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Short communication

ALP, TRAcP and cathepsin K in elasmoid scales: a role in mineral metabolism?

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Summary

Elasmoid scales from the common carp (and other teleostean fishes) appear to be an exciting new model in the research of mineralized tissues. The presence of alkaline phosphatase (ALP), a marker of mineralization, on both sides of the scale was demonstrated by means of enzyme histochemistry. Tartrate-resistant acid phosphatase, a marker for mineral degradation and osteoclasts, was observed along the radii, at the same location as the ALP activity on the episquamal side. This points towards an active mineral metabolism, were scale cells are involved in both formation and degradation of the mineralized matrix. Cathepsin K staining revealed the presence of multinuclear osteoclasts along the grooves of the scale. Interestingly, the scales were taken from growing control fish; they were not induced to resorb their matrix. Presence of these enzymes in scale cells, together with the demineralized regions in the centre of the scale suggest a more dynamic mineral metabolism in cyprinid scales then previously observed in other species. Scales are derived from odontode tissues, their formation relies on many the same underlying mechanisms and genes as other mineralized tissues. Moreover, a single scale offers the possibility to culture scale-forming and -degrading cells together on their original matrix. All of the these unique properties substantiate the potential of scales to yield new insights on osteoclasts and regulation of tissue mineralization.

Introduction

To study the biology of mineralized tissues, bone cell cultures or pieces of foetal bone like calvaria are mostly used. Hence, animals have to be killed to obtain cells or tissues for the experiments. This generally yields only one or two samples from each animal because a specific part of the skeleton is used. Another option is the use of isolated cells, with the disadvantage that the cells tend to dedifferentiate when dissociated from their natural matrix. Scales of teleostean fishes offer a unique solution to (some of) these problems as fish possess several hundreds of these (partly) mineralized collagen structures of dermal origin. Scales can be traced back in evolution to odontodes, tooth-like structures embedded in the dermis (Sire and Akimenko, 2004). Odontodes show a close morphological resemblance to teeth as they contain a layer of dentin, enamel and a pulp cavity (Sire et al., 1998). Early scales and odontodes also comprise a bony layer, the basal plate. In the course of evolution, scales acquired different traits; the pulp cavity was lost and the basal plate replaced by the partly mineralized elasmodine. Although early scales may contain osteocyte-like cells in the basal plate, in elasmoid scales the basal plate is fully acellular. However, some traits like the type and arrangement of collagens in the basal plate, are still reminiscent of that of bone (Meunier, 1984; Weiner et al., 1999).

Zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) are two popular model species with elasmoid scales. These scales can easily be removed with forceps without causing major harm to the fish and a new scale is formed subsequently. Scales, with its associated scale-forming cells (scleroblasts) can be cultured *ex vivo* for up to 2 weeks. The formation of a scale occurs in an enclosed environment, aptly named the scale pocket. Scale forming cells, called scleroblasts (Sire and Géraudie, 1984), are presumed to be of neural crest origin, just as the odontoblast and osteoblasts of the dermal skeleton (Smith et al., 1994). In scale regeneration, the first mineral layer is already deposited within the first week. Regeneration of the entire scale may take up to 30 days, depending on the ambient temperature. Scale formation and regeneration have been described extensively in literature (Sire, 1989; Sire et al., 1997; Bereiter-Hahn and Zylberberg, 1993).

Scales are an ideal reservoir of phosphorus and calcium since they contain up to 20% of the total body calcium, mainly as calcium phosphate crystals (Flik et al., 1986; Schönbörner et al., 1979). It has been demonstrated that this pool of calcium is utilized in goldfish exposed to estrogen whereas calcium resorption could not be detected in the rest of the skeleton (Mugiya and Watabe, 1977). The mineralized side of the scale (the episquamal side) possesses concentric ridges (circuli) and grooves (radii) radiating from the centre (focus) to the edges of the scale (Schönbörner et al., 1979). Along these ridges, episquamal scleroblasts are found, which are thought to play a role in mineral metabolism. The cells on the hyposquamal side form a flattened monolayer and appear to function similarly to fibroblasts as they only synthesize a collagenous matrix. These unique properties make fish scales highly suitable to study mineral deposition and degradation directly on the intact mineralized tissue and its associated cells.

Marker enzymes of mineral metabolism

Tissue non-specific alkaline phosphatase (TNAP) is a well established marker of osteoblasts and bone formation in mammals and teleostean fishes (Kaunitz and Yamaguchi, 2008; Witten, 1997). But the role of ALP in mineralization is

