Organization and characterization of fibrillar collagens in fish scales in situ and in vitro

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Summary

The characterization of the fibrillar collagens and the cellular control of their spatial deposition were studied in fish scales using immunofluorescence, electron microscopy, electrophoretic and HPLC analyses, immunoprecipitation and hybridization with cDNA probes. This study was carried out on undisturbed and regenerating scales in situ and in organ and cell cultures from regenerating scales. The hyposquamal scleroblasts forming a pseudoepithelium show an apico-basal polarization and synthesize thick collagen fibrils (100 nm) organized in a plywood pattern as long as the integrity of the cell-cell and cell-collagenous matrix contacts are preserved. In culture, scleroblasts become fibroblast-like and produce an unordered meshwork of thin collagen fibrils (30 nm). Comparison of the synthesized collagens in culture with those extracted from the scales indicates that culture conditions modify fibrillogenesis but do not change the expression of fibrillar collagen genes. Type I collagen, the predominant component, is associated with the minor type V collagen. Type III collagen was not present. In type I collagen, a third chain, \( \alpha_3 \) chain, was identified. The ratio between the 3 chains suggests the coexistence of two heterotrimers (\( \alpha_1(1) \) \( \alpha_2(1) \) and \( \alpha_1(1) \) \( \alpha_2(1) \) \( \alpha_3(1) \)). Analysis by HPLC and electrophoresis of the cyanogen bromide-derived peptides obtained from the purified \( \alpha_3 \) chain support the hypothesis that \( \alpha_1(1) \) and \( \alpha_3(1) \) chains are encoded by two different genes. The presence of the two types of heterotrimers in vivo as well as in vitro could correspond to an innate property of the goldfish scleroblasts. Despite the fact that teleost cyanogen bromide-derived peptides differ from those of higher vertebrates, homologies with the mammalian collagen genes (human, for example) are sufficient to allow the detection of mRNA transcripts for \( \alpha_1(1) \), \( \alpha_2(1) \) and \( \alpha_2(1) \) from confluent scleroblast cultures with human probes.

Key words: collagens, microtubules, culture, fish scales.

Introduction

The structure of collagen fibrils and the precise three-dimensional arrangement of these fibrils within the extracellular matrix are essential for a connective tissue to function normally. Fibrillar collagen deposition is considered to be a multistep process regulated by a variety of factors such as the types of collagen produced (Miller, 1976; Birk and Silver, 1984; Adachi et al., 1989), the interactions of the different collagen types present (reviewed by Fleischmajer et al., 1990; Linsenmayer et al., 1990; van der Rest, 1991), the sequence of propeptide processing (Fleischmajer et al., 1981; Hulmes, 1983; Miyahara et al., 1984), the interactions with other extracellular components such as proteoglycans (Scott, 1988) and glycosaminoglycans (Flint et al., 1984; Merrilees et al., 1987), collagenase activities (Unemori and Werb, 1986) and cellular mechanisms (Trelstad et al., 1982; Hay, 1983; Birk and Trelstad, 1984; Zylberberg et al., 1988; Ploetz et al., 1991).

The aim of the present study was to analyse the production and the organization of fibrillar collagens in teleost scales in situ and in vitro: the scale structure is known to be responsible for the swimming performance of fish (Burdack, 1979). The interactions of scale-forming cells with collagen-fibril synthesis, fibrillogenesis and spatial arrangement have been studied using undisturbed tissues, regenerating scales and cultures. The scales of the goldfish Carassius auratus L. are thin, transparent mineralized dermal plates. In previous studies, a close relationship between the organization of microtubules (MTs) and actin microfibrils with the formation of a plywood-like pattern of the collagen fibrils forming the basal plate has been shown in scales
in situ (Byers et al., 1980; Zylberberg et al., 1988; Zylberberg and Bereiter-Hahn, 1991). These scale-collagen fibrils are thicker than those of the surrounding dermis (Onozato and Watabe, 1979). They are produced by peculiar fibroblasts considered to be neural-crest-derived mesenchymal cells termed scleroblasts (Klaatsch, 1890). The scleroblasts form an uninterrupted pseudoepipithelium, the hypopusquama (Waterman, 1970), lining the basal surface of the basal plate. In this study, the capacity of the scleroblasts to produce collagens in cultures and the identification of these collagens have been achieved by using electron microscopy, electrophoretic and HPLC analyses, immunoprecipitation and hybridization with cDNA probes.

