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Short Contribution

Alterations in biosynthetic accumulation of collagen types I and III during growth and morphogenesis of embryonic mouse salivary glands

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ABSTRACT We examined the biosynthetic patterns of interstitial collagens in mouse embryonic submandibular and sublingual glands cultured *in vitro*. Rudiments explanted on day 13 of gestation and cultured for 24, 48, and 72 h all synthesized collagen types I, III, and V. However, while the total incorporation of label into collagenous proteins did not change over the three-day culture period, the rate of accumulation of newly synthesized types I and III did change. At 24 h, the ratio of newly synthesized collagen types I:III was approximately 2, whereas at 72 h, the ratio was approximately 5. These data suggest that collagen types I and III may be important in initiation of branching in this organ, but that type I may become dominant in the later stages of development and in maintenance of the adult organ.

KEY WORDS: *submandibular gland, sublingual gland, interstitial collagen, extracellular matrix*

The importance of collagens in branching morphogenesis has been shown by experiments in which the synthesis or accumulation of these molecules was manipulated. Grobstein and Cohen (1965) found that salivary epithelia cultured in the presence of collagenase exhibited total loss of branching morphogenesis. Subsequently, it was demonstrated that lung and kidney epithelial branching also are perturbed in the presence of collagenases (Wessells and Cohen, 1968). These early results are questionable as the enzyme preparations used may have been contaminated with glycosaminoglycan degrading activity (Bernfield and Wessells, 1970). However, Nakanishi *et al.* (1985, 1986a) found that pure bacterial collagenase inhibited the formation of new branches in the salivary system, but did not cause loss of established branches. A specific interstitial collagenase that degrades types I and III collagens, but not types IV and V, also inhibits branching (Fukuda *et al.*, 1988), indicating that these interstitial collagens are required for branching morphogenesis.

Similar results are obtained by perturbation of collagen synthesis or secretion. L-azetidine-2-carboxylic acid (LACA), a proline analogue which prevents formation of the triple helix of mature collagen, and α,α'-dipyridyl, a prolyl hydroxylase inhibitor, dramatically inhibit formation of new branches in lung and salivary gland rudiments *in vitro* (Alescio, 1973; Spooner and Faubion, 1980). These morphogenetic effects correlate with effects on collagen synthesis and accumulation (Spooner and Faubion, 1980). In marked contrast to the effect of reducing collagen accumulation, treatment with collagenase inhibitors leads to supernumerary branch-points in submandibular glands (Nakanishi *et al.*, 1986a,b).

Krachtochwil *et al.* (1986) examined organogenesis in the Mov13 collagen type I-deficient mouse. The phenotype of this mutant is recessive lethal and homozygous offspring die before birth. However, some embryos survive until day 15 or 16 of gestation. Salivary gland, lung, kidney, and pancreas rudiments removed from surviving embryos on the appropriate day of gestation and grown *in vitro* exhibit normal growth and branching morphogenesis, indicating that other interstitial collagens are sufficient to support these activities. Metabolic labeling and immunohistochemistry confirmed the absence of type I collagen in these organs, and correspondingly high levels of collagen types III and V.

Despite the clear importance of the interstitial collagens in salivary gland development, there has been no evaluation of the biosynthetic patterns of these molecules during normal morphogenesis. Our results show that there is an alteration in the rates of accumulation of newly synthesized collagen types I and III. We propose that this alteration reflects changing requirements by the developing organ over developmental time.

Embryonic submandibular and sublingual gland rudiments exhibit normal branching morphogenesis when grown in culture, as shown in Fig. 1. Collagenous proteins in pepsin/acetic acid-treated

Abbreviations used in this paper: ECM, extracellular matrix; LACA, L-azetidine-2-carboxylic acid; HBSS, Hank's balanced salt solution; ß-APN, ß-aminoproprionitrile; TCA, trichloroacetic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ARG, autoradiogram.

