Cartilage, Bone and Intermandibular Connective Tissue in the Australian lungfish, *Neoceratodus forsteri* (Osteichthyes: Dipnoi)

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INTERMANDIBULAR ATTACHMENT TISSUE IN LUNGFISH

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ABSTRACT The connective tissue that links the bones of the mandible in the Australian lungfish, *Neoceratodus forsteri*, has been described as an intermandibular cartilage, and as such has been considered important for phylogenetic analyses among lower vertebrates. However, light and electron microscopy of developing lungfish jaws demonstrates that the intermandibular tissue, like the connective tissue that links the bones of the upper jaw, contains fibroblasts and numerous bundles of collagen fibrils, extending from the trabeculae of the bones supporting the tooth plates. It differs significantly in structure and in staining reactions from the cartilage and the bone found in this species. In common with the cladistian *Polypterus* and with actinopterygians and some amphibians, lungfish have no intermandibular cartilage. The connective tissue linking the mandibular bones has no phylogenetic significance for systematic grouping of lungfish, as it is present in a range of different groups among lower vertebrates.

KEY WORDS: intermandibular connective tissue in lungfish; cartilage; bone; phylogenetic implications

INTRODUCTION

In one of the earliest papers on the Australian lungfish, *Neoceratodus forsteri* (Krefft, 1870), Greil (1913) described a fibrous connective tissue that links the prearticular bones of the mandible in this species. A similar fibrous connective tissue holds the pterygopalatine bones and the vomers together. In the lower jaw the connective tissue is thick and wide, and more extensive than it is in the upper jaw. Subsequent authors have reconstructed the connective tissue as a bulky triangular intermandibular cartilage (Bartsch, 1994; Fox, 1965), and suggested phylogenetic implications for this structure.

This paper reports the results of histological and ultrastructural examination of the material linking the symphyses of the prearticular bones in lungfish, compares the structure with adjacent bone and cartilage, and shows that it is a connective tissue rich in fibroblasts and with numerous parallel bundles of collagen. It does not resemble the cartilage or the bone found in this species of lungfish.

MATERIALS AND METHODS

Specimens of *N. forsteri* were collected as eggs or embryos in the upper reaches of the Brisbane River in southeast Queensland, during the spawning season of 1998, and reared in the laboratory until they reached the stages required for analysis (Kemp, 1981). Morphological stages follow Kemp (1982, 1999). Material was collected and processed with the permission of the Queensland Fisheries Management Authority, permit number PRM03012K, and the University of Queensland Animal Ethics Committee, approval number CMM/013/03/ARC.

Jaw tissue from a series of ten *N. forsteri* hatchlings, covering developmental stages from 48 to 58 (Kemp, 1982, 1999), was prepared for transmission electron microscopy by fixing in cold Karnovsky's fixative for two hours (Karnovsky, 1965). Tissue was washed several times in 0.1M cacodylate buffer, immersed in 1% osmium tetroxide for one hour, cleaned in 0.1M cacodylate buffer, rinsed in several changes of distilled water, dehydrated in a graded series of alcohols, cleared in acetone and soaked overnight in equal parts of acetone and epon araldite resin. The tissue was transferred to fresh epon araldite resin for 16 hours, then embedded after one further change of resin. Blocks were allowed to set at 60 ^{O}C. Sections were cut with a diamond knife at 500 Å, and were mounted on coated copper grids before staining with uranyl acetate and lead citrate. Procedures used for light microscopy follow Kemp (1992, 1999, 2003).

RESULTS

Components of the jaws

The mandible of the living Australian lungfish, *N. forsteri*, consists of three bones and a single cartilaginous element, known as Meckel's cartilage (Fig. 1A). This forms from paired elements in young hatchlings (Kemp, 1999), which fuse early in development. The cartilage persists throughout life. Two parachondral bones, the angular and the splenial, cover the cartilage on the lateral surface. The angular bone supports the articulation of the mandible with the quadrate, and the splenial underlies the anterior process of Meckel's cartilage. A single bone, the prearticular, which carries the lower tooth plates, protects the cartilage on the medial aspect. This bone reinforces the articulation of Meckel's cartilage with the quadrate medially. Two long processes extend ventromedially from the prearticular bone and articulate in the

midline, linked by a bulky mass of connective tissue. The processes form a deep groove, which accommodates the tongue during suctorial feeding.

