A New Approach to Assessing Collagen Turnover by using a Micro-Assay

A HIGHLY EFFICIENT AND RAPID TURNOVER OF COLLAGEN IN RAT PERIODONTAL TISSUES

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Measurement of [^3H]proline incorporation into newly synthesized and mature collagen in connective tissues was used to compare rates and efficiency of collagen turnover. The approach minimizes label-recycling problems. By using a micro-assay to determine hydroxyproline specific radioactivities, a highly efficient and rapid collagen turnover in rat periodontal tissues was demonstrated.

Collagen is the most abundant vertebrate protein and also the most important tensile component of connective tissue. The tensile strength of collagen fibres is derived from the uniform packing of tropo-collagen subunits held rigidly together by inter-molecular cross-links (Bailey *et al.*, 1974). The collagen fibres provide a framework for the extra-cellular matrix of connective tissues, but, because of their rigid nature, the fibres must be remodelled by resorption and synthesis when changes in the size, shape or biophysical properties of connective tissue occur. Numerous studies, mostly carried out in rats, have shown that collagen turnover is quite rapid in connective tissues of growing animals (Kao *et al.*, 1961a; Tanzer & Gross, 1964). However, turnover rates in the adult are greatly decreased (Kao *et al.*, 1961b), except in the uterus during pregnancy (Harkness & Harkness, 1954), in areas of wound healing (Grillo *et al.*, 1958) and in some pathological conditions (Woessner, 1968), where collagen turnover is high. There is also some evidence suggesting a high collagen turnover in the periodontal tissues that support the teeth. This evidence comes from radio-autographic studies (Carneiro & Leblond, 1966; Skougard *et al.*, 1970) and from the observation that in vitamin C deficiency the teeth loosen and may be lost (Hunt & Paynter, 1959). However, the radio-autographic procedures lack specificity (Melcher & Eastoe, 1969) in labelling, and there are problems in good quantification (Rogers, 1973), whereas the evidence provided by vitamin C deficiency is indirect and is based on the knowledge that vitamin C is necessary for collagen synthesis (Barnes *et al.*, 1970).

To measure collagen turnover directly, radio-actively labelled amino acids are used to label the collagen. If non-specific amino acids such as glycine

are chosen, the collagen must be purified for assay. Alternatively, if radioactively labelled proline is used, labelled hydroxyproline, which is peculiar to collagen, can be measured. The metabolism of collagen can then be followed by observing changes of specific radioactivity with time. The most popular method for collagen-turnover studies has been to prelabel the collagen and then follow the loss of collagen specific radioactivity over extended time-periods. However, besides being time-consuming and subject to the effects of tissue growth, it has been shown in skin that the approach is highly inaccurate, because of the recycling of proline label (Jackson & Heininger, 1974). To avoid these problems I have followed the rate of incorporation of [^3H]proline into newly synthesized collagen and mature collagen fractions over short time-periods. By using this approach in combination with a micro-analytical method for measuring hydroxyproline specific radioactivities, I have shown that not only is collagen turnover in adult rat periodontal tissues rapid, but also there is a much more efficient maturation process in operation when compared with skin collagen.

Experimental

Male CBL Wistar rats weighing 205 ± 10 g were given $10 \mu\text{Ci}$ of [^3H]proline (NET-323; New England Nuclear Corp., Boston, MA, U.S.A.)/g body wt. by intraperitoneal injection. Animals were killed by cervical dislocation at various times up to 27 h. Immediately after death a blood sample was taken by cardiac puncture. Tissues for analysis were dissected from the mandibles under a dissecting microscope. Attached gingiva was removed from the buccal and lingual aspects of the molars, and representative

samples were processed for histological examination to determine the presence of inflammation. Inflamed tissues were not analysed. Periodontal ligament was scraped from the apical two-thirds of the roots of extracted first and second molars which were fully erupted and functional. Bone, free from soft connective tissue, was excised from the alveolar process mesial, buccal and lingual to the first molar. A sample of subepithelial connective tissue of skin was taken from the thoracic region to serve as a control. The tissue samples from each animal were extracted with 0.45M-NaCl followed by 0.5M-acetic acid to provide the three characteristic collagen fractions (Jackson & Bentley, 1960).

