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Original Research Article

Ultrastructural and immunofluorescence features of the epidermal cells and its secretory granules in the amphioxus *Branchiostoma lanceolatum* L.

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Abstract

Background and purpose: *In the vertebrate family, the epidermis of terrestrial animals is keratinized while in aquatic forms and amphibians can produce mucus. Amphioxus, a cephalochordate, is an important animal model in the study of chordate phylogeny. Major cytoskeletal and extracellular matrix proteins in epidermal cells of amphioxus (*Branchiostoma lanceolatum *L.) were investigated using transmission electron microscopy and immunofluorescence.*

Materials and Methods: *Amphioxus specimens were fresh caught in the Adriatic Sea. Tissue samples were fixed and prepared for transmission electron microscopy and immunofluorescence techniques.*

Results: *The epidermis consisted of one-layered columnar epithelium. Epithelial cells contained voluminous nucleus, a well-developed supranuclear Golgi apparatus, abundant vesicles and apical secretory vesicles surrounded by cytokeratin filaments. A single row of smaller vesicles also appeared close to the basal cell membrane. Strong positive immunolocalization of hyaluronic acid and collagen type I was observed in the apical domain of the plasma membrane of amphioxus, respectively. The positive signal of hyaluronan and collagen proteins referred to the secretory granules in the apical domain of the epidermal cell. Strong positive labelling of aggrecan was seen around the cell nucleus, deep below the apical domain, suggesting that aggrecan is not located in the secretory granules. A strong signal of the cytokeratin 10 protein was seen throughout the cytoplasm of the epidermal cells.*

Conclusion: *Due to its morphology and histochemical composition, epidermal cells of amphioxus may contribute to course surface protection and the mucous layer in amphioxus may serve as a physical barrier between the body and its environment.*

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INTRODUCTION

The cephalochordate amphioxus (*Branchiostoma sp.*) is an important animal model in the study of chordate developmental mechanisms of evolution (Evo-Devo) *(1)* and as the invertebrate chordate is one of the closest living relatives of the vertebrate. Amphioxus is like vertebrates in many morphological and genomic respects, but simpler. Both have a hollow nerve cord dorsal to a notochord, a postanal tail, an endostyle (the homolog of the vertebrate thyroid gland) and pharyngeal gill slits at some stage in their life. However, amphioxus lacks vertebrate-specif-

ic characters such as paired sensory organs or a cartilaginous or bony skeleton *(2,3)*. Recent studies showed that amphioxus diverged early during chordate evolution, and it is the sister group of both tunicates and vertebrates *(4,5)*. The new phylogenetic position of cephalochordates makes amphioxus not only a good model for studying the evolution of vertebrates, but also for the better understanding the elaboration of the entire chordate lineage *(1)*.

Branchiostoma lanceolatum can be recognized by the name lancelet or amphioxus, a cephalochordate alteration from invertebrates to vertebrates *(6)*. Its skin consists of a single-layer epidermis covered by a mucus layer containing mucopolysaccharides, protein lectin and protease inhibitor *(7)* which may serve as a defense system. Histochemical study of enzyme phenoloxidase (PO) in the epidermal cells in the epidermis of *Branchiostoma belcheri tsingtauense* has shown its presence, and both phenoloxidase and prophenoloxidase (its precursor) has been shown in the mucus on the body surface of the amphioxus *(6)*. Phenoloxidase is an important agent in the humoral immune system of crustaceans and insects. The presence of this enzyme prophenoloxidase (proPO) in the epidermal cells and the mucus is physiologically important when amphioxus is in contact with pathogens from its habitat, the proPO would be excreted into the body mucus and transformed into PO *(6)*. The epidermis acts as a final barrier between the organism and its environment and one of its main functions is to protect the organisms against external pathogens *(8)*.

Fish proteins have long been known as one of the most important sources of nutrients needed for proper growth and development of the human body. In recent years, in addition to food purposes, more and more attention has been paid to various potential applications of proteins derived from fish sources for medical, pharmaceutical, and cosmetic purposes. Currently, most research on the potential uses of proteins from fish sources is focused on collagen, a structural protein of the extracellular matrix, which due to its high biocompatibility and biodegradability is an excellent alternative source of collagen *(9)*.

Collagen is the main structural protein of the extracellular matrix and one of the most abundant proteins in the vertebrate body. So far, 28 different types of collagen have been discovered, the most important of which are in the skin, cartilage, smooth muscle and basal lamina *(10)*.