Intermandibular tissue.

Tissue between the prearticular bones includes large cells, surrounded by apparent lacunae within a fibrous matrix (Fig. 1A). Bundles of collagen fibres extend from the collagen matrix of the surrounding bone and are continuous with it, and bind the bone and intermandibular tissue to Meckel's cartilage. The fibres stain red in Sirius red, and pale blue in toluidine blue. They are contained within a ground substance that does not stain with Alcian blue. Tissue between the vomers and the pterygopalatine bones is identical in structure and in staining reactions to the tissue linking the mandibular bones (Fig. 1B), although it is less bulky.

In the transmission electron microscope, cells of the intermandibular tissue are highly irregular in shape, with little cytoplasm, and large elongate nuclei (Fig. 2A). The cytoplasm has mitochondria, rough endoplasmic reticulum, vesicles and ribosomes. Collagen spreads out from bone trabeculae around the connective tissue (Fig. 2B). Long cytoplasmic processes extend from the cells and make contact with a dense secretion of collagen fibres, many arranged in parallel bundles with single fibrils or small groups oriented in different directions, and interspersed with cytoplasmic processes (Fig. 2C). The collagen lies in an amorphous ground substance, containing a few fragments of calcified material.

Cartilage and bone

In the light microscope, the cartilage appears hyaline, and cells are enclosed in a diffuse matrix, apparently surrounded by lacunae (Fig. 1A, B). The cells may have a

regular arrangement in rows, or they may be scattered. Most cells are single, with an occasional pair of recently divided cells. The nucleus is large and cytoplasm minimal. The cartilage matrix stains faintly pink with Sirius red (Fig. 1A), suggesting the presence of collagen within the matrix, although fibrils are not evident in the light microscope. Cartilage matrix is metachromatic when stained with toluidine blue, and stains bright pink (Fig. 1 B). Alcian blue reacts strongly with the matrix (Kemp, 1999), suggesting a high content of mucopolysaccharide. In this species of lungfish, the Meckel's cartilage and the chondrocranium does not ossify.

Ultrastructural analysis of the tissue confirms the presence of cells with a large, rounded, granular nucleus, diffuse cytoplasm with numerous mitochondria, vesicles of several sizes and abundant caveoli (Fig. 3A, B), containing presecretory materials. The rough endoplasmic reticulum is highly structured but not particularly dense (Fig. 3A). The cytoplasmic membrane is convoluted, with short branching processes entering the extracellular matrix of the cartilage. There is little space between the cell membrane and the extracellular matrix (Fig. 3B, C). In most places they are in actual contact. The extracellular matrix includes numerous short, fine fibres of collagen, large, coarse, electron dense granules and diffuse matter consisting of fine granules (Fig. 3B, C).

Cells associated with developing lungfish bone may lie outside the bone, or may be enclosed in a large, clear lacuna within the mineralised tissue. They have a dark nucleus, with minimal cytoplasm. The bone trabeculae stain heavily with Sirius red, indicating the presence of a large amount of collagen (Fig. 1A). The bone trabeculae are homogeneous in appearance, and not obviously fibrous in the light microscope. The staining reaction of decalcified bone is pale blue with toluidine blue (orthochromatic). Trabeculae do not stain with alcian blue.

Transmission electron microscopy shows that osteocytes within the bone are enclosed in lacunae with few canaliculi (Fig. 4). The cells have little cytoplasm and a large nucleus. Mitochondria and rough endoplasmic reticulum are present, and there are occasional caveoli and cytoplasmic vesicles. The cells have a few short unbranched cytoplasmic processes entering the canaliculi. Unmineralised collagen fibrils lie within the lacuna.

