for γ -chains (mol.wt. 300 000), suggesting the presence of inter- α -chain disulphide bridges characteristic of type-III collagen (Chung & Miller, 1974).

When collagen synthesis was analysed in cultures labelled in the presence of 10% foetal calf serum, the elution pattern from DEAE-cellulose showed a dramatic change in the relative sizes of the three peaks (Fig. 3). The collagen peak, peak 1, was absent, the type-I procollagen peak, peak 2, increased approximately 3-fold, and the type-III procollagen peak, peak 3, was more or less the same. Two additional peaks were also obtained, which were eluted in a position corresponding to type-I procollagen chains (Uitto & Lichtenstein, 1976). The type-III procollagen produced under these conditions was approx. 20% of the total collagen recovered from the DEAE-cellulose column.

Analysis, by salt fractionation, of α -chains of collagens synthesized by monkey periodontal-ligament and skin fibroblasts

The nature of the collagens synthesized by monkey periodontal-ligament and skin fibroblasts was also analysed by the salt-fractionation procedure originally described by Chung & Miller (1974) and modified by Narayanan & Page (1976) for analysing collagens synthesized by gingival fibroblasts. The results of this fractionation are shown for collagens in both medium and cell layer in Table 2. The 5 M-NaCl supernatant contained a small amount (10%) of labelled protein that was apparently resistant to pepsin digestion. In the cell layer this fraction was 50% of the total labelled protein, but in both fractions

Table 2. Analysis of salt-fractionated radioactive collagens synthesized by monkey periodontal-ligament and skin fibroblasts

Labelled proteins of both medium and cell layers of monkey periodontal-ligament and skin fibroblast cultures were digested with pepsin, separated into NaCl fractions, then fractionated by CM-cellulose chromatography. Numbers in parentheses are results obtained from monkey skin fibroblasts derived from the same animal and treated identically with the periodontal-ligament fibroblasts. The percentage of type-III collagen in each fraction was calculated by measuring the radioactivity under the $\alpha_1(\text{III})$ peak and comparing this with radioactivity measured under the type-I collagen $\alpha_1(\text{I})$ and $\alpha_2(\text{I})$ peaks from the CM-cellulose chromatography. The $\alpha_1(\text{I})$ and $\alpha_1(\text{III})$ peaks from the monkey periodontal-ligament medium were analysed by amino acid analysis and shown to have hydroxyproline/proline molar ratios of 1:1.54 and 1.15:1 respectively. The average number of skin fibroblasts per flask was approx. 2.0×10^6 .

	$10^{-6} \times$ Labelled protein (d.p.m./flask)	Percentage of total protein label	Type-III collagen (% of total)	α_1/α_2 -chain ratio
Medium				
5 M-NaCl supernatant	0.265 (0.348)	9.8 (17.2)	—	—
2.5 M-NaCl supernatant	0.004 (0.004)	0.2 (0.2)	—	—
1.5 M-NaCl supernatant	1.870 (1.230)	68.8 (60.8)	5.2 (8.2)	3.4 (2.8)
1.5 M-NaCl precipitate	0.579 (0.441)	21.3 (21.8)	21.3 (21.0)	3.0 (2.4)
Cell layer				
5 M-NaCl supernatant	1.210 (1.410)	48.7 (58.1)	—	—
2.5 M-NaCl supernatant	0.003 (0.003)	0.1 (0.1)	—	—
1.5 M-NaCl supernatant	0.730 (0.335)	29.4 (13.8)	4.5 (8.3)	2.7 (2.7)
1.5 M-NaCl precipitate	0.542 (0.679)	21.9 (28.0)	16.4 (16.0)	2.1 (3.0)

Fig. 4. CM-cellulose elution profiles of salt fractions prepared from media and cell extracts of monkey periodontal-ligament-fibroblast cultures

Labelled collagenous proteins from the media and cell-layer extracts of monkey periodontal-ligament-fibroblast cultures were digested with pepsin and the resulting collagen molecules fractionated by differential salt precipitation. The collagen α -chains in four fractions were resolved by CM-cellulose chromatography by using a 40 mM-sodium acetate buffer, pH 4.8, containing 4 M-urea, and a linear gradient of 0–160 mM-NaCl in the same buffer (---). Solid bars indicate fractions pooled for analysis and measurement of radioactivity for quantification of type-I and type-III collagen. (a) 1.5 M-NaCl supernatant of the medium; (b) 1.5 M-NaCl precipitate of the medium; (c) 1.5 M-NaCl supernatant of the cell extract; (d) 1.5 M-NaCl precipitate of the cell extract. —, ^{14}C radioactivity.