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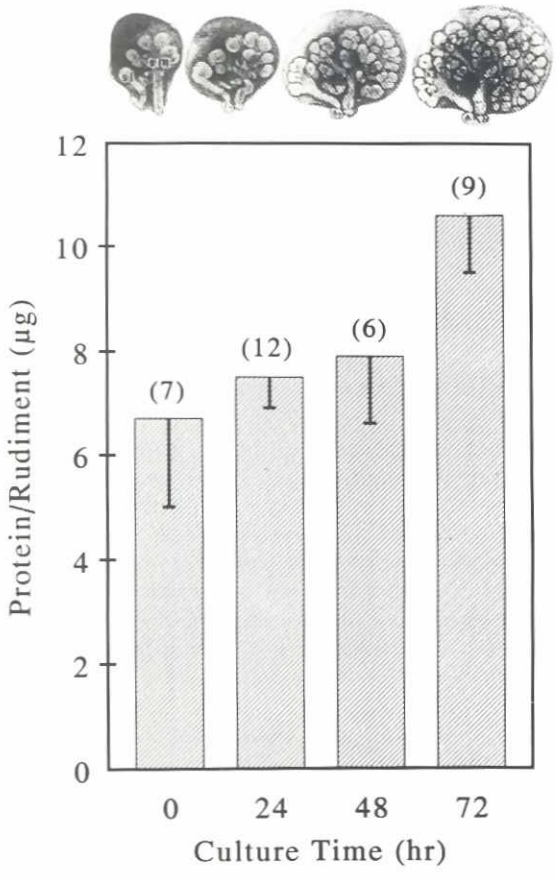

Fig. 1. Salivary gland growth and morphogenesis *in vitro*. *The top panel shows the same living submandibular (sm) and sublingual (sl) glands after 0, 24, 48, and 72 h (left to right) in culture. The graph demonstrates that the increase in size is accompanied by increased total protein per rudiment. Submandibular and sublingual glands were cultured for 0, 24, 48, and 72 h, pooled, and assayed for total protein. Pool size varied from 6 to 24 rudiments; the number of determinations for each time point are shown in parentheses. The bars represent the mean ± standard error.*

salivary gland extracts were precipitated with TCA. Fig. 2 shows that radiolabel was incorporated into collagens (pepsin/acetic acid-resistant protein) at a constant rate throughout the culture period. Radiolabel incorporation into total TCA-precipitable protein also occurred at a similar rate at all times examined.

Extracts from rudiments cultured for 24, 48, or 72 h and labeled for the last 24 h of the culture period, were subjected to SDS-PAGE under non-reducing and reducing conditions. ARGs prepared from gels run under non-reducing conditions had 3 main bands (Fig. 3A), these co-migrated with standard collagen type III trimer and with the α1(I) and α2(I) chains of the type I standard. Reduced samples gave only 2 major bands corresponding to α1(I) plus α1(III) polypeptides and α2(I) polypeptide (Fig. 3B). Small amounts of type V collagen were present consistently in our preparations. The α1(V) chain of this collagen migrates behind α1(I) chains. A band corresponding to the α2(V) chain of type V could not be distinguished under the conditions used, as this molecule migrates with or very slightly behind α1(I) chains. We did not allow for the presence of this molecule when calculating the type I to III ratios as, based on the density of the α1(V) band, it accounted for a very small portion of the material seen on the ARGs and was present in consistent amounts (Fig. 3). In addition to these bands, several minor bands were seen on most ARGs. These included a doublet corresponding to $ß_{1,1}$ and $ß_{1,2}$ dimers of type I collagen.

Visual comparison of ARGs indicated differences in the relative band intensities of type I and III collagens in 24, 48, and 72 h extracts. This was confirmed by scanning densitometry of ARGs (Fig. 4). Peak areas were used to calculate the ratio of newly accumulated collagen types I to III α1(I) plus α2(I):III. Fig. 5 shows that while the amount of type I was always greater than type III, the ratio of labeled type I:III increased from about 2 at 24 h to about 5 at 72 hr. The differences between 24 and 72 h and between 48 and 72 h were significant ($p= 0.0001$ and $p= 0.0022$, respectively). The difference between 24 and 48 h extracts was almost significant ($p= 0.0725$). No differences were observed in any of the other bands.

