The cytoskeleton of chondrocytes of *Sepia officinalis* (Mollusca, Cephalopoda): an immunocytochemical study

F. Leone, A. Bairati, L. Vitellaro-Zuccarello
Dipartimento di Scienze Biomolecolari e Biotecnologie, University of Milan, Italy

Our previous electron microscope study showed that chondrocytes from cephalopod cartilage possess a highly developed cytoskeleton and numerous cytoplasmic processes that ramify extensively through the tissue. We have now carried out a light microscope immunocytochemical study of chondrocytes from the orbital cartilage of *Sepia officinalis* to obtain indications as to the nature of the cytoskeletal components. We found clear positivity to antibodies against mammalian tubulin, vimentin, GFAP, and actin, but not keratin. The simultaneous presence of several cytoskeletal components is consistent with the hypothesis that cephalopod chondrocytes have the characteristics of both chondrocytes and osteocytes of vertebrates, which endow the tissue as a whole with some of the properties of vertebrate bone. We confirm, therefore, the presence in molluscs of the ubiquitous cytoskeletal proteins of metazoan cells that have remained highly conserved throughout phylogenetic evolution.

Key words: cytoskeleton molecules,chondrocytes, *Sepia officinalis*, immunocytochemistry.

Correspondence: Prof. Aurelio Bairati, Dip. Scienze Biomolecolari e Biotecnologie, via Celoria 26, 20133 Milan, Italy.
Phone: international +39.02.50314884.
Fax: international +39.02.50314881.
E-mail: aurelio.bairati@unimi.it

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A unique feature of the chondrocranial cartilage of cephalopods is the presence of chondrocytes possessing long ramifying cytoplasmic processes. These cells were described by Del Rio-Hortega (1918) and considered to be similar to neurons, glia and osteocytes of vertebrates. Furthermore, the metal staining techniques he used revealed the presence of filaments within the cytoplasm similar to those observed in vertebrate neurons. Rio-Hortega, in fact, concluded that these filaments were analogous to neurofibrils.

We described the ultrastructural morphology of these chondrocytes in a comprehensive study (Bairati et al., 1998) noting the presence of microtubules and filaments. While the microtubules and microfilaments had ultrastructures that enabled us to conclude confidently that they were composed of tubulin and actin respectively, the nature of the intermediate filaments was unclear from their morphology, although by analogy with vertebrate chondrocytes (Benjamin et al., 1994) we presumed that they contain vimentin.

We have now carried out a detailed light-microscope immunocytochemical study of the cytoskeleton of Sepia chondrocytes using antibodies against the typical cytoskeletal constituents of vertebrate cells (anti-tubulin, anti-vimentin, anti-GFAP, anti-keratin, and anti-actin). We also used tetramethylrhodamine isothiocyanate (TRITC)-labelled phalloidin to investigate filamentous actin. Our aim was to determine the extent of homology between the cytoskeletal components of *Sepia* chondrocytes and those in the taxonomically distant vertebrates, hoping to gain insights into the evolution of these macromolecules.

Materials and Methods

Tissue preparation

Seven adult specimens of *Sepia officinalis* (20 cm) were processed as soon as possible after catch-

ing. Small fragments (about 2 mm thick) dissected from the orbital cartilage were fixed at room temperature in either 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4 (PBS) for 6 h, or 2% glutaraldehyde plus 0.7% ammonium hexachlororuthenate (RHT) in 0.1 M phosphate buffer (PB), pH 7.2 for 2 h.

Some blocks were cryoprotected with increasing concentrations (10%, 15%, 20%) of sucrose solution and with 20% sucrose/5% glycerol in 0.1 M PB, pH 7.4 and embedded in Tissue-Tek OCT Compound (Miles Scientific, IL, USA). These blocks were then frozen by immersion in liquid nitrogen and stored at -80°C. Sections were cut at 10 µm on a cryostat (Leica) and collected on slides coated with 3-aminopropyltriethoxyethylsilane, air-dried and kept frozen pending use. Other cartilage blocks were dehydrated in acetone and embedded in paraffin.

