Acta Histochemica xxx (2013) xxx-xxx



Contents lists available at SciVerse ScienceDirect

Acta Histochemica



journal homepage: www.elsevier.de/acthis

Soft sheets of fibrillar bone from a fossil of the supraorbital horn of the dinosaur *Triceratops horridus*

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ARTICLE INFO

Article history: Received 9 December 2012 Received in revised form 28 December 2012 Accepted 3 January 2013 Available online xxx

Keywords: Osteocytes Fossil Dinosaur Triceratops Horn Ancient soft tissue

Introduction

Previous studies have reported soft tissues and cell-like microstructures in fossilized dinosaur bones from Tarbosaurus bataar, Tyrannosaurus rex, Brachylophosaurus canadensis, and Triceratops horridus (Pawlicki, 1978; Pawlicki and Nowogrodzka-Zagorska, 1998; Schweitzer and Horner, 1999; Armitage, 2001; Zylberberg and Lauren, 2011), as well as other extinct organisms such as certain marine turtles (Cadena and Schweitzer, 2012). Light and electron microscopic studies have tentatively identified tissue components of dinosaur remains as red blood cells, endothelial cells, osteocytes and collagen fibers (Schweitzer et al., 2005, 2007a, 2009). Isolation of dinosaur peptides and proteins has also helped to confirm the cellular nature of these fine structures (Schweitzer et al., 2007a, 2009; Lindgren et al., 2011; San Antonio et al., 2011). Exceptions to these findings have been offered by Kaye et al. (2008), however recent analyses seem to confirm that original soft tissues and possibly original molecules do exist in incompletely fossilized remains of extinct animals, including dinosaurs (Schweitzer et al., 2009, 2013; Lindgren et al., 2011; Cadena and Schweitzer, 2012).

Furthermore, a wide variety of specimens yielding soft tissues has bolstered the fact that soft tissue is not limited to specific

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ABSTRACT

Soft fibrillar bone tissues were obtained from a supraorbital horn of *Triceratops horridus* collected at the Hell Creek Formation in Montana, USA. Soft material was present in pre and post-decalcified bone. Horn material yielded numerous small sheets of lamellar bone matrix. This matrix possessed visible microstructures consistent with lamellar bone osteocytes. Some sheets of soft tissue had multiple layers of intact tissues with osteocyte-like structures featuring filipodial-like interconnections and secondary branching. Both oblate and stellate types of osteocyte-like cells were present in sheets of soft tissues and exhibited organelle-like microstructures. SEM analysis yielded osteocyte-like cells featuring filipodial extensions of 18–20 µm in length. Filipodial extensions were delicate and showed no evidence of any permineralization or crystallization artifact and therefore were interpreted to be soft. This is the first report of sheets of soft tissues from *Triceratops* horn bearing layers of osteocytes, and extends the range and type of dinosaur specimens known to contain non-fossilized material in bone matrix.

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fossil sites or fossil species, thus, a major focus of recent work has been the sampling of fossils from various taxa (dinosaur and otherwise), depositional environments, and geological time frames to determine the extent of soft tissue presence in Devonian, Triassic and Cretaceous strata in comparison with recent specimens (Schweitzer et al., 2007b; Zylberberg and Lauren, 2011).

The aim of this paper was to examine fresh fossil specimens of adult supraorbital horn and rib remains of *T. horridus* for the presence of soft tissues and to characterize any soft tissues found.

Materials and methods

An intact *Triceratops* horn (HCTH-00) was recovered on May 12, 2012, from a well-sorted fluvial sandstone within the Hell Creek Formation at a previously unexcavated site on a private ranch within the Hell Creek Formation (a portion of land located at E 1/2 of the SW 1/4 of the NE 1/4 Section 14, T. 15 N., R. 56 E., Dawson County, Glendive, MT, USA). The recovered horn was jacketed and removed. The length, girth and external morphology of the fossil was consistent with other *Triceratops* horns recovered from the Hell Creek Formation. Disarticulated *Triceratops* ribs (HCTR-11) and vertebrae (HCTV-22) found within a mile of the horn were also recovered for analysis.

Hand-sized pieces of HCTH-00 were fixed in 2.5% glutaraldehyde solution, buffered with 0.1 M sodium cacodylate buffer at 4 °C for 5 days, rinsed in distilled water and buffer and stored in phosphate

^{0065-1281/\$ -} see front matter © 2013 Elsevier GmbH. All rights reserved. http://dx.doi.org/10.1016/j.acthis.2013.01.001

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buffered saline (PBS). Individual pieces of roughly 20 cm² were removed by pressure fracture (HCTH-01, 02, 03), examined under a dissecting microscope and probed with sterile forceps to identify and collect soft material. Soft materials recovered were washed in double distilled water and stored in PBS awaiting further analysis.