Type I collagen is a fibrillar type collagen, and most likely the best investigated collagen *(11)*. The potential role played by fibrillar collagens in vertebrate evolution has not been considered previously largely because the family has been around since the sponge, and it was unclear precisely how and when those members now found in vertebrates first arose. Classical vertebrate fibrillar collagens share a single common ancestor that arose at the very dawn of the vertebrate world and prior to the associated genome duplication events *(12)*. Type I collagen is the most abundant collagen and is the key structural composition of several tissues like bone, skin, and connective tissue. It is expressed in almost all connective tissues and the predominant component of the interstitial membrane. Type I collagen mutations have documented important roles in a range of diseases, with particular focus on bone and connective tissue disease. Several biomarkers of type I collagen have been developed, of both type I collagen degradation and formation, as surrogate makers of bone degradation and formation, respectively. The formation of type I collagen is also associated with fibrosis, and fibrogenesis *(11)*.

The posttranslational modifications of the type I collagen have been hosted, some formed during synthesis to ensure mechanical competence of the fibrils, such as interhelical and interfibrillar crosslinks, and some formed as a function of aging and disease, such as cleavage and glycation, and often resulting in reduced competence of the fibrils. Biomarkers of both type I collagen synthesis and degradation have proven of great utility, particularly in the osteoporosis field and to monitor anti-inflammatory responses *(13)*.

The ground substance is made up of proteoglycans, glycosaminoglycans and multiadhesive glycoproteins. One of the most important proteoglycans in the extracellular matrix is aggrecan. Aggrecan is a large proteoglycan that is most abundant in cartilage. It consists of a central protein chain to which more than one hundred glycosaminoglycan (GAG) chains of chondroitin sulphate are covalently linked. By noncovalent interactions, aggrecan molecules bind to long chains of hyaluronic acid, thus forming aggregates that are trapped in the collagen network in the extracellular cartilage matrix which allow the osmotic pressure to be made. Hyaluronic acid or hyaluronan is a glycosaminoglycan which, like aggrecan, is most abundant in cartilage. It is the only glycosaminoglycan that is not covalently linked to the central protein chain of proteoglycans, but comes in the form of single polysaccharide chains that can form aggrecan aggregates *(10)*.

The cytoskeleton of eukaryotic cells is a complex network of microtubules, actin filaments and intermediate filaments *(14)*. Intermediate filaments are a group of filamentous proteins with an average diameter of 10 to 12 nm. Based on gene structure, protein composition, and cell distribution, they are divided into six main classes. Some of the most well-known representatives are: keratins (cytokeratins) found in epithelial cells, vimentin in cells of mesenchymal origin, desmin in muscle cells, glia-filaments in glia-cells, and neurofilaments in nerve cells *(15)*.

One of the basic markers for epithelial differentiation are cytokeratins (CKs), intermediate filaments that form the cytoskeleton of almost all eukaryotic cells. To date, twenty different cytokeratin polypeptides have been detected in human epithelium. Cytokeratins are generally divided into acidic (type I) and neutral-basic (type II). Cytokeratin 10 (CK10), acidic type I medium-sized protein with a molecular mass of 56.5 kDa is expressed in all

suprabasal epithelial cells together with cytokeratin 1. Cytokeratin 10 is not present in the lower layers, in basal cells, where the lower molecular weight cytokeratins are present. It is used as a tumor marker (differentiates in squamous epithelial cells), but also for various other studies *(16,17)*.

In this study, the epidermal cells of the amphioxus *(Branchiostoma lanceolatum* L.) were investigated morphologically by transmission electron microscopy (TEM) and using immunofluorescence techniques for detection of the major cytoskeletal and extracellular matrix proteins.

MATERIAL AND METHODS

Animal preparation

The study was done on ten adult amphioxus (*Branchiostoma lanceolatum* L.) specimens, fresh-caught in the Adriatic Sea at an average depth of 5 m. The specimens were preserved immediately after they were caught. The average length of the animals was 27.47 mm. The specimens were fixed in 4% paraformaldehyde in phosphate buffer, dehydrated in an ascending series of ethanol, cleared in xylene, and then embedded in paraffin wax. Paraffin sections of 6 µm thickness were cut on a rotatory microtome (Leica RM 2155m) and mounted on glass slides *(18,19)*.