Immunocytochemistry

Cryostat or paraffin-embedded dewaxed sections were treated sequentially with 0.05 M NH_4Cl in PB for 1 h, 0.3% H_2O_2 in methanol for 30 min, 10% bovine serum albumin (BSA) in PBS for 1 h, and incubated overnight at 4°C with primary antibodies. Microwave pretreatment (frequency 2.45 GHz, 750 W) was applied to improve tubulin immunoreactivity in paraffin sections.

The antibodies used were mouse monoclonal antibodies against: chicken gizzard actin (1:100, Amersham, UK), pig vimentin (1:50), human glial fibrillary acid protein (1:200, GFAP, Boehringer, Mannheim, 1:200), human pan cytokeratin (1:200); and rabbit polyclonal antibodies against: human vimentin (1:40), human GFAP (1:150), chicken tubulin (1:50). Unless otherwise indicated, antibodies were from Sigma.

Antigen-antibody reactions were visualized using the avidin-biotin-peroxidase complex (ABC) technique (Hsu et al., 1981). This involved sequential incubation in biotinylated secondary antibodies (1:200, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature, followed by avidin-biotin complex (Vectastain ABC kit, Vector Laboratories) for 75 min. The reaction was visualized with diaminobenzidine tetrahydrochloride (DAB, 0.075% in 0.05 M TRIS-HCl buffer, pH 7.3, with addition of 0.02% H_2O_2) as a chromogen.

For immunofluorescent revelation, methanol-H_2O_2 treatment was omitted, and the antigen-antibody reaction was revealed using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:50, Sigma). Sections were mounted in PBS-glycerol 1:3 and examined under a Leica DMR fluorescence microscope equipped with the following combinations of filters: BP 450-490, FT 510,LP 520 for FITC; BP 546,FT 580, LP 590 for TRICT.

Fluorescence microscopy of F-actin

Cryostat sections of cartilage segments fixed in 4% paraformaldehyde were stained with TRITC-labelled phalloidin (5 U/mL in PBS, Sigma) for 10 min at 4°C. Excess TRITC-phalloidin was removed by several washes with PBS and sections were mounted in PBS-glycerol.

Controls

In control sections, incubation with primary antibody was omitted. The specificity of the antibodies was tested on appropriate vertebrate tissue: anti-actin on chicken skeletal muscle; anti-pan cytokeratin and anti-vimentin on human skin; and anti-tubulin and anti-GFAP on rat cerebellar cortex.

Results

Chondrocyte staining was highly variable and sometimes extremely weak, so various preparation methods were tried in order to demonstrate the location of a given antigen.

TRITC-phalloidin and anti-actin

After treatment with TRITC-phalloidin (Figure 1), both globular chondrocytes of the inner zone, and finely elongated cells of the outer zone of the orbital cartilage showed fluorescence located mainly at the periphery of the cell body and the origins of the cytoplasmic processes (Figure 1A). Furthermore, numerous small fluorescent spots were observed along cell surfaces (Figure 1A, 1B). Staining with monoclonal antibody against chicken actin produced more intense and extensive staining of chondrocyte cell bodies, and was also present to variable extents in the cytoplasmic processes (Figure 1C).

Anti-vimentin antibodies

The staining reaction of chondrocytes to anti-vimentin antibodies varied in intensity with the type of antibody (anti-pig or anti-human vimentin) and the sample preparation method (Figure 2A-D). In