Other horn specimens (HCTH-04, 05) were processed through a decalcification protocol. Several pieces about 20–50 cm in size were rinsed in double distilled water after fixation and were incubated in a solution of 14% sodium EDTA at room temperature. EDTA was exchanged every 2–4 days for a period of 4 weeks. Significant bone mineral remained after 4 weeks, therefore it was unknown whether complete decalcification would yield soft and transparent, vessellike tissues such as previously reported (Schweitzer et al., 2005, 2007a,b, 2009; Lindgren et al., 2011).

Decalcified bone was air-dried, affixed to aluminum stubs, sputter-coated with gold for 60 s at 20 mA and imaged at 20 kV on a Hitachi S2500 scanning electron microscope (SEM). Digital images were recorded on a 4Pi Spectral Engine II (Hillsborough, NC, USA) running under NIH ImageJ.

Large strips of thin, light brown, soft material (20 cm by 10 cm) were recovered from the innermost interior sections of other fixed and unfixed, non-decalcified horn bone pieces. Strips were post-fixed in 2% osmium tetroxide, dehydrated in a graded series of acetone and infiltrated with a polymer resin (EMBed-812, Electron Microscopy Sciences, Ft. Washington, PA, USA). Other strips of soft material were cut into 2 cm by 2 mm pieces, embedded in OCT (Sakura Tissue Tek, Tokyo, Japan) and sectioned on a Microm (Thermo Scientific, Waltham, MA, USA) HM550 cryostat at -23 °C. Cryosections of 9–11 µm thickness were affixed to glass slides, and cover slipped for light microscopy. Photo-documentation was performed using a Jenoptik (Jena, Germany) C14+ camera on a Nikon (Tokyo, Japan) 80i microscope.

Triceratops rib specimens were allowed to air dry and were subsequently fractured with mechanical pressure yielding 5–10 mm pieces suitable for SEM. Pieces of rib with their internal surfaces exposed were sputter-coated and examined with SEM.

Results

The *Triceratops* horn (Fig. 1) was approximately 58 cm long, 22 cm in diameter and 9 kg in total mass. No keratin was found. The horn had been partially buried under 30 cm of homogeneous, but loosely packed sandstone and rock. The rock required fracturing by hammer and chisel to free the distal part of the horn. Rib fragments (Fig. 2), located separately from the horn, were approximately 15 cm long and had no visible moisture when removed. Horn material was not completely desiccated, but appeared somewhat moist during excavation (Fig. 3). Soft, moist, muddy material can be seen surrounding pores of bone vessels on inner horn surfaces (Fig. 4).

Subsequent to jacketing and removal, the horn fractured into several large pieces (Figs. 3 and 4). Individual large pieces were wrapped separately in aluminum foil, sealed in containers and transported to the lab for analysis. Fixed horn material did not seem as friable as unfixed material, possibly because interior soft tissues were stabilized by fixatives. Small (2–5 mm) red and brown plant roots were loosely attached to exterior surfaces and extended into fractures in the horn (see left side of bone in Fig. 4). Pieces of small fixed material from the inner core of the horn came apart with moderate hand pressure and were found to contain thin, elastic, reddish-brown flaps of soft material (Fig. 5, white arrow) which could be peeled away from the bone in sheets and stretched to almost double the original size. It was initially thought that this soft material might represent the remains of a biofilm and/or plant material due to the many tiny plant roots associated with it (Fig. 6, black arrows). These reddish-brown flaps of soft material and the off-white to milky-white pieces of soft material (Fig. 7, black arrows) collected from pre and post-decalcified bones yielded innumerable small sheets of lamellar bone matrix with clearly visible microstructures consistent with lamellar bone osteocytes (Fig. 8). Osteocyte-like structures also exhibited internal microstructures consistent with cellular organelles (Figs. 9 and 10, arrows). The matrix of parallel fibers densely populated with microstructures (Figs. 8–10) was identical to osteocytes found in compact bone of *T. rex* femur (Schweitzer et al., 2005, 2013), *B. canadensis* femur (Schweitzer et al., 2009), and *Prognathodon* sp. femur (Lindgren et al., 2011).

Flexible vessels were not present but demineralization was halted on specimens after 4 weeks. Both processed and unprocessed horn specimens exhibited many clear to milky-white or reddish brown pieces of soft material, which swayed gently upon bone surfaces when solutions were disturbed (Fig. 7, black arrows). Soft material processed for polymer thin sectioning disintegrated during sectioning, possibly as an artifact of dehydration or incomplete infiltration.