DISCUSSION

The paired tooth bearing bones of lungfish jaws, that is, the vomers and pterygopalatine bones of the upper jaw, and the prearticular bones of the lower jaw, are linked by strong fibrous connective tissue. In the upper jaw the bones are held close together, and the fibres are short. In the mandible, the connective tissue is bulky, and the bones are separated in early stages by a solid triangular mass of tissue. Reconstructions of the developing lungfish head have suggested that the connective tissue joining the prearticular bones of the lungfish is actually cartilage (Fox, 1965; Bartsch, 1994) and the appearance of the tissue under certain conditions can certainly be misleading. In semi-thin sections stained by toluidine blue, which does not reveal the fibrous nature of the tissue, the connections between the bones look as if they are made of cartilage. The tissue however never stains with alcian blue, regarded as diagnostic of cartilaginous material (Kelly and Bryden, 1983). When histological sections are stained with Sirius red, collagen fibres are visible. This is confirmed in transmission electron micrographs of the tissue, where it is obviously different from both cartilage and bone in lungfish, as Greil (1913) pointed out many years ago.

Lungfish cartilage is unusual in that it appears to be hyaline cartilage, but it contains granular matter and short collagen fibrils, although otherwise the staining

reactions are as would be expected of cartilage. In the Australian lungfish, the cartilaginous skeleton persists unchanged in Meckel's cartilage, and in the chondrocranium, in juveniles and in adults. Perichondral bone forms around the ceratohyal, the exoccipital bones and the ribs. However, the centre of ossification in these structures is not part of the cartilage, but lies in the thick perichondral membrane around the cartilaginous element. Parachondral bones like the prearticular use the cartilage as a template when they develop (Kemp, 1999), but this does not represent calcification of cartilage.

Lungfish bone is based on an extracellular matrix of collagen, mineralised with calcium hydroxyapatite, with the staining reactions expected of a tissue with this content (Kemp, 1992). Bone in *N. forsteri* is cellular, and cells within the bone are often arranged in layers. Much of the dermal bone in a lungfish skeleton is dense and solid, with nothing resembling cortical bone or canals containing blood vessels. Occasionally a thick bone may include wide cavities containing cells, or be made up of a dense mass of strong trabeculae, as are found in the symphyses of the prearticular bones. In places, cells that secrete fresh bone surround the hard tissue, such as around the bone in the pulp cavity below the tooth plate.

The tissue that links the prearticular bones, and the connective tissue between the bones of the upper jaws, extends from bone trabeculae, and consists of masses of parallel bundles of collagen fibrils secreted by fibroblasts within the tissue. The collagen stains deep red with histological stains specific for collagen. The scanty ground substance around these cells and fibrils does not stain in a way that suggests a cartilaginous matrix of mucopolysaccharides, and the tissue cannot be described as a form of cartilage, despite its shape in the mandible. Nor does it ossify, even in older adult fish. As in Characidae (Bertmar, 1959), in cladistians (Clemen et al., 1998) and

in caecilians (Clemen and Opolka, 1990; Muller et al., 2005; Muller, 2006), it is a form of connective tissue, not a block of cartilage that ossifies and forms the dentition as reconstructed and described by Fox (1965). Bartsch (1994) describes the structure as an intermandibular cartilage with perichondral ossifications connecting to the spongy tissue extending from each prearticular bone, but this interpretation is also not correct. Bertmar (1966) does not consider the intermandibular tissue in *N. forsteri*. He illustrates the mandible in lateral view in the course of his work on the snout and olfactory organ of lungfish, but gives no details on the mandible.

The tissue linking the prearticular bones of *N. forsteri* has no phylogenetic implications. It is not a separate part of the primary cartilaginous endoskeleton, but a mass of a fibrous connective tissue developing to link the prearticular bones that support the lower jaw tooth plates. Equivalent tissue, but not so massive, is present between the vomers and the pterygopalatine bones of the upper jaws. The bulky mass linking the prearticular bones has no particular relationship to durophagy in lungfish because juvenile lungfish are not durophagous (Kemp 1977, 1995). They feed on invertebrates such as small worms. The bulk of the tissue is reduced as the fish grow older, and begin to eat hard food such as small clams and water snails. Similar arrangements of connective tissues between bones are found in several lower vertebrates from widely different groups. Phylogenetic considerations and comparisons of homologies are important to a complete understanding of relationships among early vertebrates, but they need to be based on factual information and not on misinterpretation.