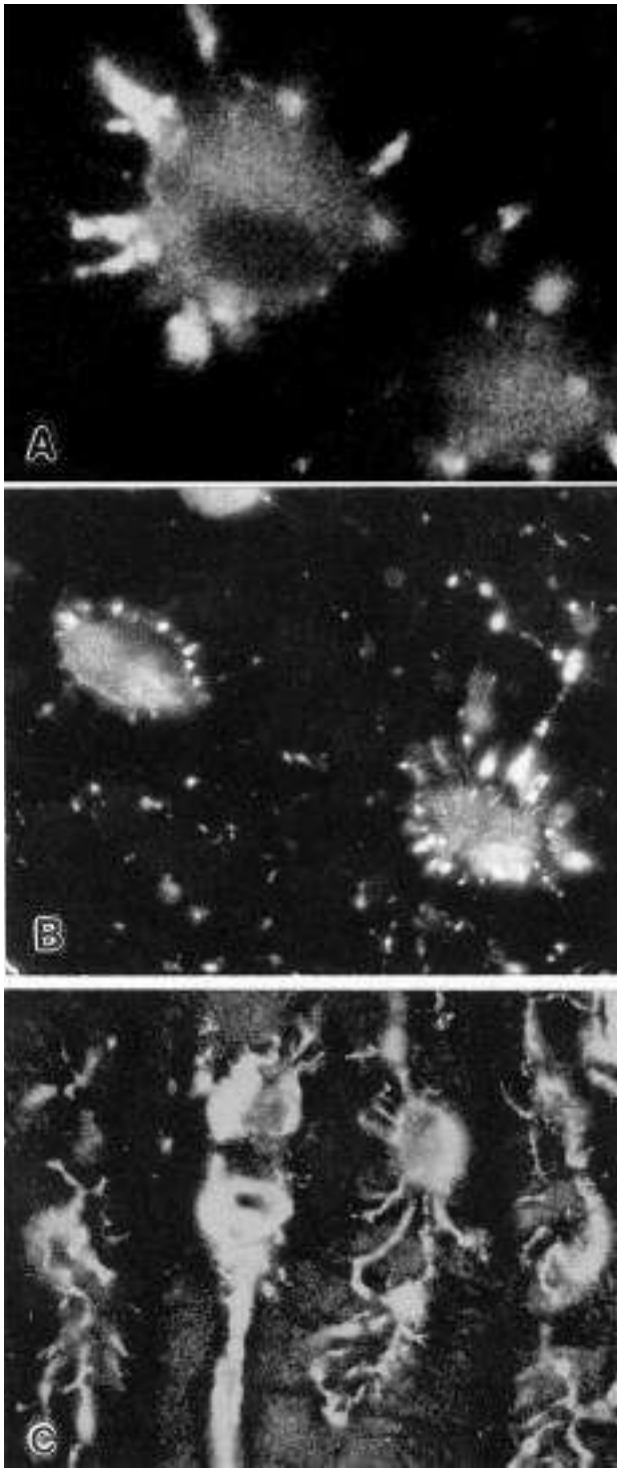

Figure 1. Cryosections of Sepia orbital cartilage fixed in paraformaldehyde. A (x 300), B (x 200): staining with TRITC-phalloidin viewed under fluorescent microscope. C: indirect immunostaining with antibody against chicken actin (x 200).

paraformaldehyde-fixed cryostat sections treated with monoclonal antibodies against pig vimentin, the initial tracts of the largest cytoplasmic process, as well as the cell bodies, were intensely stained; within the cell bodies areas of reticular staining were visible (Figure 2 A). Use of polyclonal anti-human vimentin on glutaraldehyde-fixed cryosections produced weak background staining in chondrocytes against which short tracts around the nucleus and at the cell periphery stood out as more intensely stained (Figure 2B). The flattened chondrocytes of the outer zone of the cartilage were more uniformly and intensely stained (Figure 2C). Use of polyclonal anti-human vimentin on glutaraldehyde-fixed, paraffin-embedded sections produced intense staining both in cell bodies and cytoplasmic processes; staining was particularly intense in samples fixed with glutaraldehyde/RHT (Figure 2D).

Anti-GFAP antibodies

Monoclonal antibody against human GFAP on paraformaldehyde-fixed cryostat sections did not produce any reaction with DAB. Polyclonal anti-GFAP antibody produced a weak granular (dusty) positivity in chondrocyte cell bodies.

On glutaraldehyde fixed cryosections, anti-GFAP polyclonal antibodies intensely stained elongated tracts at the cell body periphery (Figure 3A), but did not stain cytoplasmic processes.

Much more intense and extensive staining was obtained in cell bodies and cytoplasmic processes on glutaraldehyde/RHT-fixed cryosections, particularly after intensification with the ABC method (Figure 3B). On paraffin-embedded samples fixed with glutaraldehyde/RHT, staining was even more intense, with areas of reticulated positivity both in cell bodies and cytoplasmic processes (Figure 3C).