Fractured rib specimens contained well-preserved Haversian systems (osteons) with many visible lamellae and lacunae (Fig. 11). The Haversian canals were sometimes filled with many spherical microstructures (Fig. 12), which are consistent with the size and shape of red blood cells. So many spherical cell-like structures were present that vessel structure was often obscured. Many soft, hollow cylindrical tubes were seen to project from Haversian canals (Fig. 13) and are consistent with blood vessels.

Non-decalcified horn specimens showed good preservation of mineralized vessel-like structures surrounded by dense cortical bone. Decalcified horn specimens were characterized by vessels present in vertical conformation (as they would be in Haversian canals) and interconnecting Volkmann's canals are evident (white arrows, Fig. 14), yet all vessel-like structures studied were fully permineralized. The fractured end of a vessel-like structure in Fig. 15 (white arrow) is further magnified in Fig. 16 and it is clear that microstructures within it (possibly vessel products, e.g. blood and lymph) are also fully permineralized. It therefore appeared that permineralization was selective or at the least, that some soft tissues were sequestered from the process of fossilization. Further study of the horn must be done to determine if this seemingly selective permineralization is related to anatomical differences within the horncore (Happ, 2010).

SEM analysis of decalcified portions of horn shows a large number of osteocyte-like microstructures with very fine filipodial projections lying along the same layer of fibrillar bone (Fig. 18).

Discussion

The Hell Creek Formation has been a well-characterized and studied rock unit since first described in the early 1900s (Brown, 1907). It is exposed by the well-known Cedar Creek Anticline at Glendive, MT and encompasses nearly 700 km (Johnson et al., 2002). Many valuable fossils have been recovered from the Hell Creek Formation exposed at Glendive, and *Triceratops* remains (including brow horns) are frequently found at that location (Horner, 2001).

This is the first report of soft tissues from a *Triceratops* horn, and thus offers a unique opportunity to understand their form and function. Horn anatomy has been rarely studied, thus much remains to be known about their structure. Anatomically, *Triceratops* horn offers a unique fossil structure with differentiated inner layers of varying thickness and porosity. Below the keratin sheath (altered and mineralized by the fossilization process) lies an outer bone layer (OBN) of approximately 1–5 mm thickness, composed

Please cite this article in press as: Armitage MH, Anderson KL. Soft sheets of fibrillar bone from a fossil of the supraorbital horn of the dinosaur *Triceratops horridus*. Acta Histochemica (2013), http://dx.doi.org/10.1016/j.acthis.2013.01.001

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Figs. 1–7. (1) *Triceratops* supra-orbital horn specimen, in matrix prior to coating in methylmethacrylate, Hell Creek Formation, Glendive, MT. (2) *Triceratops* rib specimen, post-extraction, Hell Creek Formation MT. (3) *Triceratops* supra-orbital horn, 20 cm by 20 cm unfixed specimen. Note moisture on exterior surface and rootlets penetrating lower, interior surface. Scale bar = 2.5 cm. (4) Close up of bottom surface of horn from (3). Note porosity of vascular channels in horn bone. Scale bar = 1.8 cm. (5) Light micrograph, flap of fixed soft tissue (white arrow) slightly peeled away from undecalcified *Triceratops* bone specimen (black arrow), 10× magnification. Scale bar = 0.75 mm. (7) Light micrograph of decalcified portion of *Triceratops* horn bone, 20× magnification. Note white portions of non-fossilized, soft tissues which adhere to permineralized vessel elements (black arrows). Scale bar = 0.5 mm.

of compact Haversian bone extensively embedded with vascular tissues (Happ, 2010). Within the OBN is the horncore made up of highly vascularized trabecular bone. The trabecular horncore extends almost the entire length of the horn but terminates into a cornusal sinus at the base of the horn where it joins the skull. *Triceratops* fossils are considered common within the Hell Creek Formation (Happ, 2010).

Discovery of soft tissue in *Triceratops* horn provides additional insight into the nature of fossilization, and extends our understanding on the prevalence of preserved original dinosaur tissue.

No tissues or vessels floated away from bone in solution, yet solutions were not examined for the presence of very small, freefloating osteocytes or pieces of cortical bone matrix. Decalcification was only performed for 4 weeks, which was sufficient to expose soft tissues for collection but not long enough to completely free all possibly trapped organic material. Further study is required to determine if intact, soft vessels might be recovered from completely demineralized horn specimens.