Our data demonstrate that normal growth and branching morphogenesis of mouse embryo salivary glands *in vitro* are accompanied by changes in the rates of accumulation of newly synthesized interstitial collagen types I and III. The differences in the ratio of radiolabeled type I to type III collagen after 1 day and after 3 days is dramatic. The results indicate that the overall rate of incorporation of ^{14}C-glycine into collagenous proteins did not change over the culture period. Thus, the change in the ratio of type I to III is not simply due to increased type I synthesis, but must reflect alterations in the synthesis and/or degradation of both molecules. In agreement with Krachtochwil *et al.* (1986) we also found type V collagen in our preparations. There was no change in the relative synthetic rate of this component during the culture

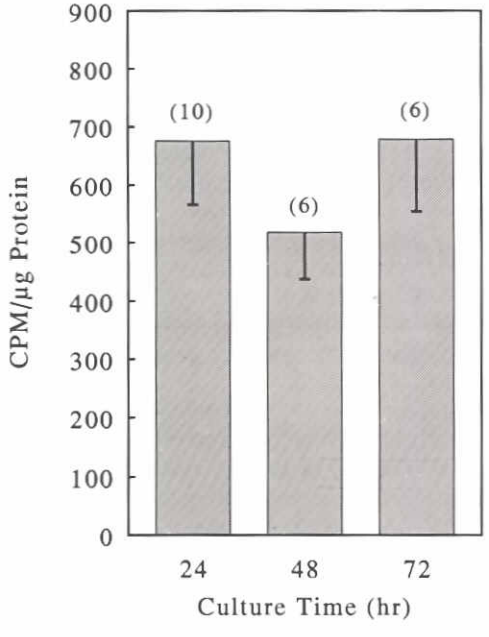

Fig. 2. Incorporation of ^{14}C-glycine into collagenous proteins (pepsin/acetic acid-resistant). *Pooled rudiments were digested as described in Materials and Methods and labeled proteins were precipitated with TCA and counted. A minimum of 7 and a maximum of 57 rudiments were used for each pool; the number of determinations for each time point are shown in parentheses. The bars are the mean ± standard error.*

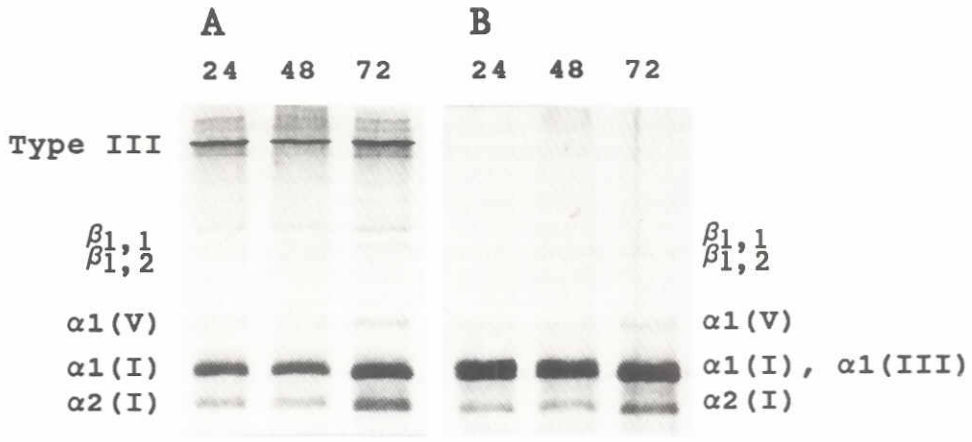

Fig. 3. ARG of radiolabeled collagens in extracts prepared from rudiments cultured for 24, 48, and 72 h. *Extracts were subjected to SDS-PAGE under non-reducing* **(A)** *and reducing* **(B)** *conditions and ARGs prepared. Notice the differences in the intensity of the α1(III) and α1(I) bands in the 3 extracts. The migration positions of collagen standards are shown.*

period. At present, nothing is known about the role of type V collagen in this system. It should be noted that these results show accumulation of newly synthesized collagen and do not comment on total accumulation of any of the collagen types.