Immunofluorescence staining

After deparaffinization and rehydration, sections were heated for 10 minutes in a citrate buffer (pH 6.0) in a water steamer and afterward, cooled down to room temperature. Blocking buffer (ab 64226, Abcam, Cambridge, UK) was applied for 30 minutes to exclude unspecific staining (20*)*. Sections were then incubated in a humid chamber overnight with primary antibodies (Table 1). After washing in PBS, secondary antibodies (Table 1) were applied, pairings with primary antibodies, for one hour and washed in PBS again. Then, nuclei were stained with 4'6'-diamidino-2-phenylindole (DAPI) for 2 minutes. After final washing in PBS, sections were mounted in Aqua-Poly/Mount (Polysciences, Europe, Germany). All slides were studied using fluorescence microscope (Carl Zeiss AxioVision 4.7.2). Images were captured under a Zeiss Axio-imager M1 epifluorescence and bright-field microscope with a high-resolution camera (Carl Zeiss Axio-Cam MR Rev3) using Axio Vision Rel. 4.7 software (Zeiss, Vienna, Austria). Other tissues in the same sections that were known to stain specifically with the primary antibodies were used as positive internal controls *(21,22)*. No significant staining was observed if secondary antibodies were applied alone, or when only primary antibodies were applied *(23)*.

Electron microscopy

The small pieces of tissue (2 mm x 4 mm) were taken from the tail region of the amphioxus. The tissue was fixed in 3.5% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.3) during 24 hours on 4°C, and then in 3% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.2) for 2 hours. The postfixation was done in 2% osmium tetroxide in the same buffer solution *(19,24)*. The tissue was embedded in Epoxy resin and cut transversally. The ultrathin sections (0.05 µm) were stained with uranyl acetate and lead citrate. The electron microscope Zeiss EM 10A was used for examination of ultrathin sections.

RESULTS

Ultrastructural characterization of the amphioxus epidermis

The epidermis of the amphioxus consisted of one-layered columnar epithelium (Figure 1A). These epithelial cells were characterized by voluminous nucleus, a well-developed supranuclear Golgi apparatus, abundant vesicles and numerous apical secretory vesicles surrounded by cytokeratin filaments (Figure 1A, B). Some vesicles con-

Table 1. *Primary and secondary antibodies.*

	Antibody	Catalog number	Host	Dilution	Produced by
primary	Anti-Fish Collagen Type I	T8917R	rabbit	1:50	Biodesign International, Saco, ME, USA
primary	Anti-Aggrecan	ab36861	rabbit	1:100	Abcam Inc., Cambridge, MA, USA
primary	Anti-hyaluronic acid	ab53842	sheep	1:300	Abcam Inc., Cambridge, MA, USA
primary	Anti-Cytokeratin 10	M7002	mouse	1:50	Dako Denmark A/S, Denmark
secondary	Alexa Fluor® 594 Donkey Anti-Rabbit IgG H&L	ab150068	donkey	1:200	Abcam Inc., Cambridge, MA, USA
secondary	Alexa Fluor® 594 Donkey Anti-Goat IgG (H+L)	ab150132	donkey	1:500	Abcam Inc., Cambridge, MA, USA
secondary	Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (H+L)	715-545-150	donkey	1:400	Jackson Immuno Research Laboratories, Inc., Baltimore, PA, USA