Anti-pan cytokeratin antibodies

Anti-pan cytokeratin antibodies – which reacted with mammalian epidermis – did not produce any reaction on any Sepia sample.

Anti-tubulin antibodies

Polyclonal antibodies against chicken tubulin produced variable staining reactions in relation to the sample preparation method.

On glutaraldehyde/RHT fixed cryostat sections treated with polyclonal antibody, staining picked out elongated tracts and dusty or granular areas at the cell body periphery; cytoplasmic processes were weakly stained (Figure 4A). Staining was more intense and extensive in paraffin-embedded sections, in both cell bodies and cytoplasmic processes

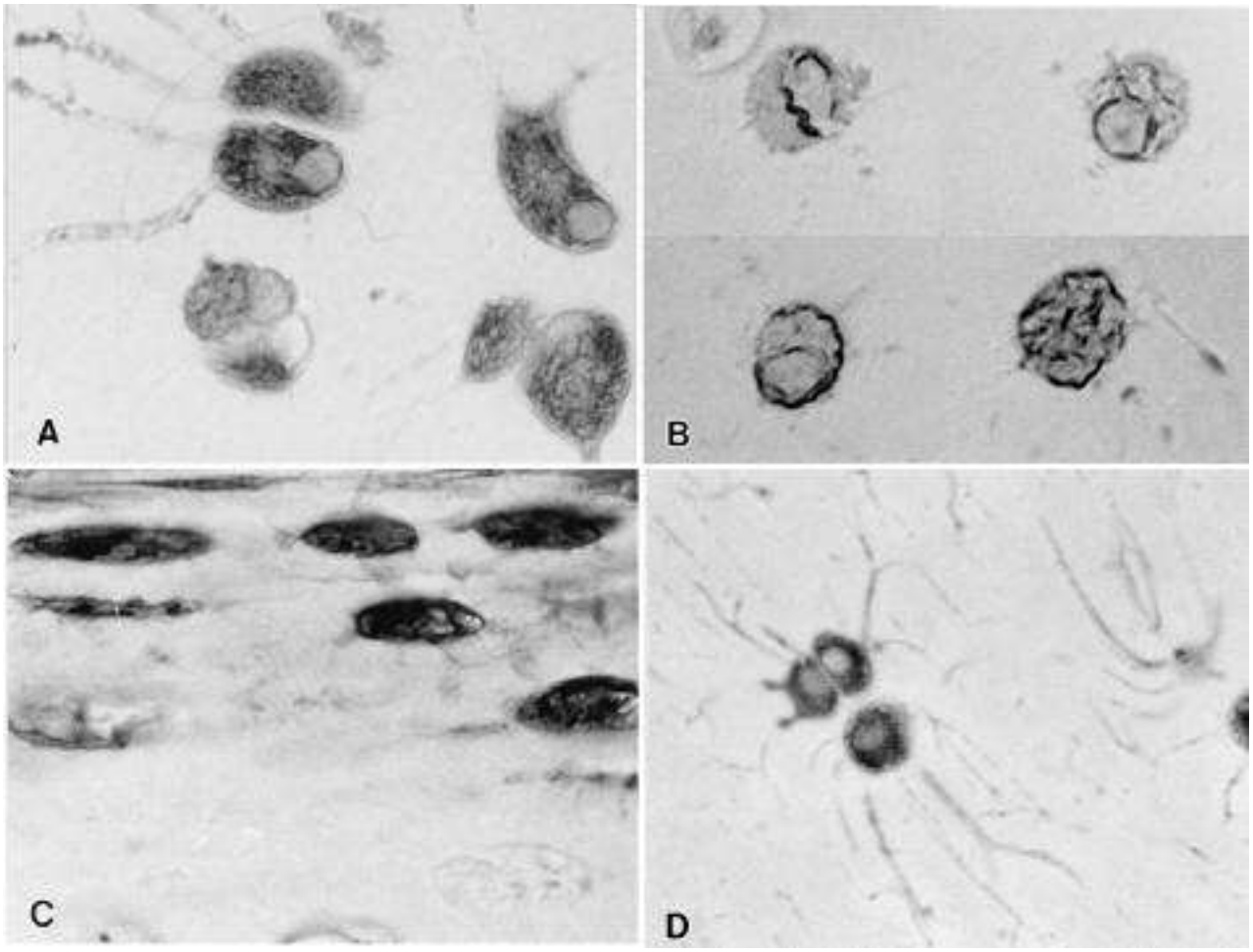

Figure 2. Sections of *Sepia* orbital cartilage treated with anti-vimentin antibodies. A: paraformaldehyde-fixed cryosections reacted with monoclonal antibody against pig vimentin (x 750). B: glutaraldehyde-fixed cryosections reacted with polyclonal antibody against human vimentin (x 750). C: chondrocytes from outer zone cartilage treated as in B (x 630). D: paraffin-embedded section fixed in glutaraldehyde RHT and treated with polyclonal antibody against human vimentin (x 550).

(Figure 4B), particularly after microwave treatment (Figure 4C).

Controls

No positivity was ever observed, either with DAB or fluorescent staining, on any of the control sections not treated with primary antibody. All antibodies were checked for specificity by reaction with the specific tissues containing the antigens used to raise the antibodies. In all cases, the expected specific cellular positivity was observed.

Discussion

General aspects

Our use of a variety of fixation and preparation methods allowed us to observe two main morphological patterns of immunoreactivity: a) tracts and filaments, the latter sometimes forming a network, and (b) extensive areas of uniform finely granular (*dusty*) staining. The morphology of the filamentous immunoreactivity corresponded closely to the filamentous entities observed in the light microscope by silver salt staining (Del Rio-Hortega, 1918), and which, in the electron microscope, are revealed as intermediate filaments, microtubules, and microfilaments (Bairati et al., 1998). Aldehydes, particularly glutaraldehyde with RHT, proved the best fixatives in that they allowed good immunostaining and at the same time conserved cell body morphology and cytoplasmic processes without shrinkage and detachment of the cells from the extracellular matrix – as occur with traditional histological methods (Del Rio-Hortega, 1918). Use of RHT is reported to have similar stabilising effects on GAGs and PGs of the pericellular extracellular matrix (Hunziker et al.,