Cardiac blood samples (150 μ l) were mixed immediately with an equal volume of phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 50 units of heparin/ml, and 10nmol of norleucine was added as an internal standard. After centrifugation in an Eppendorf Microfuge (Brinkmann Instruments, Toronto, Ont., Canada) for 10min, the serum proteins were precipitated with 6% (w/v) trichloroacetic acid. After further centrifugation the supernatant was extracted four times with equal volumes of diethyl ether to remove trichloroacetic acid, dried under vacuum and the residue washed twice with water. The residue was dissolved in 0.2M-sodium citrate buffer, pH2.2, and a portion taken for automatic amino acid analysis on a Beckman 121 instrument. A second portion, containing [14 C]-glycine as an internal standard, was separated on the same system but the effluent collected in scintillation vials to determine the radioactivity associated with the proline and hydroxyproline. Radioactivity was measured in a Nuclear-Chicago Isocap 300 liquid-scintillation system (Searle Instrumentation, Toronto, Ont., Canada) after the addition of 7 ml of Aquasol scintillation cocktail (New England Nuclear Corp., Boston, MA, U.S.A.). The counting efficiency for 3 H in these analyses was consistently around 45%.

Tissue samples were extracted three times with 300 μ l of 0.45M-NaCl in a 400 μ l micro-centrifuge tube for 24h on a rotary shaker. After centrifugation in a Microfuge for 10min, the tissue residue was washed with water and extracted with $3 \times 300 \mu$ l of 0.5M-acetic acid and finally washed again with water. The combined salt extracts and acid extracts were centrifuged at 100000g on an IEC B 60 ultracentrifuge and the supernatants exhaustively dialysed against 1% acetic acid and freeze-dried. The residues from each collagen fraction were hydrolysed in 6M-HCl for 24h at 110°C in evacuated hydrolysis tubes. Samples were taken for amino acid analysis and for scintillation counting as described for the blood samples. The assay can be used to measure specific radioactivities on tissue samples containing as little as 10pmol of collagen, provided that there is at least 100c.p.m. associated with the hydroxyproline.

Results and Discussion

The specific radioactivity of blood proline determined at various times (Fig. 1) indicates that the [3 H]proline appears to enter the vascular system in a pulse form, with a peak between 20 and 30 min after injection. Determination of the specific radioactivity of hydroxyproline in the salt-soluble collagens provides profiles for the incorporation of label into the newly synthesized collagen molecules (Fig. 1). However, to evaluate the relative rates of collagen synthesis in the tissues, it is necessary to compensate for differences in the salt-soluble pool sizes. Of the total collagen in the tissue samples, the amount that was salt-soluble was 2.8% for ligament, 3.0% for gingiva and 6.3% for skin, as determined by hydroxyproline analyses on the amino acid analyser. The mineral in bone samples precludes efficient extraction, so analysis of alveolar bone was not possible. The rate of collagen synthesis in ligament can be calculated to be twice as fast as in gingiva and four times as fast as in skin. In each case the radioisotope appeared in the salt-soluble fraction 30min after injection of [3 H]proline, which is in agreement with reports on the time required for the synthesis and secretion of collagen by cells (Weinstock & Leblond, 1974). To test that the tissues were being equilibrated

Fig. 1. Hydroxyproline specific-radioactivity profiles for salt-soluble tissue fractions

The 0.45M-NaCl tissue extract after acid hydrolysis in 6M-HCl was analysed on a Beckman 121 amino acid analyser as described in the text. Usually two-thirds of the available material containing 10–50pmol of collagen was used for colorimetric quantification of hydroxyproline, and one-third used for determination of the associated radioactivity. Hydroxyproline specific-radioactivity profiles for periodontal ligament (●), gingiva (○) and skin (▲) are shown together with the profile for blood proline specific radioactivity (△). Each point represents a single determination from the tissue obtained from one animal.

equally with radioisotope, an animal was perfused with the same amount of radioactively labelled proline through the jugular vein over a 3 h period, killed and the specific radioactivity of hydroxyproline determined in each of the tissues. Although, as expected, the specific radioactivities were lower than at the equivalent time of death for animals receiving radioisotope intraperitoneally, the ratios remained the same, suggesting equal equilibration of the tissues.