Kaye et al. (2008) maintain that the soft tissue from dinosaur fossils is polysaccharide from a microbial biofilm. They propose that the polysaccharide film forms a cast of the tissue. Once dissolved from the fossilized bone matrix this film purportedly retains the shape of vessels and osteocytes. They conclude that what has been described as intact tissue is actually biofilm polysaccharide. Furthermore, Rasmussen et al. (2003) report that some microorganisms can form collagen-like proteins, which Kaye et al. (2008) suggest might be mistaken for dinosaur collagen.

What is not made clear by Kaye et al. (2008) is the mechanism by which microbes might replicate stellate and oblate osteocytes (Cadena and Schweitzer, 2012) in such well preserved and fine detail; including internal nucleus-like spheres, primary and secondary filipodia, and cell to cell junctions as reported here. If such a microbial replication mechanism were discovered it could eclipse the exquisite siliceous production and assembly systems (not yet completely understood), employed by diatom cells to create the compelling geometric valves known by microscopists the world over (Sumper and Bruner, 2006; Tesson and Hillebrand, 2010). What is also not clear is how such biofilm structures could themselves survive the ravages of time, as once produced other microorganisms could begin to digest even these. Bone material examined in this study was found non-desiccated, therefore microbial activity could have been supported within this specimen. The most parsimonious explanation is that these are original tissues, not highly reproducible organic ghost images of original tissues.

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Figs. 8–13. (8) Light micrograph of sheet of soft, fibrillar bone material from decalcified *Triceratops* horn, magnification 800×. Note three osteocytes with fine filopodial processes which interconnect (lower right). Scale bar = 50 μ m. (9) Light micrograph of sheet of soft, fibrillar bone material from decalcified *Triceratops* horn, magnification 1100×. Note intracellular organelle-like structures (arrows). Scale bar = 50 μ m. (10) Light micrograph of sheet of soft, fibrillar bone material from decalcified *Triceratops* horn, magnification 1100×. Note multiple intracellular organelle-like structures (arrows). Scale bar = 50 μ m. (10) Light micrograph of sheet of soft, fibrillar bone material from decalcified *Triceratops* horn, magnification 1100×. Note multiple intracellular organelle-like structures (arrows). Scale bar = 40 μ m. (11) Scanning electron micrograph (SEM) of compact fractured bone material from non-decalcified *Triceratops* rib, magnification 120×. Note well-defined circular Haversian system (osteon). Also note that the center of each Haversian system is populated by possible preserved blood products. Scale bar = 200 μ m. (12) SEM of compact fractured bone material from non-decalcified *Triceratops* rib, magnification 300×. Note well-defined circular Haversian system populated at the center with possible preserved blood products and red blood cell-like micro-structures. Scale bar = 80 μ m. (13) SEM of compact fractured bone material from un-decalcified *Triceratops* rib, magnification 350×. Note hollow blood vessel emanating from Haversian canal. Blood vessels are flexible and pliable. Scale bar = 80 μ m.

The fact that any soft tissues were present in this heavily fossilized horn specimen would suggest a selective fossilization process, or a sequestration of certain deep tissues as a result of the deep mineralization of the outer dinosaur bone as described by Schweitzer et al. (2007b). As described previously, however, the horn was not desiccated when recovered and actually had a muddy matrix deeply embedded within it, which became evident when the horn fractured. Additionally, in the selected pieces of this horn that were processed, soft tissues seemed to be restricted to narrow slivers or voids within the highly vascular bone, but further work is needed to fully characterize those portions of the horn that contained soft material. It is unclear why these narrow areas resisted permineralization and retained a soft and pliable nature. Nevertheless it is apparent that certain areas of the horn were only lightly impacted by the degradation that accompanied infiltration by matrix and microbial activity. If these elastic sheets of reddish brown soft tissues are biofilm remains, there is still no good explanation of how microorganisms could have replicated the fine structure of osteocyte filipodia and their internal microstructures resembling cellular organelles (Figs. 9 and 10). Filipodial processes

show no evidence of crystallization as do the fractured vessels in Fig. 16 and some filipodial processes taper elegantly to 500 nm widths (Fig. 19).

Furthermore, if biofilms represent the sole component of these soft tissues as suggested by Kaye et al. (2008), why are they only found within compact bone? Certainly there would seem to be sufficient nutrients in the matrix surrounding buried bones to support the production of additional biofilms. Moreover, why are the decaying roots that no longer support plant functions not covered over or completely replicated themselves by biofilms? Finally, biofilm production must have taken place within years or decades after burial in order to capitalize on nutrients available in their original form and to faithfully replicate ultrastructure before autolysis. It does not seem reasonable to suggest that the original tissues would not survive through deep time but replicated structures captured in biofilms would.