Type I collagen is the major component of both scales and the surrounding dermis (Kimura et al., 1991). The type I heterotrimer α1α2α3, identified in teleost tissues only, was found in the skin of many species (Piez, 1965; Kimura et al., 1987) as well as in the scales of the carp (Kimura et al., 1991). The present study revealed the ability of the scleroblasts to produce both type I collagen heterotrimers (α1α2α3), and also type V collagen in vivo as well as in vitro, even if the fibrillogenesis is dramatically changed in vitro. In addition, procollagens and their derivatives obtained from scleroblast cultures have, for the first time, been analysed in teleost fish. Homologies between teleost and human collagen genes were found using human cDNA probes. Research on fish collagens provides an evolutionary reference point for other vertebrate-collagen studies because of evolutionary position of the fish relative to other vertebrates (Powers, 1989).

Materials and methods

Scale preparation

The present study was carried out on scales of the goldfish, Carassius auratus L. (Cyprinidae). Fish, 60 mm to 80 mm standard length (from snout to caudal penduncle), were raised at a constant temperature of 25°C. Carassius has typical elasmoid scales (Bertin, 1958) with a partially mineralized, well-developed basal plate. This basal plate is composed of thick collagen fibrils organized in a double twisted plywood-like structure (Meunier, 1984) and of thin collagen-fibril TC fibers (Onozato and Watabe, 1979) forming sheet-like structures perpendicular to the plies (Fig. 1). Regenerating scales were obtained as previously described (Zylberberg et al., 1988).

Organ culture of regenerating scales

Four-day regenerating scales were carefully removed from the fish flanks cleaned with a 1% KMT1O4 solution. The scales were then washed in Leibovitz medium containing 10% antibiotics (streptomycin and penicillin) and then deposited in 50 mm plastic tissue culture dishes containing serum-free Leibovitz medium with 1% antibiotics. The cultures were incubated at 27°C. All the chemicals (p.a. grade) were obtained from Merck (Darmstadt, Germany).

Scleroblast culture

To initiate a scleroblast culture, 4-day regenerating scales were also removed from the flanks of the fishes. The scales were carefully cut into two parts. Only the anterior part, deeply inserted within the dermis, was used, to avoid contamination with epidermal cells attached to the posterior part of the scales (Fig. 1A). The anterior part of the scales was immobilized between two glass coverslips in Leighton tubes and incubated at 37°C in DMEM supplemented with 10% FCS and antibiotics (streptomycin and penicillin). The primary culture was trypsinized, transferred to plastic flasks (25 cm²) and allowed to reach confluency. After trypsinization, the cells were incubated at 27°C or at 37°C and then regularly passed and counted with a haemocytometer in order to establish saturation densities. No signs of senescence or morphological alteration were observed after fifteen passages. For immunofluorescence labelling, the cells were grown on glass coverslips.

Transmission electron microscopy

Control, regenerating and cultured scales as well as confluent scleroblast cultures were fixed with a mixture containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. They were postfixed in 2% osmium tetroxide in the same buffer. Dehydration for isolated scales was in ethanol and for cultures in 2-hydroxyl methacrylate. All the samples were embedded in Epon. Cross-sections of the isolated scales were obtained as previously described (Allizard and Zylberberg, 1982). The thin sections were double stained with uranyl acetate and lead citrate (Reynolds, 1963). They were viewed in a Philips 300 EM electron microscope at an operating voltage of 80 kV with a cooling anticontamination device.

Indirect immunofluorescence

Indirect immunofluorescence of Tyr-α-tubulin (tyrosinated tubulin) was performed on methanol-fixed samples as described by Wehland et al. (1983) and applied to fish scales as described by Zylberberg et al. (1988). In short, a monoclonal antibody YL1/2 (a generous gift from Dr. J. Wehland, Brauschweig, Germany) directed against Tyr-α tubulin was used as the first antibody (Klimart et al., 1982). The second antibody consisted of TRITC-conjugated goat anti-rat IgG. In control stainings, the first antibody was omitted. All the controls were negative. Cells labelled for actin microfilaments were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.05% Nonidet (Sigma, St Louis, USA) and stained with TRITC-phalloidin (Sigma, St Louis, USA). All the samples were mounted in Mowiol (Hoechst AG, Frankfurt am Main, Germany) and examined with a Zeiss photomicroscope Axiosvert 35 equipped with epifluorescence. Kodak Tri-X film was developed in Emofin (Tetenal).