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FIGURE LEGENDS

Figure 1. A. Frontal section (paraffin) through the mandible of N. forsteri, stage 58, stained with Sirius red. B. Transverse section (methacrylate) through the pterygopalatine tooth plate and attached bone, cartilage and connective tissue, stained with toluidine blue. Scale bars = 30 µm. l = lower tooth plate, im = intermandibular connective tissue, ip = connective tissue between pterygopalatine bones, mc = Meckel's cartilage, t = trabeculae of prearticular bone, tc = cartilage of trabeculae cranii, u = upper tooth plate.

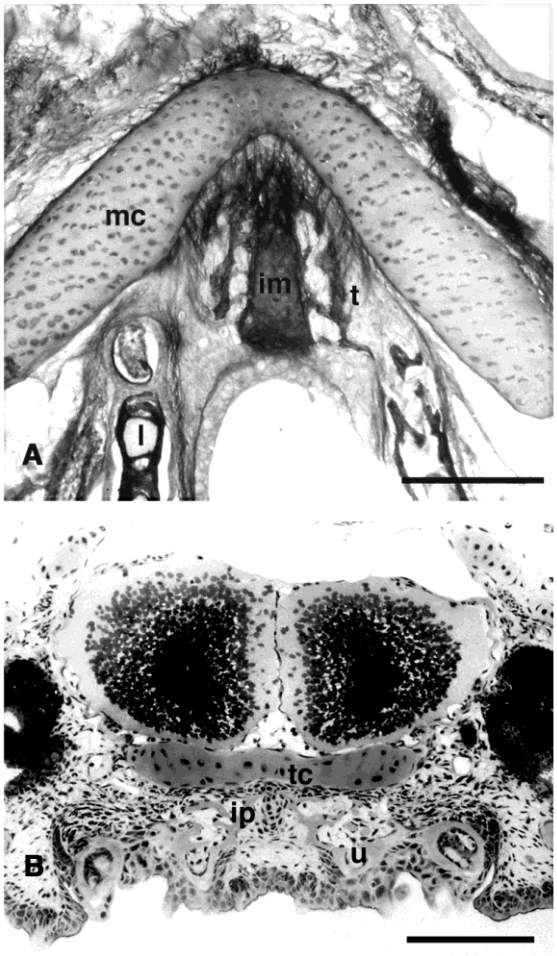

Figure 2. Transmission electron micrographs of the intermandibular connective tissue in the mandible of *N. forsteri*. A. Fibroblast and surrounding collagen fibres. Scale bar = 1 µm B. Trabeculae of the prearticular bone and collagen fibres. Scale bar = 2µm. C. Detail of the matrix of the connective tissue with collagen fibres and cell processes. Scale bar = 5µm. cf = collagen fibril, cp = cell process, f = fibroblast, t = trabeculae.