still under debate; it is thought to free phosphates which are required for mineralization but a role in breakdown of pyrophosphates, an inhibitory factor in mineralization was also suggested (Omelon and Grynpas, 2008). Enzymatic ALP activity has already been demonstrated in association with elasmoid scales (Suzuki et al., 2000). Moreover, we detected expression of the predominant bone alkaline phosphatase paralogue in the cells associated with zebrafish scales (unpublished results). Although ALP has many times been described in relation to scales, to the best of our knowledge no histological images that show the location of alkaline phosphatase have appeared in literature. Tartrate-resistant acid phosphatase (TRAcP) has been the classical marker of vertebrate osteoclasts for more than 20 years (Minkin, 1982; Witten and Huysseune, 2009). However, TRAcP is not restricted to these cells, as it is also found in e.g. immune and prostate cells (Hayman, 2008). TRAcP (from the *ACP5* gene) exerts its function in the resorption pits generated by osteoclasts, where the required acidic pH for optimal activity is realized by HCl production (Kaunitz and Yamaguchi, 2008). TRAcP is also suggested to be involved in osteoclast migration by weakening the interaction between proteins that bind the osteoclast to the matrix (Andersson et al., 2003). Vertebrate osteoclasts also contain cathepsin K (Nemoto et al., 2007). This cysteine proteinase is predominantly expressed in osteoclasts and degrades the bone matrix (Saftig et al., 1998). We searched for the presence of these marker enzymes on untreated cyprinid scales, to gain insight in their normal mineral metabolism.

Materials and methods

All scales were obtained from growing control group carp (*C. carpio*). Scale were taken from the skin with a forceps under light sedation (0.5% 2-phenoxyethanol, 10 min maximally). Scales were used for histological analysis without fixation. For alkaline phosphatase staining, intact scales were preincubated with 0.1 M TRIS / HCl pH 9.5 for 15 min. Next, samples were stained with NBT / BCIP in ALP-buffer (100 mM TRIS / HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) at 37°C. Reaction was stopped by rinsing samples with water. If samples were double-stained for tartrate-resistant acid phosphatase, they were preincubated for at least 2 h in fresh 0.2 M TRIS-buffer pH 9.0 at 37°C. Samples were stained for several minutes (until the red colour became visible by microscopic inspection) with TRAcP staining solution according to (van de Wijngaert and Burger, 1986). To stop the reaction, samples were rinsed with water.

Cathepsin K was stained with the commercially available Magic Red Cathepsin K detection kit (AbD Serotec, Düsseldorf, Germany) according to the manufacturer's protocol and counterstained with Hoechst nuclear staining.

The minerals of the scale were stained according to Von Kossa. Scales were incubated with 5% silver nitrate under bright light for 1 h. Scales were subsequently rinsed with tap water for 1 min and developed for 5 min in 5% sodiumthiosulfate. Finally, scales were rinsed thoroughly with tap water.

Results and discussion

Alkaline phosphatase activity is mainly detected in the hyposquamal cells of carp scales, although we also observed some staining at the episquamal side (Fig. 1a,b). TRAcP staining on fish scales has, to the best of our knowledge, never been combined with alkaline phosphatase staining. In the ALP / TRAcP double stained scales, TRAcP staining masks the ALP staining, which can no longer be observed after double staining (Fig. 1c). In previous studies, with different fish species, TRAcP activity could only be localized on scales from fish in experimental conditions where a higher calcium demand was indicated (Persson et al., 1995; Suzuki et al., 2000). Even after stimulation of scale resorption through starvation of hormonal treatment, TRAcP was only detected on a few locations of scale resorption. In scales from control carp, TRAcP activity was detected along the entire length of the grooves of the scale and in the focus. As the Von Kossa staining (Fig. 1d) demonstrates, the grooves and many areas in the focus are not mineralized. Presence of mineral degrading cells is further established by the cathepsin K positive cells that could be detected along the grooves of the scale (Fig. 1e–g). In

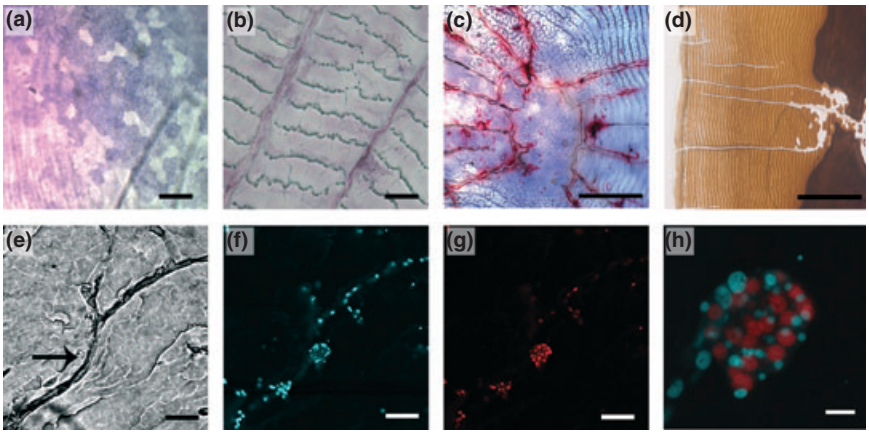

Fig. 1. (a) ALP staining, focused on the hyposquamal side. The amount of ALP varies between the scleroblasts. This results in differential staining of the cells. Scale bar = 80 μm (b) Alkaline phosphatase staining, focused on the episquamal side. Staining is observed along the radii of the scale. Scale bar = 50 μm. (c)ALP / TRAcP double staining. TRAcP is stained along the unmineralized grooves on the episquamal side of the scale. Scale bar = 500 μm. (d) The brown colour of the Von Kossa calcium staining clearly shows that the grooves are unmineralized. There are also unmineralized regions in the focus of the scale. (e) Light microscopic image of the scale that is stained for cathepsin K. The cell, indicated by the arrow, is located along one of the radii. Scale bar = 60 μm. (f) Fluorescent image of the same scale. Nuclei, stained with Hoechst, are shown in blue. Scale bar = 60 μm. (g) Same image as F, cathepsin K is demonstrated in red. Scale bar = 60 μm. (h) A multinuclear osteoclast found on the scale surface. The cell is positive for the nuclear staining (blue) and cathepsin K (red). Scale bar = 10 μm