Nakanishi *et al.* (1988) reported that collagen types I and III have distinct distributions in mouse submandibular glands from day 12 and 13 embryos. Type I is present uniformly at the epithelial-mesenchymal interface, while type III is localized differentially at indentations and clefts in the lobular surfaces. An association between type III collagen distribution and areas of morphogenetic shape change has also been found in the developing mouse molar (Thesleff *et al.*, 1979; Mao *et al.*, 1990), and in fetal rat gonads (Paranko *et al.*, 1987). It is known that in the absence of type I collagen in the Mov13 mutant, other interstitial collagens can support morphogenesis of various branching organs (Krachtochwil *et al.*, 1986). Perhaps more remarkably, these other interstitial collagens can also support the early stages of development of the cornea (Bard and Krachtochwil, 1987), a structure in which type III usually is seen only transiently before formation of the adult structure (von der Mark *et al.*, 1977).

If, as suggested by Nakanishi *et al.* (1988), collagen type III is responsible for initiating cleft formation, it is surprising that its rate of accumulation had dropped so precipitously by 72 hours in culture since clefts are continuing to form in such rudiments. However, Nakanishi and colleagues only examined rudiments from day 12 through mid-day 13 embryos and they noted that while type III collagen antigenicity continued to be associated with the epithelial-mesenchymal interface, the discontinuous pattern of distribution was less marked in the mid-day-13 glands than the younger rudiments. Bernfield *et al.* (1984) reported that type I collagen accumulated in deepening clefts between days 13 and 17 of gestation *in vivo*, and that the epithelium is essentially coated with fibrils of this molecule by day 17. We have observed increasing accumulations of type I collagen throughout the mesenchyme and particularly in clefts in rudiments cultured for up to 72 hours (Hardman and Spooner, 1992). These immunofluorescence data are consistent with our present biochemical results that type III decreases and type I increases after day 13. Yet, branching activity rates do not decline (Spooner, *et al.*, 1989).

It would be interesting to determine the type I and III biosynthetic rates in day-12 rudiments, since our data and those of Nakanishi *et al.* (1988) predict that these molecules would be synthesized at a ratio of approximately 1 at this stage. It would also be interesting