We feel it is significant that large, intact sheets of dinosaur fibrillar bone matrix seemed to be more densely populated (in the *z*-axis) with osteocytes than in previous studies of dinosaur material (Fig. 8). It was interesting that *Triceratops* soft tissues

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Figs. 14–19. (14) SEM of decalcified bone material from highly vascular portion of *Triceratops* horn, magnification $25 \times$. All the bone mineral has been dissolved away leaving intact permineralized vessels. Volkmann's canals link Haversian canal vessels together (white arrows). Scale bar = 1 mm. (15) SEM similar to (14). Magnification $30 \times$. Note possible blood products lining inner wall of hardened vessel (white arrow). Scale bar = $800 \,\mu$ m. (16) SEM enlarged image from (15). Magnification $200 \times$. Note crystallized nature of possible blood products lining inner wall of hardened vessel. Scale bar = $100 \,\mu$ m. (17) SEM of decalcified bone material from *Triceratops* horn, magnification $200 \times$. Note two large oblate osteocytes lying on fibrillar bone matrix. Scale bar = $10 \,\mu$ m. (18) SEM of decalcified bone material from *Triceratops* horn, magnification $600 \times$. Note four osteocytes lying on fibrillar bone matrix. The long white diagonal line is the edge of a thin layer of fibrillar soft tissue. Tiny white filipodial processes from cells beneath the layer can be seen extruding (especially in lower right corner of micrograph). Scale bar = $40 \,\mu$ m. (19) SEM of decalcified bone material from *Triceratops* horn, magnification $1000 \times$. Note three stellate osteocytes lying on fibrillar bone matrix. Cell filipodia are anchored into the bone matrix and have diameters approaching $500 \,$ nm (white arrows). Scale bar = $20 \,\mu$ m.

(particularly those containing multiple layers of osteocytes) did not require staining for light microscopy. Osteocytes normally display very fine structure including nucleus-like spheres and secondary branching of filipodia in freshly sectioned material. We observed these nucleus-like spheres and secondary branching of filopodia. Similarly, many transparent osteocytes and nucleus-like structures were observed in the T. rex material examined by Schweitzer et al. (2005, 2007b, 2013). We also observed transparent tissue structures. Initially it was thought that simply empty lacunae were being imaged, however SEM analysis verified the presence of oblate and stellate osteocytes (Cadena and Schweitzer, 2012) with very fine structural details (to within 500 nm). Some filipodial extensions in this study reached 18-20 µm in length, almost twice the length of those previously reported (Figs. 18 and 19), therefore our data are consistent with previous reports and further complicates a biofilm explanation.

Contrary to Kaye et al. (2008) who claimed that these structures are the remains of bacterial biofilms, the vessel-like structures emanating from many of the vascular canal walls were thick, fully cylindrical and had undulating wall surfaces consistent with extant vessel tissues (Figs. 13 and 16). Furthermore, the thin strips of soft material and the vessel-like structures we discovered extended deep into the bone, uncharacteristic of superficial biofilms.

Schweitzer et al. (2005, 2007a,b, 2009) and Asara et al. (2007) analyzed different dinosaur fossils and observed osteocytes with original transparency, extensive filipodia, and internal contents (such as a defined nuclei) within the osteocytes. This is inconsistent with the suggestion that the observed osteocytes are actually biofilm imprints mimicking the morphology of octeocytes. Such imprints would not have defined nuclei or other internal cell structures. In addition, antibodies for avian collagen I exhibited an affinity for collagen isolated from *T. rex* fossils, and this collagen

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was degraded by collagenase (Schweitzer et al., 2007a). Antibodies with an affinity for both avian and reptile proteins also had affinity for *B. canadensis* (Schweitzer et al., 2009). Bern et al. (2009) further analyzed the specimens used by Asara et al. (2007) and confirmed the presence of an avian-like collagen with no indication of microbial collagen-like proteins. Moreover, Schweitzer et al. (2013) have unequivocally detected affinities for avian collagen specific antibodies and nucleic acid specific antibodies in osteocytes recovered from decalcified dinosaur bones. Such affinity is indicative of the presence of avian collagen and nucleic acids in dinosaur tissue. Cumulatively, these characteristics are consistent with the presence intact tissue and inconsistent with the putative presence of microbial biofilm material. The most straightforward interpretation of the evidence is that intact cells and tissues have been preserved in this *Triceratops* fossil.

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