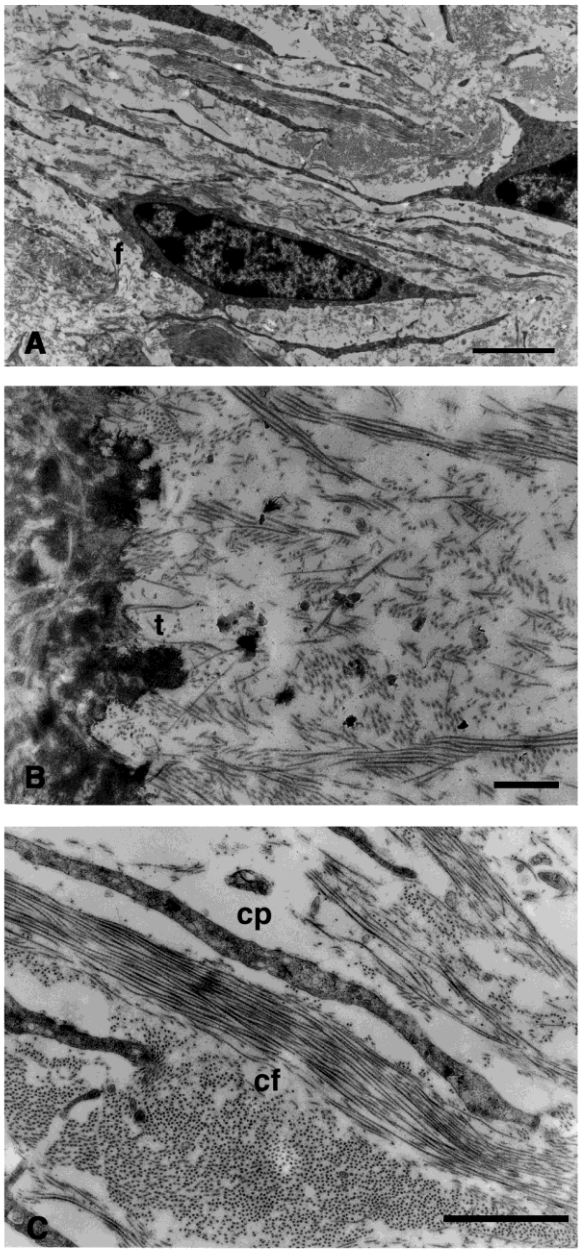

Figure 3. Transmission electron micrographs of Meckel's cartilage in *N. forsteri*. A. cell within cartilage matrix. Scale bar = 1 µm. B. Detail of contact between cell and matrix. Scale bar = 250 nm. C. fine collagen fibrils within matrix. cf = collagen fibrils. Scale bar = 125 nm.

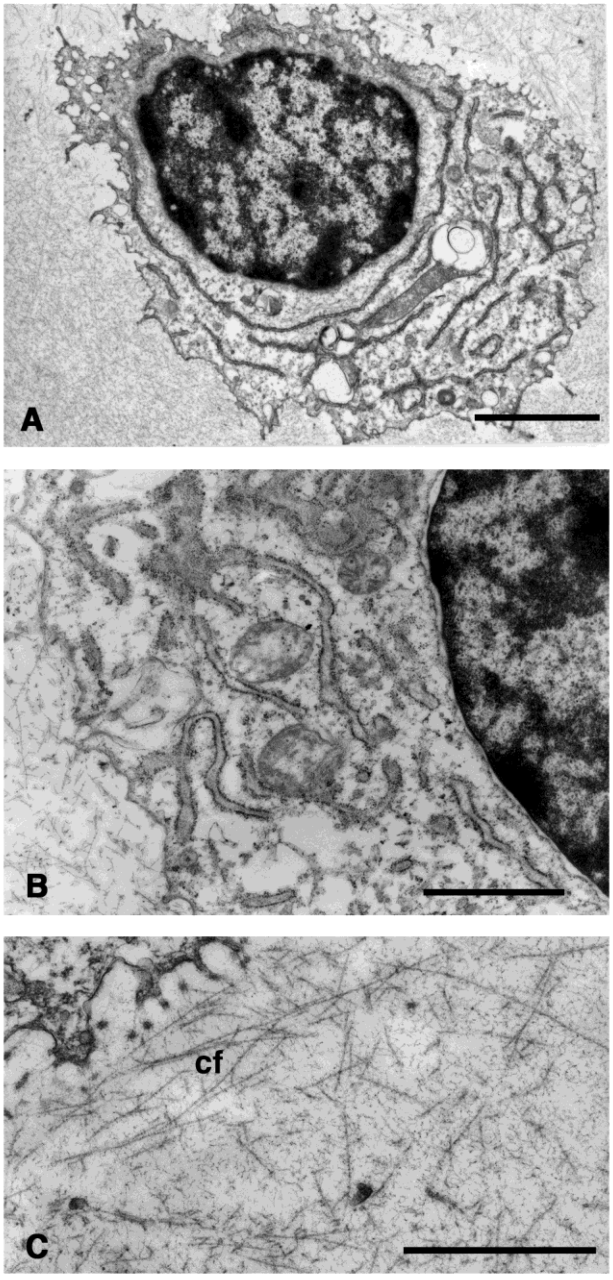

Figure 4. Transmission electron micrograph of the prearticular bone in *N. forsteri*. Osteocyte enclosed in bone. Scale bar = 1 µm.