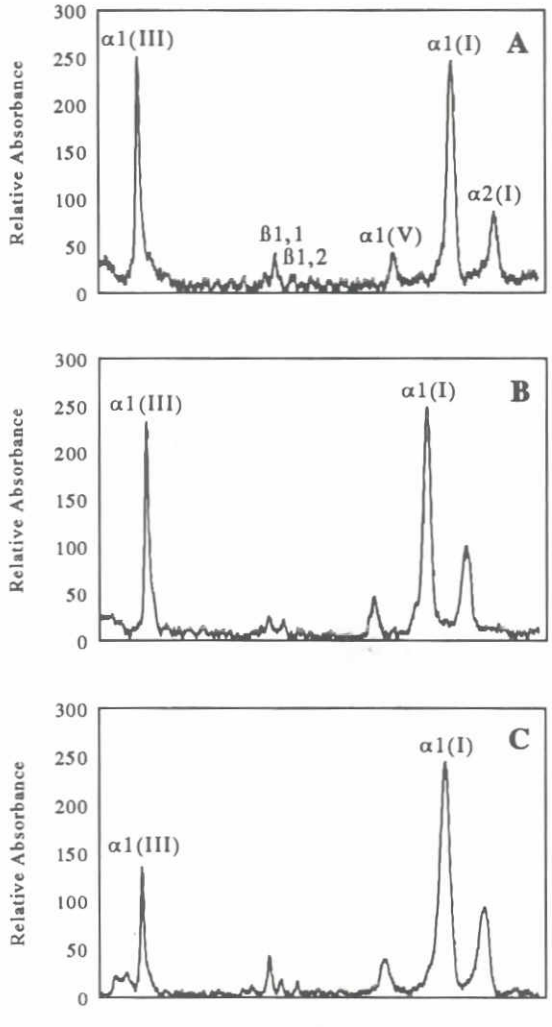

Fig. 4. Scanning densitometry of ARGs. Representative scans of profiles for 24 (A), 48 (B), and 72 (C) h extracts are shown. *There is a clear difference in the relative sizes of the α1(III) and α1(I) peaks in A, B, and C.*

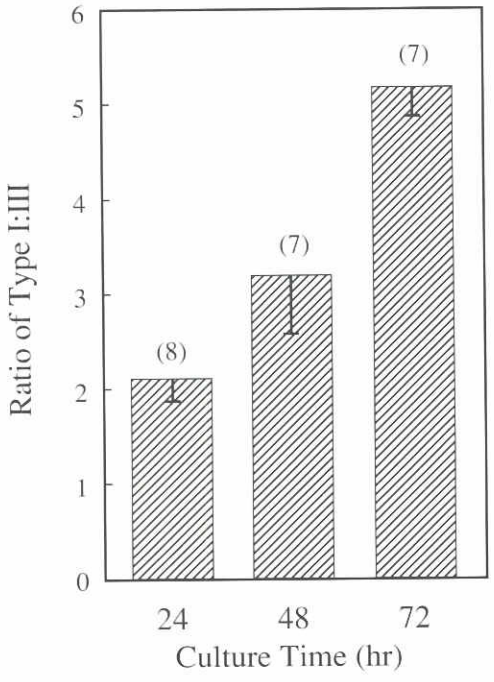

Fig. 5. Ratio of the accumulation of newly synthesized collagen types I and III. *Densitometric scans of ARGs were used to compute peak areas for the α1(I) plus α2(I) bands and the α1(III) band. Collagen type I:III ratios were calculated from these figures. The number of determinations for each time point are shown in parentheses. The bars represent the mean ± standard error.*

to assess the immunostaining patterns of type III collagen in rudiments cultured for various times.

Our work suggests that in normal branching morphogenesis in the salivary gland, type III collagen is important for initiation of branching activity, but that type I collagen becomes dominant in the continuation of morphogenesis and in maintenance of the adult structure. Resolution of respective collagen-type functions may be achieved by more molecular approaches such as addition of specific antisense RNA to the developing organ rudiment in culture. Development of a type III-deficient mutant, similar to the Mov13 mouse, would help to answer questions about the role of this molecule in development.

Experimental Procedures

Organs and organ culture

Outbred Swiss mice (Charles River Mouse Farms or Amitech Inc., Omaha, NE) were mated overnight and the morning after designated as day 0 of gestation. Early on day 13 of gestation, pregnant mice were killed by carbon dioxide asphyxiation and uteri were removed under sterile conditions and placed in Hank's balanced salt solution (HBSS). Salivary gland rudiments were isolated from embryos by microdissection and were transferred to 35 mm plastic tissue culture dishes (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) for labeling studies or to Biopore assemblies (Hardman *et al.*, 1990) for photomicroscopy.

Rudiments were cultured in F12S10 (Spooner and Hilfer, 1971) containing amphotericin B (10 μg/ml, Fungizone, GIBCO Laboratories, Grand Island, NY) and penicillin-streptomycin (100 U-100 μg/ml, GIBCO) at 37°C under high humidity in an atmosphere of 95% air/5% CO_2. The nutrient medium was changed completely every 48 h.