Extraction of collagen from tissues

Isolated scales removed from fish were lyophilized and then demineralized with 0.5 M EDTA containing protease inhibitors (phenylmethylsulfonyl fluoride (PMSF) 1 mM), N-ethylmaleimide (NEM, 10 mM), EDTA (25 mM). After centrifugation, the supernatant was discarded and the pellet washed extensively with water. Lyophilized dermis was powdered in a liquid nitrogen mill. The dry powder and the pellet issued from the scales were extracted sequentially with 0.5 M acetic acid for 48 hours at 4°C and with pepsin (Sigma, St Louis, USA) at 400 μg/ml for 18 hours at 4°C (Miller and Rhodes, 1982). Collagen from each fraction was purified by salt precipitation (0.9 M NaCl in 0.5 M acetic acid). The precipitate was collected by centrifugation and used for electrophoretic and HPLC analyses.
Collagens in fish scales

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epidermis
basal plate
hyposquamal scleroblast
scale pocket
dermis

anterior part
posterior part

Fig. 1. (a) Diagrammatic representation of a vertical section of an elasmoid scale lying in its pocket. The hyposquamal scleroblasts form an uninterrupted layer lining the basal surface of the scale. To initiate a scleroblast culture the scale was cut as indicated by the arrow. Only the anterior part was used, to avoid contamination with the epidermal cells covering the posterior part. (b) Sections of fully developed (control) and regenerating scales drawn from electron micrographs. In the basal plate of control scales, the thick collagen fibrils are organized in superimposed plies forming a double-twisted plywood-like structure crossed by sheets of vertical thin TC collagen fibrils. In regenerating scales, the plywood-like structure is orthogonal and TC fibrils are also present. The hyposquamal scleroblasts which produce the collagen fibrils forming the basal plate are very flat in the fully developed scales while they are prismatic in the regenerating scales.

Cell labelling
Confluent cells in the sixth to tenth passage were used for labelling either at 27°C or 37°C. [3H]proline (40 μCi/ml) was added to DMEM that contained 50 μg/ml ascorbic acid and 50 μg/ml β-aminopropionitrile (β-APN) (Merck, Darmstadt, Germany), after an overnight incubation in serum-free medium. Eighteen hours later, cell layer and culture medium were harvested separately in the presence of the protease inhibitors. Samples were used to measure total collagen. The analysis was done by amino acid separation on ion exchange resin as previously described (Bonaventure et al., 1986). Digestion with bacterial collagenase according to the procedure of Peterkofsky and Diegelmann (1971) was also carried out in order to confirm the presence of collagen in the fractions.

Procollagens were dialysed against 1 mM ammonium carbonate buffer supplemented with the protease inhibitors. Collagen chains were obtained by pepsin digestion (100 μg/ml for 16 hours at 4°C) in 0.5 M acetic acid.

Human procollagens and collagen chains were obtained from dermal confluent fibroblast cultures labelled with [3H]proline as previously described (Bonaventure et al., 1986).

Electrophoretic analyses
Polycrylamide (5%) gels were used for both procollagen and collagen chain analysis. The lyophilized samples were resuspended in Tris-HCl buffer (pH 6.8) containing glycerol (10%), bromophenol blue (0.001%), and heated for 3 min at 100°C under either reducing (procollagen) or non-reducing conditions (collagen) in the presence of dithiothreitol (DTT, 50 mM).

HPLC separation
Pepsin-soluble collagen isolated from scales or dermis was dissolved at 42°C for 15 min in 0.02 M sodium formate buffer (pH 3.8) containing 10% propanol-2 and 0.07 M NaCl, filtered and applied to a Mono S HR 5/5 cation exchange column (Pharmacia, Uppsala, Sweden), according to Bateman et al. (1986). Elution was achieved with a linear sodium chloride gradient to give a final concentration of 0.4 M NaCl. Absorbance was measured at 226 nm and fractions were collected at a flow rate of 1 ml/min. Samples of the fractions corresponding to the different peaks were desalted and concentrated by filtration on a PVDF membrane (Immobilon) in a slot blot apparatus. After washing the filter, small pieces of the membrane were cut and inserted in the 3% stacking gel of a 5% separating gel. This preparation was used to concentrate and to desalt the collected HPLC fractions. Coomassie blue staining of the gels allowed identification of the collagen chains. Each of the remaining HPLC fractions was desalted on Sephadex G25 mini columns, lyophilized and resuspended in 70% formic acid to allow cleavage with CNBr. Separation of the cyanogen bromide-derived peptides (CB peptides) on a Mono S HR 5/5 column was achieved by using the same gradient as for α and β chains.