scales counterstained with Hoechst, a nuclear staining, some multinuclear cells can be observed (Fig. 1h). These multinuclear cell aggregates have been described before as osteoclasts (Sire et al., 1990). As none of the scales were induced to resorb their matrix in any way, the presence of these markers for mineral formation and degradation suggests a dynamic mineral metabolism in cyprinid scales.

The exact role of alkaline phosphatase in bone formation is not established, not even in mammals. The location of the enzyme on scales only raises more questions since ALP is considered to play an important role in tissue mineralization in both mammals and fish (Kaunitz and Yamaguchi, 2008; Sindre et al., 2005). Remarkably, on elasmoid scales, ALP is mainly found on the hyposquamal side, which is never mineralized, as new collagen is slowly deposited. Therefore, it seems unlikely that these alkaline phosphatases still act in mineralization as they are always located far from the mineralized part of the scale, and the mineral layer grows only slowly or not at all in hyposquamal direction. At the mineralized part of the scale, alkaline phosphatase activity is mainly located around the grooves and on the growing edges. This may be an indication of involvement in mineral deposition. However, we realized that histochemical detection of the enzymes does not necessarily imply activity *in vivo*. In ALP/TRAcP double-stained scales, episquamal alkaline phosphatase activity can no longer be detected, as TRAcP staining masks the ALP staining. This may suggest that the presence of the enzymes on the same locations of the scale. It is difficult to identify cells in the TRAcP positive regions as TRAcP is a secreted enzyme and present in the scale matrix. We found a small number of multinuclear osteoclasts with the cathepsin K staining. Therefore the larger part of the secreted TRAcP has likely come from mononuclear cells or was released by migrating osteoclasts. Mineral degradation by mononuclear cells is observed in mononuclear osteoclasts or osteocytic osteolysis. The latter mechanism describes cells that originate from the osteoblastic cell lineage, that are now able to degrade bone (Witten et al., 2000; Witten and Huysseune, 2009). Since most cells on the episquamal side of the scale are scleroblasts, it is possible that a similar underlying switch of function could be present in these scale degrading episquamal scleroblasts. The origin of these cells may shed more light on regulation of scale degradation, as we are not aware of any osteoclast-like cells that originated directly from mesenchyme (scleroblasts). However, the dermis of fish where the scales reside is well perfused and migratory progenitor cells of ectodermic origin would abide.

Scales as a potential model for mineralized tissues?

Our results suggest a basal level of continuous deposition and resorption of scale minerals, as these carp were not subjected to any experimental condition, i.e. assumed to be in positive calcium and phosphorus balance. This requires active and controlled mineralization and demineralization by cells associated with the scale. We clearly demonstrated the presence of osteoclasts on control scales and the presence of ALP suggests episquamal scleroblasts may be involved in deposition of scale matrix.

Although scales and teeth share a common ancestor, it is clear that elasmoid scales evolved to a new structure that is neither tooth or bone. It is already known that mineralization of bone and tooth matrix shares many similar mechanisms (e.g. matrix vesicles, (Anderson, 2003)) and genes (e.g. osteocalcin, osteonectin, BMPs, MMPs (Robey, 1996; Chen et al., 2008; Rawadi et al., 2003; Krane and Inada, 2008; Bildt et al., 2009)). Moreover, various experiments have shown that scale cells are affected by starvation of the fish and hormonal treatments; conditions that are known to alter mammalian bone density (Rigotti et al., 1991; Kim et al., 2007). Indifferent to their origin, several papers have appeared in literature where scales are already in use as a model to study bone formation. Scales show the same response as mammalian bone to a number of treatments (Suzuki et al., 2000; Yoshikubo et al., 2005; Redruello et al., 2005). The use fin rays, which are also part of the dermal skeleton, is described as a model to study direct ossification. (Marí-Beffa et al., 2007; Quint et al., 2002). Compared to fin rays, elasmoid scales benefit from the presence of cells with scale-forming and -degrading (osteoclast-like) properties, and these cells can be cultured together on their original substrate (Brittijn et al., 2009). Moreover, many scales can be obtained from a single fish, formation of the mineral layer is fast and ongoing in growing fish. Also, the ectothermic fish allows to study these aspects of scales at different temperatures set by the experimenter, and thus likely at different paces, to define the readout resolution. The future will point out whether or not scales are suitable for use as an osteogenesis model. However, our results show that all the required components for mineral deposition and degradation are present on normal carp scales. To study these may offer unique insights inscale mineral metabolism, and possibly in mineralization of biological tissues in general.

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