Original

Histochemical Analyses of Glycosaminoglycans in the Dermal Connective Tissues of Indian Air-breathing Freshwater Teleosts by Means of a Sensitized High Iron Diamine (S-HID) Technique

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Abstract: In the dermal connective tissues of Indian air-breathing freshwater teleosts, the molecular species of glycosaminoglycans have been studied by means of newer histochemical staining and enzyme digestion procedures with or without urea treatment. The staining procedure used was a sensitized high iron diamine (S-HID) procedure, whereas the enzymes employed were chondroitinase ABC and keratanase. According to the results obtained, isomeric chondroitin sulfates and keratan sulfate were the primary glycosaminoglycan molecular species contained in the dermal connective tissues of the freshwater teleosts and the former molecular species were apparently masked by collagens in the tissues. These findings are taken to suggest that the glycosaminoglycan molecular species and their specific interactions with collagenous fibers are associated with the assistance of cutaneous respiration and protection of the bodies from hostile environments in the fishes.

Keywords: histochemical analyses, glycosaminoglycans, dermal connective tissues, freshwater teleosts, sensitized high iron diamine

INTRODUCTION

In recent years, a series of reports have been made available on the precise histochemical and physiological properties of carbohydrates in the epidermis of fish skin1,2. In contrast, however, hardly any significant data have been obtained on the precise histochemical nature of carbohydrates in the dermal connective tissues of fishes3,4. Such gaps in information as to the nature of carbohydrates in these tissues are due, in part, to their unstainability with conventional histochemical techniques for the detection of carbohydrates involved, and such an unstainability could be ascribed to the linkage of the carbohydrates to proteins5. Previously, Banerjee and Yamada6 attempted to study the histochemical properties of collagen bound glycosaminoglycans in the dermal connective tissues of a fish species by unmasking with urea treatment6. In the study by Banerjee and Yamada6, conventional histochemical techniques, such as alcian blue (AB) pH 1.0 and high iron diamine (HID), were used for the demonstration of sulfated glycosaminoglycans. With these techniques, however, it was hardly possible to visualize exceedingly small amounts of glycoconjugates6. To overcome such limitations, a sensitized HID (S-HID) method has been developed and established as a more sensitive and efficient histochemical technique for sulfated glycoconjugates6, and it has been widely used for the detection of acidic glycoconjugates in a variety of tissues7,8,9,10,11,12,13,14,15,16,17,18,19.

In the present study, attempts have been made to histochemically analyze glycosaminoglycans by means of the combined S-HID and enzyme digestion procedures in the dermal connective tissues of two Indian freshwater teleost species, some of which were subjected to urea treatment. Since these freshwater teleosts are known to be air-breathing and wriggling and burrowing in mud1,2, the results obtained in the present study could be helpful to the precise recognition and elucidation of the histophysiological roles played by glycosaminoglycans in the connective tissues of these fish species.
MATERIALS AND METHODS

Preparation of tissues
Live specimens of Mastacembelus pancalus (119 ± 17 mm in length) and Monopterus cuchia (407.5 ± 24.8 mm in length) were collected from the river Ganges at Varanasi and Ramgarh lake at Gorakhpur respectively. The fishes were cold anaesthetized and skin pieces were excised from their back and fixed in Carnoy’s fluid. Following fixation, the tissues were dehydrated in an ascending ethanol series, cleared in xylene and embedded in paraffin wax. Sections were cut at a thickness of 4 to 6 μm and mounted on glass slides without any adhesives. Dewaxed and hydrated sections were subjected to the following staining and enzyme digestion procedures. The stained sections were dehydrated in an ascending ethanol series, cleared in xylene and mounted in HSR.

Histochemical procedures
1) Sensitized high iron diamine (S-HID) procedure
The S-HID procedure was similar to that described previously. Briefly, tissue sections were incubated in high iron diamine solution at 37°C for 60 minutes, immersed in 0.5 mM potassium trichloro(ethylene)platinum solution at room temperature for 60 minutes, reacted with 0.2% sodium borohydride solution at room temperature for 10-30 seconds and then subjected to a physical development procedure at 20°C for 3-10 minutes.

2) Digestion with glycosaminoglycan-degrading enzymes
Prior to the S-HID staining procedure, some hydrated sections were subjected to digestion with glycosaminoglycan-degrading enzymes as follows:
(a) Digestion with chondroitinase ABC (a modification of Hirabayashi’s method). Prior to the staining by the S-HID method, sections were subjected to digestion with chondroitinase ABC and keratanase under conditions of duration and temperature (buffer/S-HID).
(b) Double digestion with chondroitinase ABC and keratanase. Prior to the staining by the S-HID method, sections were subjected to digestion with chondroitinase ABC mentioned above, and then incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing 2.0 units of keratanase (Pseudomonas sp.) (Seikagaku Corp., Japan) at 37°C for 7-12 hours (Chase ABC/Kase/S-HID).