Immunoprecipitation
Polyclonal antibodies were raised in rabbit against acid-soluble type I collagen extracted from the dermis of Carassius
Total RNA was extracted from confluent scleroblast cultures electrophoresis buffer and then analysed on a 5% polyacrylamide gel containing 2.2 M Drs M. L. Chu and F. Ramirez and labelled with pPJdCTP by ion exchange chromatography, was used to precipitate the A/antibody complex was recovered by 5 min boiling in the electrophoresis buffer and then analysed on a 5% polyacrylamide gel by electrophoresis and visualized by autoradiography.

RNA preparation and analysis
Total RNA was extracted from confluent scleroblast cultures using the guanidinium chloride procedure (Sambrook et al., 1989). Integrity of the preparation was tested by ethidium bromide staining of rRNAs separated on a non-denaturing agarose gel. Samples of 2 and 4 µg of total RNA were deposited on 1% denaturing agarose gel containing 2.2 M formaldehyde and 20 mM borate buffer (pH 8.3) and run for 16 hours in borate buffer. Transfer to Hybond N membrane was achieved according to the recommendations of the supplier (Amersham). The following probes: H1040 (α1 cDNA), HSf (α2 cDNA), H934 (α1I cDNA), OK 2.5 (α2 V cDNA) and p18 (α1 V cDNA) were kindly provided by Drs M. L. Chu and F. Ramirez and labelled with [35S]dCTP (Amersham) by nick-translation. Filters were washed twice at room temperature for 10 min, then once at 65°C with 2SSC/0.1% SDS and exposed for 2.4 days at -60°C.

Results

Fibrillar collagen deposition in the scales in situ
In the well-developed control scales, the hyposquamal scleroblasts are flat cells (3 to 5 µm thick at the level of the nucleus) (Figs 1B, 3). Because of their flat shape, these cells were not clearly seen in phase-contrast whereas the direction of the collagen fibrils forming the innermost ply in contact with the hyposquamal scleroblasts was obvious (Fig. 2A). Tyr-α-tubulin antibody labelling revealed the distribution of MTs, which were roughly aligned parallel to each other and with the direction of the collagen fibrils of the innermost ply (Fig. 2A,B). A similar coalignment of the actin filaments and the collagen fibrils was observed in scales stained with TRITC-phalloidin (not shown). Electron micrographs revealed that the collagen fibrils forming the plywood-like structure reached their final diameter (about 100 nm) in close proximity to the cell (Fig. 4). The thin TC collagen fibrils (about 30 nm in diameter) were organized in a vertical sheet-like structure inserted among the thick collagen fibrils of the plywood-like structure (Figs 3, 4, 17).

Regenerating scales were observed from days 4 to 8 after the initial removal. The basal plate of these regenerating scales was also composed of thick collagen fibrils of about 100 nm in diameter (Fig. 17) but the plywood-like structure is orthogonal. Also the thin TC fibrils were present in the regenerating scales (Fig. 17). The hyposquamal scleroblasts synthesizing the basal plate differ dramatically from the undisturbed scales. In the regenerating scales, they appeared as tall prismatic cells (15 µm high) (Fig. 1C). RER and Golgi areas were well developed (Fig. 6). In most hyposquamal scleroblasts of regenerating scales, MTs (Fig. 5A,B) and actin filaments (stained with TRITC-phalloidin, not shown) were coaligned with the innermost collagen fibrils. Electron microscopic examination confirmed this co-alignment (Figs 6, 7). The collagen fibrils reached their final diameter of about 100 nm in the vicinity of the cells, as previously described for the control scales (Fig. 4).