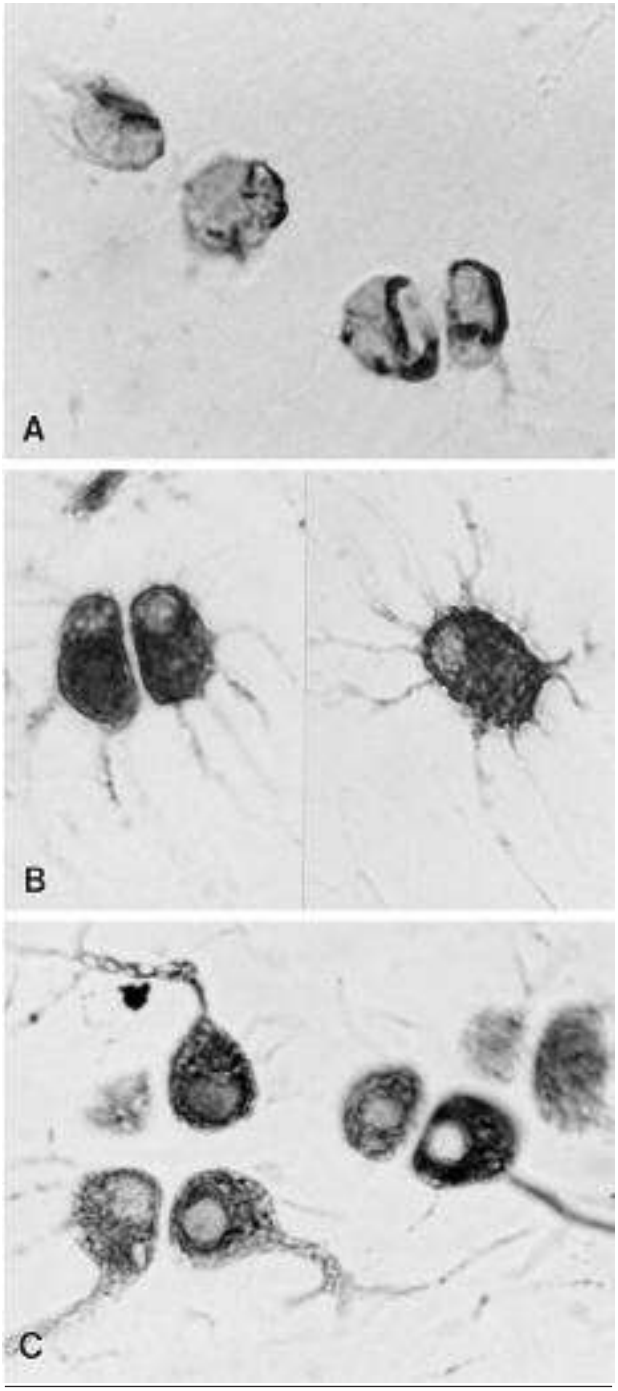

Figure 3. Sections of *Sepia* orbital cartilage treated with anti-GFAP antibodies. A: glutaraldehyde-fixed cryosections and polyclonal antibody against human GFAP (x750). B: glutaraldehyde/RHT-fixed cryosection reacted with polyclonal antibody against human GFAP (x750). C: paraffin-embedded section fixed and stained as in B (x750).