Urea treatment (a modification of Hirabayashi and Yamada’s method)
Prior to the histochemical procedures described above, some sections were treated with 6M urea solution at 60-62°C for 10-12 minutes to unmask glycosaminoglycans chemically bound with proteins. Control tissue sections were either kept intact or incubated in distilled water (medium of urea solution) under the same conditions of duration and temperature.

RESULTS

Mastacembelus pancalus (Mp)
As in many other species of fishes, the skin of the Indian freshwater fish, Mp consisted of three main layers, the epidermis, dermis and subcutis. The epidermis was the outermost layer, and the dermis was the subjacent layer consisting of two zones, a relatively thin upper sublayer of loose vascular connective tissue and a thick compact sublayer of dense connective tissues. The lower compact sublayer was characterized by the presence of bundles of coarse collagenous fibers compactly arranged, and a few collagenous fiber bundles ran vertically at intervals. The subcutis was a layer beneath the dermis and was composed mainly of arrays of large adipose cells. The skin of Mp was provided with rudimentary scales, which were embedded in the thin upper sublayer of the dermis.

In the skin of Mp stained by the S-HID procedure, weak or moderate positive reactions in brownish shades were obtained in the connective tissues of the entire dermis (Fig. 1). In the...
tissue sections subjected to the Chase ABC/S-HID sequence, digestion with chondroitinase ABC slightly decreased the intensity of S-HID reactions in these connective tissues (Fig. 2). In the tissue sections stained by the Chase ABC/Kase/S-HID procedure, digestion with keratanase following that with chondroitinase ABC tended to slightly diminish the intensity of S-HID reactions in the connective tissues of the entire dermis exhibiting markedly stronger S-HID reactions (Fig. 4) than those in the control tissue sections for urea treatment. In the urea-unmasked Mp skin stained by the Chase ABC/S-HID procedure, digestion with chondroitinase ABC pronouncedly diminished the intensity of S-HID reactions in the connective tissues of the dermis (Fig. 5). In the urea-unmasked skin stained with Chase ABC/Kase/S-HID, digestion with keratanase following that with chondroitinase ABC resulted in a further diminution of the S-HID reactivity of the connective tissues in the dermis (Fig. 6), as compared with that in the tissues stained with the control procedure (Chase ABC/Buffer/S-HID). It was of particular note that such a diminution was most prominent in the linear matrix surrounding each bundle of collagenous fibers (Fig. 6).

*Monopterus cuchia* (Mc)

The skin of this Indian air-breathing swamp eel, *Mc* consisted likewise of three main layers, the epidermis, dermis and subcutis. The epidermis was the outermost layer and thinner than that in *Mp*, whereas the dermis was a subjacent layer composed of two zones, an upper sublayer of loose vascular connective tissue and a lower compact sublayer of dense connective tissues. The lower sublayer was characterized by the presence of bundles of coarse collagenous fibers arranged compactly and a few collagenous fiber bundles vertical in direction at intervals. The subcutis was a layer beneath the dermis and consisted mainly of groups of large adipose cells. In the skin of *Mc*, rudimentary scales were found embedded in the thin upper sublayer of the dermis.

In the skin of *Mc* stained by S-HID procedure, varying degrees of positive reactions in brownish shades were obtained in the connective tissues and the reaction was most pronounced in intensity in those parts of the tissues facing the scales in the upper dermis (Fig. 7). In the lower compact sublayer of the dermis stained by the S-HID method, alternate strong and weak positive reactions were obtained in collagenous fiber bundles (Fig. 7). In the tissue sections stained by the Chase ABC/S-HID procedure, digestion with chondroitinase ABC either diminished slightly or hardly affected the reaction intensity in all the S-HID reactive histologic structures (Fig. 8). In the fish skin subjected to the Chase ABC/Kase/S-HID procedure, digestion with keratanase following that with chondroitinase ABC moderately reduced the S-HID reactivity in the dermic connective tissues, even though it failed to affect the reactions in those parts of the tissues facing the scales (Fig. 9).