Fibrillar collagen deposition in regenerating scales in organ culture
Four-day regenerating scales were cultured for 48 hours and then examined by light and electron microscopy. The general organization of the scales was preserved (Fig. 9). However, the hyposquamal scleroblasts did...
not appear as tall as those of scales in situ at the same regenerating stage (Fig. 6). In the cultured scales, the direction of the MTs, which was continuous throughout the hyposquamal scleroblasts, was coaligned with the orientation of the innermost collagen fibrils as in scales in situ (Fig. 8A, B), and actin filaments stained by TRITC-phalloidin showed a similar coalignment (not shown). Electron micrographs confirmed this coalignment between the cytoskeletal elements and the innermost collagen fibrils. When a new ply was initiated these cytoskeletal elements were the first to change their direction (Fig. 10). As in the scales in situ, the collagen fibrils of the plywood-like structure reached 100 nm in diameter whereas the TC fibers present had a diameter of about 30 nm. However, after a 3-day culture, some flattened scleroblasts were detached from the basal plate, separated from their adjacent cells by more or less prominent spaces, and surrounded by thin collagen fibrils of about 30 nm in diameter.

Fibrillar collagen deposition by cultured scleroblasts

A cell monolayer was obtained in Leighton tubes after a 30 day culture of regenerating scales at 37°C. The cells were fibroblast-like (Figs 12A, 13, 14). After trypsinization, the cells grew exponentially for 6-8 days. At 37°C, a saturation density of 6 x 10⁶ cells/cm² was reached after 8-10 days. In extensive cultures (>20 days), scleroblasts progressively detached from the plastic support. When the cells were cultured at 27°C, the saturation density was higher (8 x 10⁶ cells/cm²) (Fig. 11). Cell detachment from the plastic support was less marked at 27°C than at 37°C. Electron microscopic examination revealed that these fibroblast-like cells were very flat (Fig. 13). The MTs, which emanated from the MTOC, transversed the whole cell (Fig. 12B) and actin fibrils formed straight stress fibres (not shown). In confluent cultures, cells were in close contact (Fig. 14); however, desmosomes were scarce and small (Fig. 16). Meshworks of thin collagen fibrils of about 30 nm in diameter were found in the intercellular spaces (Fig. 15). The characteristic striation which was obvious in the thick collagen fibrils of the basal plate of scales in situ (Fig. 17) was less clear (Fig. 19). These thin fibrils resembled those forming the vertical sheet-like structures (Fig. 17) and those of the surrounding dermis (Fig. 18).

Isolation and characterization of collagen from scales and from cultured scleroblasts

The percentage of total [3H]proline-labelled collagen secreted by scleroblasts in confluent cultures was about 10% of total secreted proteins at 27°C. However, when the scleroblasts were cultured at 37°C, this value dropped to 5-6%. This temperature-dependent decrease was confirmed using two different techniques (Table 1).

Electrophoretic profiles of the procollagens isolated from the culture medium and the cell layer showed a series of bands. By comparison of their mobilities with those of human procollagen chains produced by cultured dermal fibroblasts (Fig. 20, lane 5) they were identified as pro-α1 and its partially processed derivatives (pN α1(1) and pC α1(1)) and fully processed derivatives (Fig. 20, lanes 1, 2, 3, 4). The relative intensity of the pro-α1(1) band in fish was weak compared to that of the human counterpart, indicating a high amount of processing of procollagen to collagen in fish scleroblast cultures. At 27°C and 37°C, no differences were found in collagen processing (Fig. 20, lanes 1, 2, 3, 4). Incubation in the absence of ascorbic acid slightly decreased the procollagen amount but it had no influence on the processing.

Pepsin digestion of culture medium and cell-layer fractions produced two major bands (Fig. 20, lanes 10, 11) with an electrophoretic mobility close to the α1 and α2 chains of mammals (human, for example, Fig. 20, lanes 10, 11).

Table 1. Total collagen synthesis by scleroblasts in culture

<table>
<thead>
<tr>
<th>Temperature of incubation (°C)</th>
<th>Hydroxyproline/ proline (culture medium)</th>
<th>% Collagen/total protein in culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>0.220</td>
<td>9.8</td>
</tr>
<tr>
<td>37</td>
<td>0.132</td>
<td>5.8</td>
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Cells were labelled for 18 hours with [3H]proline.

*Culture media were harvested and hydroxyproline was separated from proline by elution through an ion exchange column. Radioactivity was counted in each fraction. The formula of Wiestner et al. (1979) was used to calculate the percentage of collagen.

†Culture media were digested with purified bacterial collagenase and the collagenase-resistant proteins were precipitated with 10% TCA.

‡Not done.
Fig. 12. (a,b) Same field. (a) Phase-contrast. Scleroblasts in cell culture show a fibroblast-like aspect. (b) Immunofluorescence (same MT labelling as in Fig. 2). MTs arising from the MTOC do not show any preferential orientation. $\times650$; bar, 10 $\mu$m.