negligible amounts of labelled hydroxyproline could be detected. The 2.5 M-NaCl supernatant contained low amounts of radioactivity in material from both medium and cell layer. This fraction has been shown to contain type-II collagen and/or the type-I α_1 -trimer collagen (Narayanan & Page, 1976). The 1.5 M-NaCl supernatant contained most of the radioactive proteins in the medium (68.6%). Analysis of this material by CM-cellulose chromatography gave the typical

Fig. 5. Densitometric tracing of radioautographic band patterns produced by CNBr digestion of α -chains separated by CM-cellulose chromatography

Radioactive collagen α -chains produced by monkey periodontal-ligament fibroblasts and separated by CM-cellulose chromatography (Fig. 4) were digested with CNBr in 70% (v/v) formic acid. The digests were freeze-dried and separated on a 10% (w/v) polyacrylamide slab gel. The slab gel was vacuum-dried, then exposed to X-ray film, which was developed and scanned at 550 nm. The resulting densitometric scans are shown. Electrophoresis was carried out from negative to positive electrode (from left to right). Numbers above the peaks represent known CNBr peptides. Identification of each peptide was made by comparison with the scanning patterns of CNBr peptides prepared by Hance *et al.* (1976). CNBr digest of: (a) $\alpha_1(\text{I})$ -chain; (b) $\alpha_1(\text{III})$ -chain; (c) α_2 -chain.

profile for type-I collagen α -chains (Fig. 4a), from which an α_1 -/ α_2 -chain ratio of 3.4:1 was calculated from the radioactivity in the corresponding peaks. A small amount of type-III collagen (5.2% of the total collagen in this fraction) was also recovered, and its identity, together with that of the type-I α_1 - and α_2 -chains, was confirmed by SDS/polyacrylamide-gel electrophoresis of the intact chains (results not shown) and of their CNBr fragments (Fig. 5). The 1.5M-NaCl pellet was enriched (21.3%) in type-III collagen, but, as shown by CM-cellulose chromatography (Fig. 4b), most of the collagen was still type I. A significant amount of radioactivity in the pellet could not be solubilized for CM-cellulose chromatographic analysis, even when the urea concentration was raised to 8M. This was approx. 35% of the radioactivity in the pellet prepared from the medium. Similar distributions of type-I and -III collagen were found in the corresponding fractions prepared from the cell-layer material. Calculation of the total amount of type-III collagen produced gave values of 9% in the medium and 10% in the cell layer and an average of approx. 9.5% for the whole culture.

An identical analysis of collagen synthesized by monkey skin fibroblasts obtained from the same animals demonstrated a distribution of radioactivity in the collagens similar to that found for the periodontal-ligament cells (Table 2). In the skin cultures, type-III collagen accounted for approx. 12.5% of the total collagen synthesized.

Discussion

Measurement of the incorporation of labelled amino acids into proteins secreted by monkey periodontal-ligament fibroblast-like cells demonstrates, as reported previously (Sodek *et al.*, 1977), that substantial amounts of collagen are produced by these cells. The nature of the collagens in both the medium and cell layer was analysed by the salt-fractionation procedure described by Chung & Miller (1974) for separating the various collagen types. The results of this analysis (Table 2) demonstrate that almost all the labelled collagen is recovered in the 1.5M-NaCl pellet and supernatant. From the CNBr-digestion patterns of the collagen α -chains (Fig. 5) isolated by CM-cellulose chromatography the pellet was shown to be enriched with type-III collagen, and the supernatant was mostly type-I collagen. The proportion of type-III collagen calculated by this method was approx. 10% of the total collagen synthesized. This value is, however, a conservative estimate, since we were unable to solubilize more than 65% of the radioactivity from the 1.5M-NaCl pellet. In addition, accurate quantification from the CM-cellulose chromatography profiles is difficult because of poor recoveries and the

incomplete separation of the type-III α -chains from the α_2 -chains. No appreciable amounts of collagen were found in the other salt fractions, although the small amount of radioactivity in the 2.5M-NaCl supernatant and the abnormally high ratio of type-I collagen α_1 - to α_2 -chains in the 1.5M-NaCl supernatant indicate that trace quantities of the type-I α_1 -trimer, $[\alpha_1(I)]_3$, found in other culture systems (Narayanan & Page, 1976; Mayne *et al.*, 1975; Benya *et al.*, 1977) may be produced.

A more accurate quantification of the proportion of type-III collagen synthesized was obtained from the profiles of collagen precursors separated by DEAE-cellulose chromatography. When cultures were labelled in the presence of foetal calf serum all the collagen synthesized was recovered in the precursor forms (Fig. 3), and type-III collagen synthesis could be calculated to represent 20% of the total collagen synthesis.