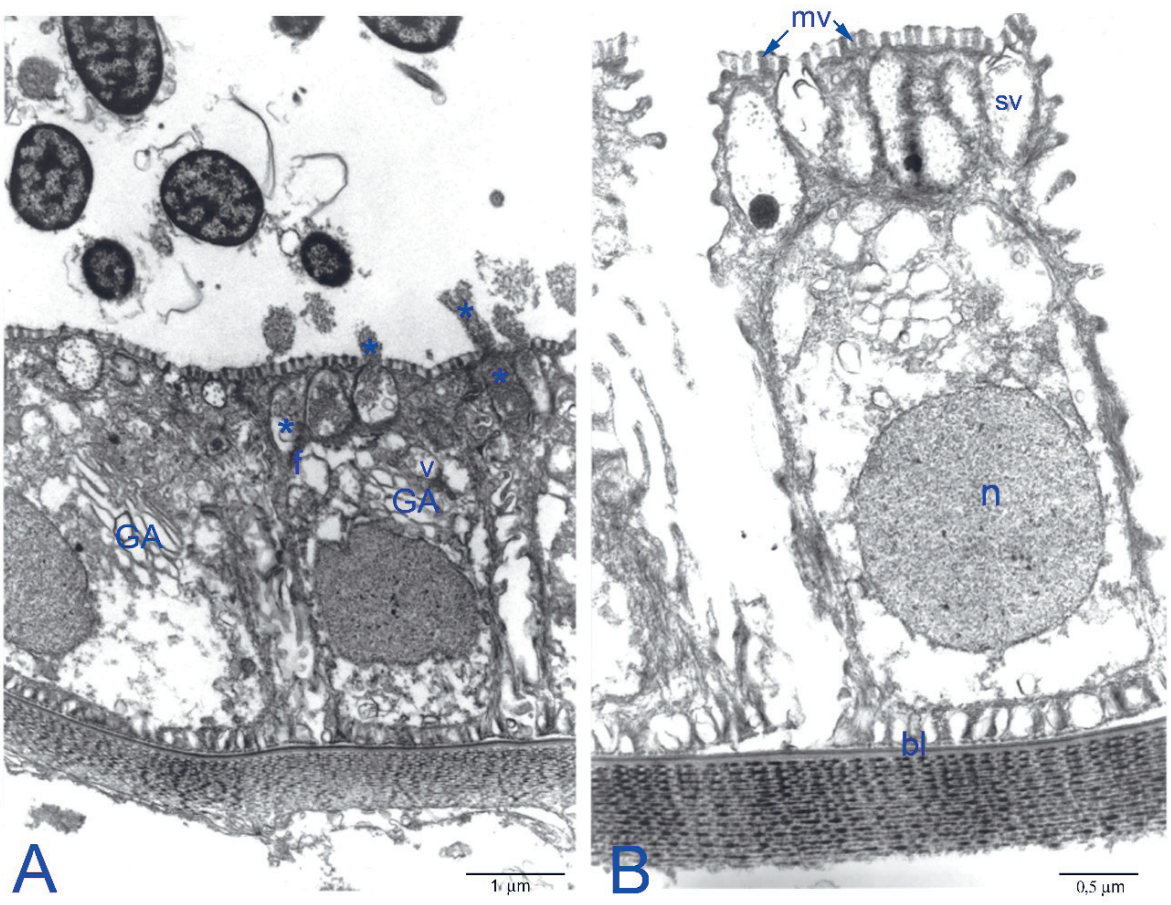

Figure 1. *Transmission electron micrographs of amphioxus epidermis. A: epidermis; x12500, B: epidermal cell; x20000. GA – Golgi apparatus; V – vesicles; f – filaments; mv – microvilli; sv – secretory vesicles; n – nucleus; bl – basal lamina; * - granulated matrix from the secretory vesicle.*

tained a sparse and irregular granulated matrix (Figure 1A, B). A single row of smaller vesicles also appeared close to the basal cell membrane (Figure 1B). Short microvilli were seen on the apical domain of the epidermal cells (Figure 1B). The plasma membrane of adjacent cells strongly intertwined on its lateral domains in the form of finger-like protrusions (Figure 1A).

Immunolocalization of the major cytoskeletal and extracellular matrix proteins in the epidermal cells of the amphioxus

The positive and strong expression of hyaluronan protein was visible as a continuous line or layer at the apical domain of the epidermal cells (Figure 2a1–a3). Intense immunofluorescence signal of hyaluronic acid and collagen type I proteins was observed in apical domain of plasma membrane of amphioxus, respectively (Figure 2a1–a3; c1–c3). This positive signal could probably refer to the numerous secretory granules in the apical domain of the epidermal cell shown on the TEM. Strong immunolocalization of aggrecan was seen around the cell nucleus, deep below the apical domain, suggesting that aggrecan is not located in secretory granules (Figure 2b1–b3). Cytokeratin 10 protein was strongly expressed throughout the cytoplasm of the epidermal cells (Figure 2d1–d3). The distribution of investigated cytoskeletal and extracellular matrix proteins in the different parts of the amphioxus epidermal cells are shown in the Table 2.