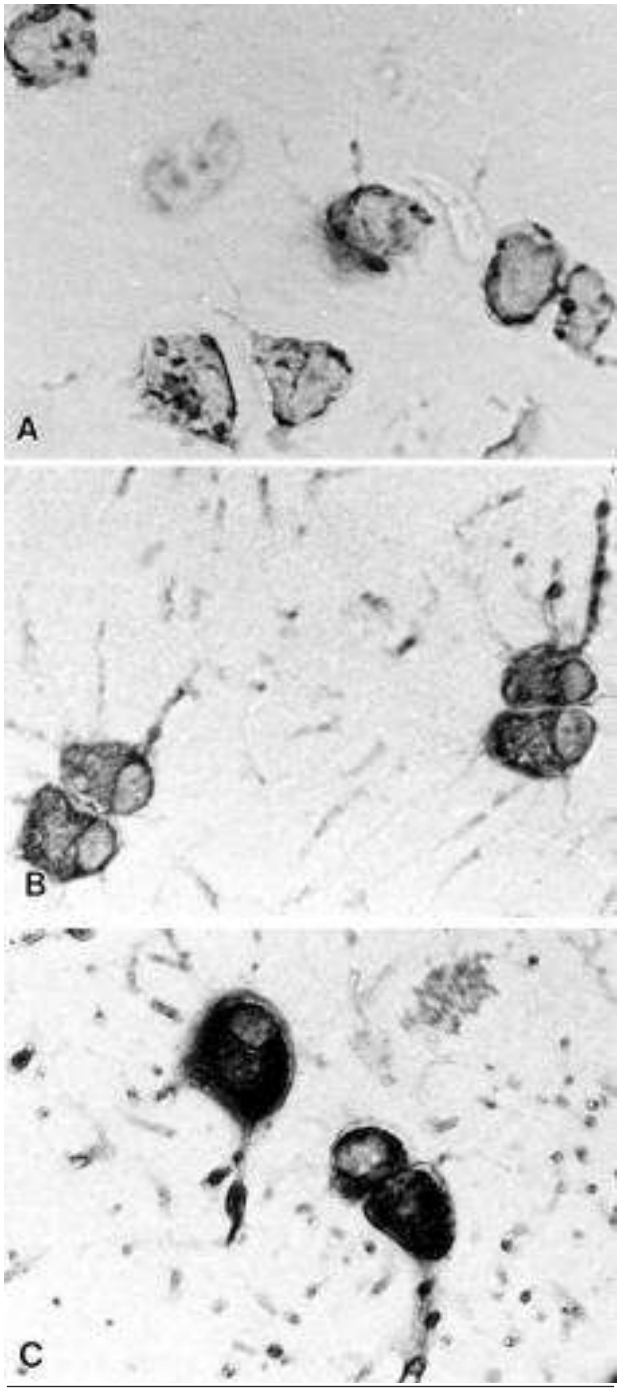

Figure 4.Sections of *Sepia* orbital cartilage treated with polyclonal anti-tubulin antibodies. A: glutaraldehyde/RHT-fixed cryosection (x 750). B: paraffin embedded section fixed as in A(x 750). C: microwave-treated paraffin section of sample fixed as in A (x 750).*