When cells were labelled in the absence of serum, considerable procollagen peptidase activity was evident from the recovery of large amounts of collagen, and the type-I and type-III procollagens were recovered in approximately equal amounts (Fig. 1). This observation is in contrast with results obtained from human skin fibroblasts in culture (Lichtenstein *et al.*, 1975), where procollagen conversion over a 24h culture period was negligible. The activity is not unique to the periodontal-ligament cells, since similar results were obtained with first subcultures of monkey skin and gingival fibroblasts (H. Limeback, J. Sodek & D. M. Brunette, unpublished work).

Although the type-III procollagen recovery in the serum-free labelled cultures was close to that obtained in cultures labelled in the presence of serum, the type-I procollagen recovery was considerably decreased. The decrease in type-I procollagen in these cultures correlated with the appearance of the large collagen peak, which, as shown by SDS/polyacrylamide-gel electrophoresis of the α -chains, was almost entirely type-I collagen. These results indicate that either the procollagen peptidase activity is specific to type-I procollagen or that type-III procollagen is a much poorer substrate. The existence of specific procollagen peptidases for each procollagen type has been suggested by Lenaers & Lapiere (1975) and is also supported by the studies *in vitro* of Benya *et al.* (1977).

The presence of the proteinase inhibitors α_2 -macroglobulin and α_1 -trypsin inhibitor in serum are probably responsible for preventing the conversion of procollagen into collagen in cultures labelled in the presence of serum by blocking the procollagen peptidase activity. However, the appearance of two new labelled peaks on DEAE-cellulose chromatography, one of which corresponds to type-I procollagen (Uitto & Lichtenstein, 1976), indicates

that not all the procollagen peptidase activity is blocked. Since it has been shown that several enzymes are responsible for the complete conversion of type-I procollagen into collagen (Davidson *et al.*, 1975), it is probable that some of these enzymes may not be completely inhibited by serum.

The procollagen profile obtained from cultures labelled in the presence of foetal calf serum showing type-III collagen synthesis to be approx. 20% of the total collagen synthesis demonstrates that the monkey periodontal-ligament fibroblast-like cells *in vitro* exhibit much the same collagen-synthetic capabilities as do periodontal-ligament fibroblasts *in vivo*. However, when the pattern of collagen synthesis is compared with that obtained from skin corium fibroblasts (Table 2), there are no significant differences in the amount of collagen synthesized nor between the relative amounts of the two types of collagen. Similar ratios of type-I and type-III collagen synthesis *in vitro* have also been reported in human lung fibroblasts (Hance *et al.*, 1976) and in smooth-muscle cells from human (Layman *et al.*, 1977) and pig (Barnes *et al.*, 1976a) aortae. Although type-III collagen has been found in all of these tissues, the relatively higher amounts of type-III collagen in aorta (Rauterberg & von Bassewitz, 1975) and periodontal ligament (Butler *et al.*, 1975) compared with skin (Epstein & Munderloh, 1975) are not reflected in the respective activities of their cells *in vitro*. In addition, there is no indication of lower metabolism of collagen in skin fibroblast cultures compared with periodontal ligament, as might be expected from studies *in vivo* (Sodek, 1976, 1977).

Attempts to correlate patterns of collagen synthesis *in vitro* and *in vivo* are complicated by the fact that cultured cells are removed from the environment of a functioning tissue. Also, it is possible that considerable cell selection can take place during cell isolation and culture procedures, so that the cultured cells may not be representative of all of the populations of cells functioning *in vivo*. By using thymidine labelling, it has been found that most fibroblast-like cells initiating a culture from explants of periodontal ligament originate from the vicinity of blood vessels (Brunette *et al.*, 1976). A similar location has been demonstrated from cells that divide in response to wounding of periodontal ligament *in vivo* (Gould *et al.*, 1977). Similarly, cultured skin cells probably also have a paravascular origin, despite some biochemical and cultural differences with ligament cells (Marmary *et al.*, 1976). As a result, the similarity in the collagen-synthetic patterns of the ligament and skin cells *in vitro* might be expected. Further, the similarity between the collagen-synthesis patterns *in vivo* shown for embryonic and wound-healing tissue (Barnes *et al.*, 1976b) and these patterns *in vitro* might also be expected, since in all these situations the cells

producing the collagen have recently divided, migrated and differentiated into functional forms.

However, it is noteworthy that the cells of functioning periodontal ligament *in vivo* also exhibit a collagen-synthesis pattern resembling that of embryonic connective and wound-healing tissue. The turnover of collagen is high, the proportion of type-III collagen is high and the dehydrodihydroxy-lysineonorleucine cross-link, which is predominant in healing wounds and embryonic connective tissue (Barnes *et al.*, 1976b), is also the major cross-link in functioning and developing periodontal ligament (Pearson *et al.*, 1975). In this case, it is conceivable that this unusual collagen-synthetic pattern may be the result of mechanical stresses derived from intermittent occlusal forces that cause micro-trauma in the periodontal ligament.

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