Table 2. *Qualitative expression of the of major cytoskeletal and extracellular matrix proteins in the different compartments of the epidermal cell of the amphioxus. +++ – strong intensity; -– no intensity*

	Collagen type I	Aggrecan	Hyaluronan	Cytokeratin 10
Apical domain	+++	–	+++	–
Below the apical domain	–	+++	–	–
Cytoplasm	–	–	–	+++

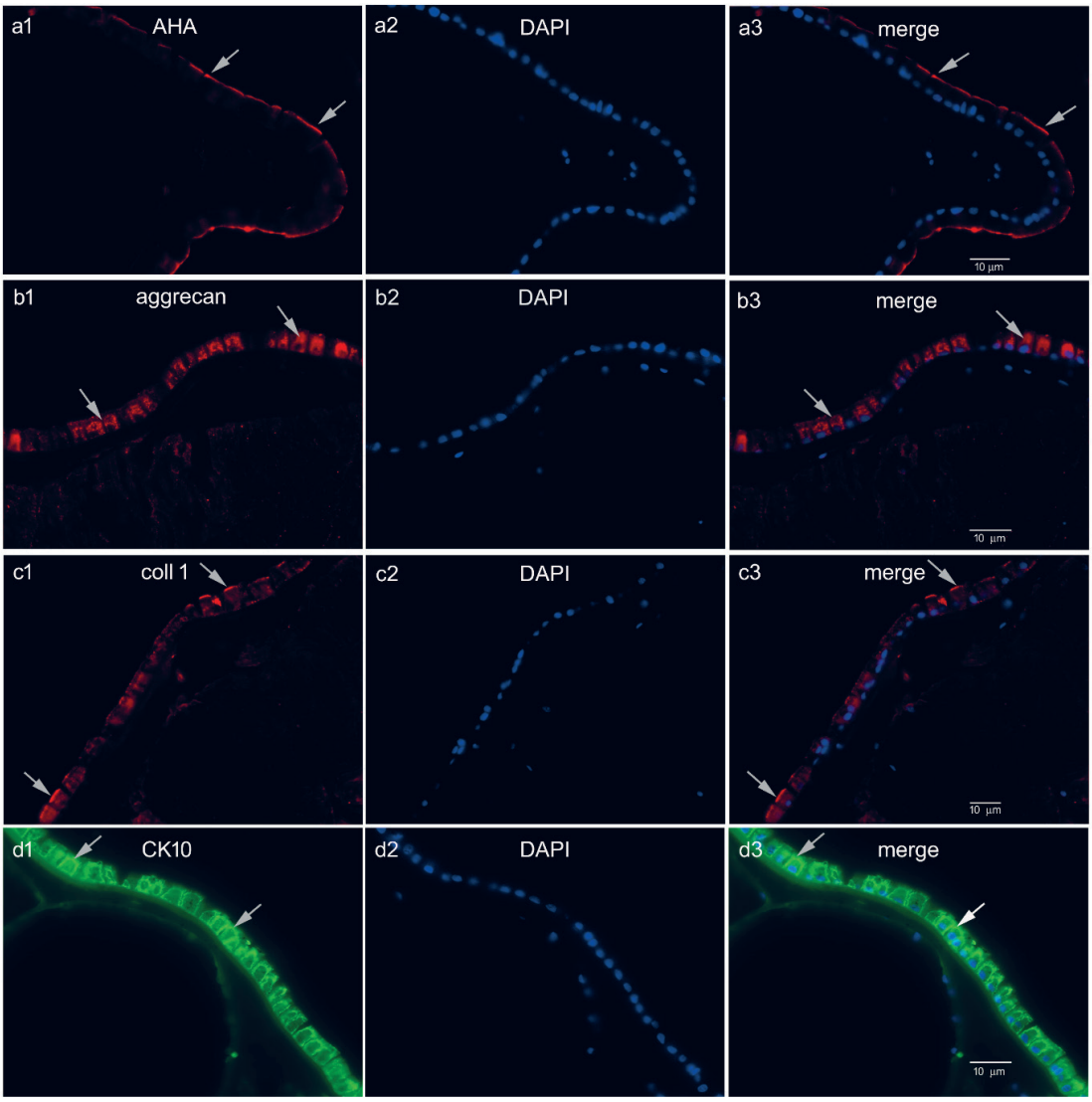

Figure 2. *Positive immunolocalization of major cytoskeletal and extracellular matrix proteins in amphioxus epidermal cells with DAPI nuclear staining: a1–a3 expression of hyaluronic acid (arrows), x63; b1–b3 expression of aggrecan (arrows), x63; c1–c3 expression of collagen type I (arrows), x40; d1–d3 expression of cytokeratin 10 (arrows), x63.*