Labeling of rudiments and extraction of collagens

Organ rudiments were cultured for 0, 24, or 48 h in F12S10. The medium was then replaced with serum-free medium containing ß-aminoproprionitrile (ß-APN, 50 μg/ml) and 5 μCi ^{14}C-glycine/ml (U-^{14}C glycine, specific activity >100 mCi/ml; Amersham, Arlington Heights, IL) and culture was continued for an additional 24 h. It has been previously demonstrated that ß-APN concentrations of 50 μg/ml have no effect on salivary gland morphogenesis (Spooner and Faubion, 1980). Rudiments were detached from dishes, washed three times with cold (4°C) HBSS, and collected by brief centrifugation.

Labeled rudiments were sonicated in distilled water (10 μl/rudiment) as follows; microfuge tubes containing the rudiments were taped to the bottom of a large beaker, covered with ice and water, and the tubes were stroked with the tip of a sonifier (model W185, Heat Systems Ultrasonics, Plain View, NY) set at 7 for 3-4 min (Weins and Spooner, 1983). Collagens were extracted by adding an equal volume of freshly made 0.6 mg/ml pepsin in 1 *M* acetic acid pH 2.0 (to give a final concentration of 0.3 mg/ml pepsin in 0.5 *M* acetic acid; Conrad *et al.*, 1980) and incubating the mixture at 4°C overnight. The triple helix in collagens renders them resistant to pepsin digestion. Thus, treatment of whole rudiment sonicates with pepsin/acetic acid effectively degrades almost all other proteins and the pepsin-resistant molecules are assumed to be mostly collagenous.

Protein determination and incorporation of radiolabel

The protein contents of tissue sonicates were measured by micro-Lowry analysis (Rutter, 1967) with minor adjustments to double the volumes of all reactants.

Measurements of total protein-incorporated radioactivity and collagen-incorporated radioactivity (pepsin/acetic acid-resistant protein) were made on sonicates and pepsin/acetic acid extracts, respectively, by trichloroacetic acid (TCA) precipitation. Samples of sonicate or extract were added to 100 μl of 1 mg/ml bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) in microfuge tubes and an equal volume of cold (4°C) 20% TCA containing 2 m*M* glycine (to reduce non-specific precipitation; Peterkovsky, 1982) was added. Tubes were placed on ice for 30 min and then centrifuged at 10,000 rpm for 3 min. Precipitates were washed twice with 10% TCA containing 1 m*M* glycine and then redissolved in 200 μl 0.2 *M* sodium hydroxide. Scintillant was added to fill the tubes (Readysafe; Beckman Instruments Inc., Fullerton, CA) and the samples counted using a Beckman LS 7500 Liquid Scintillation System.

Electrophoresis

Samples equivalent to 2 rudiments were dried using a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY), redissolved in sample buffer with or without ß-mercaptoethanol and heated at 70°C for 2 h. SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE; Laemmli, 1970) was performed utilizing 7x8 cm «minigels» with a 3% stacking gel and a 5.5% running gel. Standard collagen types I, IV, V (Sigma) and III (Calbiochem Corporation, San Diego, CA) were included in every gel. Gels were stained with Coomassie Brilliant Blue R-250 (Biorad Laboratories, Richmond, CA).

Gels were dried and autoradiograms (ARGs) were prepared by exposing Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) to the gels for 48 h. ARGs were scanned using a Hoefer densitometer (Hoefer Scientific Instruments, San Diego, CA) and the areas under the peaks calculated by integration (using a scanning program kindly provided by D.J. Roufa). Peak areas were used to calculate the ratio of radiolabeled type I:III collagen for each culture time.

Photomicroscopy

To photodocument morphogenesis, rudiments were grown on filter assemblies using Biopore membrane (Hardman *et al.*, 1990; Millipore Corporation, Bedford, MA). Photographs of living organs were taken at 0, 24, 48, and 72 h through a 4X Planapo objective on a Zeiss photomicroscope II (Carl Zeiss Oberkochen, Germany).

Statistical analysis

Overall differences in the collagen type I:III ratios between 24, 48, and 72 h culture periods were analyzed by one-way analysis of variance, and means separations were done on least square means.

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