Fig. 13. Electron micrograph of a section perpendicular to the culture surface showing the thinness of the cultured scleroblast. $\times6,500$; bar, 1 $\mu$m.

Fig. 14. Electron micrograph of a section parallel to the surface of a confluent culture of scleroblasts. $\times6,500$; bar, 1 $\mu$m.

Fig. 15. Section parallel to the surface of a confluent culture. A meshwork of thin collagen fibrils (co) is located in the intercellular space. $\times20,000$; bar, 0.5 $\mu$m.

Fig. 16. Two adjacent cells are connected by a small-sized desmosome (d). $\times40,000$; bar, 0.25 $\mu$m.
In order to confirm that the collagen produced in the cultured scleroblasts contained type I collagen similar to that of the dermis, an immunoprecipitation assay was done using the polyclonal antibody raised against type I collagen purified from the dermis of *Carassius auratus*. The two major bands visualized on the gel co-migrated with the α chains of the dermal type I collagen (Fig. 20, lane 12). The additional band comigrating with α1(V) could be due to some cross-reactivity of the antibody with type V collagen.

Analysis of CB peptides by two-dimensional electrophoresis and HPLC

When pepsin-digested collagen extracted from the scales was separated on a Mono S column, 4 major peaks were detected (Fig. 21A). Electrophoresis of the collected fractions showed that the dimer β1-2 was adequately separated in fraction d but fraction c contained both β1-1 and −2(I) (Fig. 21B). Peaks a and b had the same electrophoretic mobility as that of the α1(I) chain. In order to test whether peak b corresponded to an additional chain or to a modified version of α1(I), the fractions containing the purified peaks were desalted and submitted to CNBr treatment. Profiles of the peptides eluting from the Mono S column are shown in Fig. 22. At least 7 major CB peptides were detected in the first peak (Fig. 22) whereas only 6 peptides were derived from the second peak (Fig. 22).

The striking differences between these two peaks strongly support the idea that the major band observed on the one-dimensional gels consisted of two different chains, α1(I) and α3(I), with the same electrophoretic mobility but with different charges. In agreement with previous reports in other teleost fishes, this additional chain was designated as α3(I). The intensity of the α3(I) peak was not equal to that of the α1(I), suggesting that two types of heterotrimers ((α1(I))2 α2(I)) and (α1(I) α2(I) α3(I)) could be present in the collagen fibrils.

Northern blot analysis of mRNA from cultured scleroblasts

Hybridization of mRNA isolated from cultured scleroblasts with 4 different cDNA probes specific for the genes coding for the fibrillar collagen chains α1(I), α2(I), α1(III) and α2(V) and the non-fibrillar collagen chain α1(VI) allowed the identification of several transcripts (Fig. 23). Their size was generally different from that of human fibroblasts used as control. In the case of α1(I), the sizes of the two transcripts were, respectively, 5.7 and 5.3 kb. For α2(I), the deduced values were 5.0 and 4.2 kb, the latter being predominant. Two transcripts were obtained with the α2(V) probe. Their size and intensity were almost the same as in human fibroblasts. As expected, no transcript was detectable even after overexposure when the filter was hybridized with Hf 934, an α1 III cDNA probe (result not shown). This result is consistent with the failure to detect the (α1 III)3 trimers on polyacrylamide gels (Fig. 20). A single transcript of approximately 4.5 kb was hardly detectable with the α1 VI cDNA probe, the signal was weak and required overexposing to become distinguishable.
Fig. 20. Electrophoretic analysis of procollagen (lanes 1-5) and pepsin-digested collagen chains (lanes 6-11). Scleroblasts were cultured at either 27°C (lanes 1, 2) or 37°C (lanes 3, 4, 10, 11) and labelled with [3H]proline for 18 hours. Culture media (M) and cell layers (C) were harvested separately and run on 5% polyacrylamide gels. Human fibroblasts (hu.) incubated at 37°C in similar conditions were used as control (lanes 5 and 9). Total collagens extracted from goldfish dermis (lane 7) and scales (lane 8) were pepsin-digested and analysed on 5% gels stained with Coomassie blue. Human dermis served as control (lane 6). In lane 12, the bands correspond to immunoprecipitates obtained with tritium-labelled type I collagen extracted from goldfish scales and reacted with antibodies raised in a rabbit against dermal pepsin-digested goldfish collagen.