DISCUSSION

An epithelium is a layer of closely connected cells covering the body or lining a body cavity *(25)*. Epithelial cells make vacuolar apical compartment (VACs) within themselves in response to stimulation by external cell matrix (ECM). In a single epithelial cell surrounded by ECM, VACs fuse with each other to make a large vacuole, and the cytoplasm divides. The main function of epidermis is surface protection. The epidermal cells are covered with a layer of mucus in the amphioxus *(26)*. The epidermis is not keratinized, and the mucous layer might serve as physical barrier between body and its environment *(8)*. Adult amphioxus body surface mucus contains lectin that could agglutinate human red blood cells *(8)*. Mao *et. al. (8)* has found evidence of endocytosis of larval epidermal cells indicating that the epidermal cells of amphioxus larvae could be involved in digesting of antigens from the surface and thus cells could play a role in the body's defenses due to animal habitat, or these cells might also be involved in gas exchange or supplying the body with the required nutrients.

Epidermal cells of amphioxus at different developmental stages were investigated by electron microscopy and colloidal carbon tracing experiments *(8)* where different ultrastructural characteristics at larval and adult stages were shown. In the present study, we have shown epidermal cells with many crater-like protrusions on their surface containing secretory granules in the apical part suggesting that these cells were capable of secretion. Mao *et. al. (8)* has also reported that numerous superficial vesicles

with variable electron density matrix in the surface layer of the epidermal cells in the amphioxus larvae. Flood *(26)* has found two kinds of vesicles investigating epidermal cells in the tail of two weeks old amphioxus and named them secretory vesicles placed at the surface of the cells. These cells have a low electron density opposite to pigment granules in the apical part of the cells with a very dense matrix *(25)*. In our study, all cells containing secretory vesicles were showing a very dense matrix and well-developed Golgi apparatus in the cell cytoplasm. These secretory amphioxus epidermal cells can secrete the external cell matrix, namely collagen, hyaluronic acid and aggrecan which our results have confirmed. It is well known that collagen is a cofactor for the synthesis of vitamin C *(27)* and is usually found together with HA in connective, muscle and epithelial tissue. We have confirmed a presence of both proteins in the amphioxus epidermis which possibly imply their role in lubrication (hydration), wound healing, and tissue regeneration as well an anti-viral and anti-bacterial role *(28,29)*. The collagen matrix-based connective tissues secreted by the somite-derived cells in amphioxus likely represent the rudimentary skeletal tissues in chordates *(30)*. It was found that cells at the lateral wall of the amphioxus somite express SPARC (a crucial gene for tissue mineralization) and various collagen genes *(31)*. During development, some of these cells expand medially to surround the axial structures, including the neural tube, notochord and gut, while others expand laterally and ventrally to underlie the epidermis. Eventually these cell populations were found closely associated with the collagenous matrix around the neural tube, notochord, and dorsal aorta, and with the dense collagen sheets underneath the epidermis. The finding of SPARC-expressing skeletal scaffold in amphioxus further supports previous hypothesis regarding SPARC gene family expansion in the elaboration of the vertebrate mineralized skeleton *(31)*. Type I collagen was observed in the mineralized cartilage of the vertebral bodies, the notochord, the fibrocartilage of intervertebral disc, and the perichondrium *(23)* of the dogfish *Scyliorhinus canicula* (Linnaeus, 1758) a primitive cartilaginous fish, evolutionary placed among cephalochordates (Cephalochordata) and teleost fish (Osteichthyes). Nevertheless, the three-layered amphioxus notochordal sheath strongly expressed fish collagen type I in its outer and middle layers, while in the innermost layer expression did not occur. Collagen type I was proven to be the key extracellular matrix protein that forms the amphioxus notochordal sheath *(21)*. The notochord and notochordal sheath of the adult amphioxus were investigated ultrastructurally and histochemically *(24)*. Histochemical staining showed that the notochordal plates resemble neither the connective tissue, notochordal sheath nor the typical muscular structure myotomes. The outer and middle layer were composed of collagen fibers of different thickness and course, that correspond to collagen type I and collagen type III in vertebrates, respectively, and the inner layer was amorphous, resembled basal lamina, and was closely attached to the notochord by hemidesmosome junctions. These results confirmed the presence of collagen fibers and absence of elastic fibers in amphioxus *(24)*. X-ray diffraction and electron microscopy were used to compare the molecular and higher order structure of collagen fibrils in three tissues of the lamprey: the dermis, perinotochord and notochord sheath *(32)*, tissues that are known to contain five distinct genetic types of fibrillar collagen suggesting the molecular-packing motifs for collagen fibrils were in place at the dawn of vertebrate evolution and have been conserved since *(30,32)*. The molecular evolutionary history of chordate collagen genes is well investigated and examined how gene duplications gave rise to the collagen genes used for skeletons. Deuterostome collagen genes, including one amphioxus gene that was identified, suggested that the common ancestors of deuterostomes possessed three fibrillar collagen genes *(33)*.