In the skin of *Mc* treated with urea, the connective tissues of the dermis containing collagenous fiber bundles showed stronger S-HID reactions (Fig. 10) than those in the control tissue sections for urea treatment. In the urea-unmasked *Mc* skin stained by the Chase ABC/S-HID procedure, digestion with chondroitinase ABC notably diminished the intensity of S-HID reactions in the connective tissues (Fig. 11), but the enzyme digestion failed to affect the reactivity in those parts of the tissues facing the scales (Fig. 11). In the urea-unmasked skin subjected to the Chase ABC/Kase/S-HID procedure, digestion with keratanase following that with chondroitinase ABC gave rise to a further decline in S-HID reactivity of the connective tissues in the dermis (Fig. 12), as compared with the reactivity in the control tissues subjected to the Chase ABC/Buffer/S-HID procedure. The double digestion with the two enzymes, however, hardly affected the S-HID reactivity in the parts of the tissues facing the scales (Fig. 12).

**DISCUSSION**

In the present study, the molecular species and distribution of glycosaminoglycans in the connective tissues of the skin of Indian air-breathing freshwater teleosts were precisely analyzed by means of the combined S-HID and enzyme digestion procedures in tissue sections with or without urea treatment.

As reported previously⁵, the treatment of connective tissues with urea prior to fixation significantly increases the intensity of alcianophilia of glycosaminoglycans in the tail tendon
of adult rats. Such effects of the urea-treatment on the stainability of glycosaminoglycans could be ascribed to unmasking of the carbohydrates bound to collagen. Subsequently, treatment with urea was found to be more reliable in fixed and sectioned tissues than in unfixed fresh tissue blocks, in terms of the preservation of the histologic structures.

In the fish skins without urea treatment stained by the Chase ABC/S-HID procedure, digestion with chondroitinase ABC either slightly diminished or hardly affected the intensity of S-HID reactions in the dermal connective tissues. In the same skins treated with urea, however, digestion with chondroitinase ABC greatly diminished the S-HID reactivity in collagenous fiber bundles of the dermal connective tissues. These results are taken to indicate that protein bound isomeric chondroitin sulfates (A, B, C, D and/or E) are contained in the tissues in view of the staining selectivity of S-HID method and the substrate specificity of the enzyme.

In a previous histochemical study, keratan sulfate could not be detected in the dermal connective tissues of the eel, *Anguilla japonica*, but Ito et al. have demonstrated biochemically that keratan sulfate is distributed widely in the connective tissues of a variety of teleosts. In keeping with the results obtained by these authors, the present effects of digestion with keratanase upon the intensities of S-HID stainings in the dermal connective tissues of the two fish species are regarded as implying the presence of keratan sulfate in the matrices surrounding collagenous fiber bundles.

All the present results mentioned above have revealed that in the dermal connective tissues of *Mp* and *Mc*, isomeric chondroitin sulfates were closely associated with collagenous fibers, whereas keratan sulfate was predominantly involved in matricial sheaths surrounding the fiber bundles. In developing rat tail tendon, isomeric chondroitin sulfates were postulated to determine not only the number of collagenous fibers but the diameter of these fibers. Likewise, the particular molecular species of glycosaminoglycans in the dermal connective tissues of the two fish species appear to regulate the number and diameter of collagenous fibers. In mammalian cornea, chondroitin sulfates and keratan sulfate were reported to play roles in maintaining collagenous fibril spacing in order to permit passage of visible light through the cornea without excessive scattering. In mammalian cartilage, further, keratan sulfate has a similar function to that of the cornea in keeping the collagenous meshwork inflated, and hence endowing the tissue with rigidity and elasticity. In the dermal connective tissues of the two fish species, therefore, isomeric chondroitin sulfates and keratan sulfate could play important roles in keeping the collagenous fibers apart, maintaining the right degree of swelling and endowing the skins with rigidity and elasticity, so as to adapt the tissues to cutaneous respiration and to various surrounding environments hostile to the animals.

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Fig. 1 Dermis of urea-untreated *Mp* skin, S-HID, X200
Fig. 2 Dermis of urea-untreated *Mp* skin, Chase ABC/S-HID, X200
Fig. 3 Dermis of urea-untreated *Mp* skin, Chase ABC/Kase/S-HID, X200
Fig. 4 Dermis of urea-treated *Mp* skin, S-HID, X200
Fig. 5 Dermis of urea-treated *Mp* skin, Chase ABC/S-HID, X200
Fig. 6 Dermis of urea-treated *Mp* skin, Chase ABC/Kase/S-HID, X200
Fig. 7 Dermis of urea-untreated *Mc* skin, S-HID, X200
Fig. 8 Dermis of urea-untreated *Mc* skin, Chase ABC/S-HID, X200
Fig. 9 Dermis of urea-untreated *Mc* skin, Chase ABC/Kase/S-HID, X200
Fig. 10 Dermis of urea-treated *Mc* skin, S-HID, X200
Fig. 11 Dermis of urea-treated *Mc* skin, Chase ABC/S-HID, X200
Fig. 12 Dermis of urea-treated *Mc* skin, Chase ABC/Kase/S-HID, X200
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