Discussion

Comparison of collagens produced in situ and in culture

The results of the present study comparing the production of collagen fibrils in scales in situ and in vitro demonstrate the influence of the environment on cell behaviour and consequently on the organization and fibrillogenesis of fibrillar collagens. In situ, the hypsquamous cells are not distributed within the collagenous matrix as the fibroblasts are in other connective tissues such as the cornea or the dermis, which have a similarly ordered three-dimensional organization (Birk and Trelstad, 1984; Ploetz et al., 1991). Indeed, the hypsquamous scleroblasts forming a pseudoepithelium show an apico-basal polarization. The spatial arrangement of the collagen fibrils in a plywood pattern is coupled with the continuous course of the cytoskeleton coaligned with the innermost collagen fibrils through large groups of hypsquamous scleroblasts (Zylberberg et al., 1988). In the cultured scales, a similar coalignement is observed in the hypsquamous scleroblasts which remain attached to the basal plate. However, the scleroblasts show a general tendency to flatten, to be detached from each other and to be separated from the basal plate. In primary cell cultures, the hypsquamous scleroblasts appear as flattened fibroblast-like cells. Even if they are in close proximity in confluent cultures, they do not show large areas of cell-to-cell contact as in the in situ epithelial arrangement. The collagen fibrils form an unorganized meshwork and they are thinner than those organized in a plywood-like structure. The presence of thick collagen fibrils in the basal plate of the in situ scales, which are stiffened by the deposition of a mineral phase, might be related to the protective function of the scales (Burdack, 1979). Large collagen fibril diameters characterize tissues submitted to high stress levels whereas small collagen fibril diameters are found in tissues which have the ability to resist plastic deformations (Flint et al., 1984; Parry, 1988). The finding that scleroblasts in culture synthesized thinner collagen fibrils which are not arranged in a plywood pattern might be related to the absence of mechanical stresses in vitro and/or to the altered behaviour of cultured scleroblasts.

The flattened scleroblasts separated from their usual
Fig. 21. Mono S cation exchange HPLC of fish scale type I collagen chains; 200 µg were loaded and eluted with a NaCl gradient that was increased first from 0.07 M to 0.18 M, then rapidly to 0.4 M. HPLC fractions were collected (a-g) at a flow rate of 1 ml/min and deposited on an SDS-polyacrylamide gel, which was stained with Coomassie blue.

Fig. 22. Mono S cation exchange HPLC of α1(I) and α3(I) CB peptides from fish scales. The peaks (a and b, Fig. 21) corresponding to α1(I) and α3(I) chains were collected, desalted by passage through a G25 Sephadex column and treated with CNBr prior to being loaded on the column. A linear gradient from 0.07 M to 0.4 M NaCl was used for peptide elution.

Recent studies have demonstrated that differentiated fibroblasts removed from cornea, tendon and dermis retained their tissue phenotype when grown in three-dimensional collagen gels. However, these cultured corneal fibroblasts produced proteoglycans which were not found in the in vivo corneal stroma (Doane and Birk, 1991). On the other hand, corneal and dermal fibroblasts injected into the vitreous produced a matrix which was not as well organized as in vivo (Burke and Foster, 1985). These data suggest that although the phenotype of the differentiated fibroblasts was retained, the tissue characteristics were altered in culture. Investigations of the modulation of phenotype in culture demonstrated the importance of the extracellular environment in the regulation of cellular functions and in the expression of genes (Bissel and Barcellos-Hoff, 1987). A change in cell shape, which depends upon the extracellular environment, could switch gene expression as has been demonstrated, for example, for chondrogenesis (Benya and Shaffer, 1982).

Accordingly, in the present study, fibrillar collagen types identified in the scales were compared with those found in cultures. The rate of synthesis was influenced
Collagens in fish scales

Fig. 23. Northern blot hybridization of mRNA from C. auratus scleroblasts (F) and human fibroblasts (H). Total RNA (2 or 4 μg) was hybridized to 32P-labelled cDNA probes specific for α1(I) (Hf 404), α2(I) (Hf 32), α2(V) (OK 2.5), and α1(VI) (p18). The sizes of transcripts are indicated in kilobases.

by culture temperature. It was higher at 27°C, which corresponds to the optimal growth temperature for the goldfish, than at 37°C, which is close to the lethal temperature (39°C). However, the electrophoretic mobility of the collagen chains was identical at both temperatures. Similar conclusions on the effect of temperature on proline hydroxylation have been obtained in poikilotherms in vivo (Cohen-Solal et al., 1986) and in vitro (Eichner et al., 1978).