Specific radioactivities of hydroxyproline for the insoluble collagens of the tissues (Fig. 2) indicate that label is incorporated into periodontal-ligament collagen at a rate five times as fast as the rates in gingiva and alveolar bone, and 35 times as fast as the rate in skin. However, these values must be adjusted to compensate for the differing dilution of the radioisotopically labelled collagen in the salt-soluble compartments of each tissue. Although the rate for ligament relative to gingiva is not significantly changed, and no adjustment for alveolar bone can be made, the rate relative to that in skin decreases to 15 times as fast. A similar specific-radioactivity profile as shown in Fig. 2 could be constructed for the acid-soluble collagens, indicating that this compartment is part of the mature collagen structure. Since the rate of collagen synthesis is equal to the rate of collagen degradation at zero growth, these results provide

relative turnover rates for these tissues. Although it may be argued that growth was taking place in skin, gingiva and bone, the ligament was obtained from fully erupted teeth, and even if growth was a factor during the short period used in the study, the effect on skin could be expected to be the greatest of the four tissues, which was not reflected in the results.

Assuming zero growth, the efficiency of conversion of newly synthesized collagen into mature insoluble collagen can be calculated in the following manner. The rate of decrease of hydroxyproline specific radioactivity from the salt-soluble compartment is equal to the rate of increase of hydroxyproline specific radioactivity into the mature insoluble collagen, plus the rate of degradation of the newly synthesized collagen. Thus the percentage efficiency of collagen maturation can be given by:

$$\text{Efficiency (\%)} = \frac{K_2}{K_1} \times \frac{x}{y} \times 100$$

where K_2 is the rate of formation of insoluble collagen, measured as the increase in hydroxyproline specific radioactivity in Fig. 2, and K_1 is the rate of loss of salt-soluble collagen, measured as the decrease in hydroxyproline specific radioactivity in Fig. 1, x is the percentage of collagen in the insoluble fraction, and y the percentage of collagen in the salt-soluble fraction. By using the maximum slope of the descending peak in Fig. 1 for K_1 , and the rate of specific-radioactivity increase in Fig. 2 over the same period as K_2 , the calculation for ligament becomes:

$$\frac{48}{1600} \times \frac{97.2}{2.8} \times 100 = 104\%$$

Similarly, values of 51% and 32% can be calculated for gingiva and skin respectively. Because of the problems in obtaining accurate kinetic data from this type of study, the results are most useful as relative values, which suggest that the conversion of new collagen into fibres is highly efficient in ligament, but that in gingiva, and particularly in skin, the majority of the new collagen is degraded. The highly efficient conversion of ligament collagen into fibres suggests that in this tissue there is a rapid cross-link mechanism, or that little degradation of newly formed collagen takes place.

Since the turnover of skin collagen in rats of this age is faster than for most other connective-tissue collagens (Kao *et al.*, 1961b), the even greater rates of collagen synthesis found in the periodontal tissues demonstrate that these connective tissues have a particularly high turnover. The high turnover in rat gingiva demonstrated here is consistent with results obtained in marmosets (Page & Ammons, 1974), whereas mandibular bone has been shown to have one of the highest turnovers of a number of bones analysed in rats (Firschein, 1967). Although high

Fig. 2. Hydroxyproline specific-radioactivity profiles for insoluble tissue fractions

Tissue insoluble in 0.45M-NaCl and 0.5M-acetic acid was treated as described in Fig. 1, except that usually one-sixth of the available material containing 0.15–0.17nmol of collagen was used for colorimetric quantification of hydroxyproline and five-sixths used for determination of the associated radioactivity. Hydroxyproline specific-radioactivity profiles for periodontal ligament (●), gingiva (○), bone (■) and skin (▲) are shown.

collagen turnover has been suspected in the ligaments of continually erupting teeth, because of the continual remodelling of the collagen fibres, preliminary studies in this laboratory indicate that the collagen of rat incisor ligament turns over at a lower rate than collagen in molar ligaments of the same animal (J. Sodek, unpublished work). The reason for the high turnover of collagen in periodontal tissues is obscure, but the intermittent occlusal forces acting on the collagen fibres of these tissues, particularly during mastication, may be important. It is noteworthy that in bovine periodontal ligament, it has been shown that 20% of the collagen is the embryonic type III form (Butler *et al.*, 1975). In view of this, it would be interesting to determine whether or not the high turnover in the tissues studied here bears any relation to the amount of type III collagen present.

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