Also, in the epidermal amphioxus cells we have found a strong positive fluorescence signal of aggrecan, a typical protein of the cartilage found also in the intervertebral discs, possibly due to its habitat as adult amphioxus begin to live in the sand instead of swimming in the seawater.

The apical domain of plasma membrane in the epidermis of the amphioxus in our investigation, respectively shown an intense expression of hyaluronan. The intracellular occurrence and distribution of sulphated polyanions, interpreted to represent mucins, were studied in secretory epithelial cells in the primitive chordates *Branchiostoma lanceolatum* and *B. floridae* at the electron microscopical level by using the dye cupromeronic blue (CMB) *(34)*. CMB-precipitates were mainly found within two potential types of mucin vesicles (apical and basal) and Golgi cisterns. The mucin is secreted apically but only in the epidermis it forms a dense layer covering the apical microvilli. In the *Branchiostoma* epidermal cells a layer of specialized basal vesicles occurred, containing unusually large and branched CMB-precipitates which possibly serve mechanical functions *(34)*. The distribution of proteoglycans in the sheath and epithelial cells of the lamprey notochord had been also studied using electron microscopy on material stained with the CMB dye *(35)*. CMB is a cationic dye, usually used for the histochemical estimation of glycosaminoglycans for example in cartilage *(36)*. CMB-precipitates, which indicate the presence of sulphated proteoglycans, were found in the inner collagenous but not the outer "elastica externa" regions of the notochord sheath in the lamprey. Precipitates were also found in the zone of the basement membrane of the notochord epithelial cells, in intercellular spaces within the notochord epithelium, and in intracellular spherical bodies of the epithelial cells. These results indicate that the proteoglycans in the notochord sheath are produced by the notochord cells *(35)*. Immunofluorescence and immunohistochemical techniques were widely used to define the distribution of cytoskeletal (cytokeratin 8, vimentin) and extracellular matrix components (collagen type I, collagen type II, hyaluronic

acid, and aggrecan) in the notochord of the lesser spotted dogfish *Scyliorhinus canicula* L. *(23)*. Immunolocalization of hyaluronic acid was observed in the notochord, vertebral centrum, and neural and haemal arches, while positive labelling to aggrecan was observed in the ossified centrum, notochord, and the perichondrium of the hyaline cartilage.

A major cytoskeletal and extracellular matrix proteins of the amphioxus notochordal cells and sheath were detected by immunohistochemical techniques as well *(21)*. The amphioxus notochordal sheath was reactive to applied anti-human antibodies for intermediate filament proteins such as cytokeratins, desmin and vimentin, as well as to microtubule components. These results confirmed that genes encoding intermediate filament proteins, microtubules and microfilaments are highly conserved during evolution *(21)*.

CONCLUSION

Major cytoskeletal and extracellular matrix proteins in the epidermal cells of the amphioxus (*Branchiostoma lanceolatum* L.) were investigated using transmission electron microscopy (TEM) and immunofluorescence techniques. Epidermis of the amphioxus consisted of one-layered columnar epithelium, while epithelial cells were characterized by voluminous nucleus, a well-developed supranuclear Golgi apparatus, abundant vesicles and numerous apical secretory vesicles in secretion surrounded by the cytokeratin filaments. A single row of the smaller vesicles also appeared close to the basal cell membrane. Strong positive immunolocalization of the hyaluronic acid and the collagen type I was observed in the apical domain of plasma membrane of the amphioxus, respectively. The positive signal of hyaluronan and collagen proteins could refer to the secretory granules in the apical domain of the epidermal cell. A very strong labelling of aggrecan was seen around the cell nucleus, deep below the apical domain, suggesting that aggrecan is not located in the secretory granules. A strong signal of the cytokeratin 10 protein was seen throughout the cytoplasm of the epidermal cells. Due to its morphology and histochemical composition, the epidermal cells of amphioxus may contribute to course surface protection while the mucous layer in amphioxus may serve as a physical barrier between the body of the animal and its habitat.

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