As in the scales (Kimura et al., 1991), type I collagen is the predominant component produced in cultures. The minor type V collagen was identified in both scales and cultures. Type III collagen was undetectable in the scales, in the procollagen and in the fractions either treated with DTT or not. α1(III) mRNA transcripts were not detected. Our results are consistent with previous studies which indicated that type III collagen was not found in various fish connective tissues (Sato et al., 1989).

Aspects of the evolution of collagen genes

Comparison of the electrophoretic mobilities of fish and human procollagens - to a certain degree - provides information on the conservation of biochemical properties throughout different periods of evolution. Goldfish procollagens α1(V) and α2(V) as well as the processed derivatives behave similarly to corresponding human chains. Electrophoretic migration of pro α1(I) and pro α2(I) indicates that the sizes of the whole procollagen molecules were close to those in human cells. However, the sizes of the N and C propeptides and the triple helix differed, separately, significantly from human equivalents.

In addition to the α1(I) and α2(I) chains, a α3(I) chain has been resolved. Its relative molecular mass was the same as that of α1(I) chain, but it was separated perfectly by passage through the cation exchange column, due to charge differences. Comparison of the CB peptide-HPLC profiles of α1(I) and α3(I) clearly confirmed the non-identity of the two chains, thus supporting the idea that fish type I collagen is encoded by three different genes. Since the relative intensities of the three peaks corresponding to α1(I), α2(I) and α3(I) are not in a 1:1:1 ratio, we suggest that both (α1(I))2 α2(I) and α1(I) α2(I) α3(I) heterotrimers are present. The synthesis of these heterotrimers in scales as well as in culture is an intrinsic property of teleost scleroblasts.

The presence of an α3(I) chain seems to be a characteristic of the type I collagen of teleosts, since it has not been identified in any other group. This α3 chain is also tissue specific, since it is not found in all connective tissues in the same species (Kimura and Ohno, 1987). These results are consistent with previous studies showing that, in fish, variations in the different types of fibrillar collagen chains are related not only to their evolutionary position, but also to tissue specificities (Kelly et al., 1988).

Despite the obvious differences between CB peptides of type I collagen synthesized by scleroblasts and those of type I produced by human fibroblasts, the α1(I) and α2(I) mRNA sequences in C. auratus appeared to be sufficiently conserved to allow hybridization with the corresponding human cDNA probes, but the sizes of the transcripts were not identical to the human ones. Conversely, sizes of the α2(V) transcripts were almost identical in fish and humans. This might substantiate the hypothesis that the divergence between fish and human COL 5A2 genes is less marked than that between COL1A1 and COL1A2. These observations support the idea that type I collagen genes have undergone substantial changes whereas type V collagen has been greatly conserved during vertebrate evolution.

Type V collagen is considered to represent an ancestral type occurring before type I and type III collagens. It has been identified in the body wall of the lamprey (Kelly et al., 1988) and in the muscular tissue of chondrichthyan and osteichthyan fish (Sato et al., 1989). Moreover, a heterotrimer α1α2α3 similar to type V is a major component of the mesogloea of the jellyfish, a very primitive metazoan (Miura and Kimura, 1985). The lesser abundance of type V collagen in higher vertebrates than in fish could substantiate...
the hypothesis that evolution has been accompanied by a progressive replacement of this ancestral type of collagen by type III collagen (Kelly et al., 1988; Weil et al., 1987).

The observed homologies between fish and human fibrillar collagens are in agreement with the idea that the typical multi-exon organization of fibrillar collagen genes has undergone few modifications through evolution, as shown by recent studies on the collagens of the most primitive metazoa (D’Alessio et al., 1989; Exposito and Garrone, 1990). Conversely, the non-fibrillar collagen genes belong to distinct families even within the vertebrates (Exposito and Garrone, 1990; van der Rest, 1991). The finding that the transcript coding for the non-fibrillar α1(VI) collagen chain was almost undetectable in fish scleroblasts was consistent with this lesser conservation of non-fibrillar collagen genes. The presence of type VI collagen within fish scales might be related to its stabilizing effect, as in the cornea (Linsenmayer et al., 1990). Further experiments will be required to establish its presence, its location and its function in our model.

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