1982). Glutaraldehyde is known to stabilize microtubules by inhibiting the depolymerisation that occurs during freezing (Osborne and Weber, 1982).

The technique of paraffin embedding produced more intense and diffuse staining than cryosectioning; however, the cells typically showed coarse structures indicating artefactual aggregation of cytoplasmic components. Like Shi et al. (1991), we found that prior microwave exposure intensified immune reactivity.

Actin microfilaments

Areas positive to TRITC-phalloidin were often present at the periphery of the cell body and at the origin of the cytoplasmic processes; such areas correspond to those in electron microscope preparations (Bairati et al., 1998) where irregular aggregations of microfilaments are present and where various cytoskeletal structures converge. We propose that the small fluorescent spots observed along the surface of the cells represent sites of focal adhesion between the cell and the extracellular matrix (Bairati et al. 1998, Fig. 5). At such sites, actin microfilaments interact with various actin-related proteins (Burridge et al.,1988) to form the cytoplasmic *dense bodies* visible also in the light microscope.

We found positivity to antibodies against chicken actin within cytoplasmic processes, indicating the extensive presence of microfilaments within these processes. In this respect, Sepia chondrocytes are similar to vertebrate osteocytes: actin microfilaments have been demonstrated in osteocyte cytoplasmic processes using the confocal microscope with phalloidin (Jones and Taylor, 1990) and by immunostaining (Tanaka-Kamioka et al., 1998). Actins, widespread in invertebrate cells (Goodson and Hawse, 2002), and now demonstrated immunochemically in cephalopods, constitute one of the oldest protein families of eukaryotes and one of the most highly conserved during phylogenetic evolution.

Intermediate filaments: vimentin and GFAP

Our data clearly show that vimentin- and GFAP-like proteins are expressed in Sepia chondrocytes. We propose that the uniform positivity within cell bodies represents pools of these molecules in the cytosol; while the tracts, filaments and reticular structures indicate the presence of filaments in various stages of assembly. The filaments and reticular structures picked out by these antibodies correspond well to the forms observed by Del Rio-Hortega (1918) in the light microscope, using silver staining, and those we have described in the electron microscope of the same cells (Bairati et al., 1998). The larger tracts of immunopositivity observed in the present study seem to correspond to the larger and denser bundles we observed in the electron microscope. Our electron microscope study also showed that, within the cytoplasmic processes, intermediate filaments were present as individual elements interspersed between bundles of microtubules: and these correspond well to the immunopositivity for vimentin and GFAP in cytoplasmic processes most convincingly revealed in paraffin-embedded sections using intensification.

This study therefore provides additional evidence that the highly conserved proteins that form intermediate filaments are present in cephalopods, as previously shown by the electron microscope and immunocytochemical studies of Bartnik and Weber (1989) and the molecular cloning study of Erber et al. (1998). In vertebrates, vimentin is considered to be expressed in mesenchyme-derived cells, while GFAP is expressed almost exclusively in glia. Nevertheless, GFAP has also been described in mesenchyme cells (Kasantikul and Shuangshoti, 1989; Kasper and Stosiek, 1990), mainly in certain disease conditions (Viale and al., 1988), but also in cartilage cells (Benjamin et al., 1994) where it is expressed together with vimentin and cytokeratin.

It is possible, however, that both vimentin and GFAP filaments are not present in these cells as individual elements, but only a single type of molecule exists whose conserved epitopes are recognized by both vimentin and GFAP polyclonal antibodies. In fact, Weber et al. (1991) have proposed that these proteins are derived from a single ancestral protein closely similar to nuclear lamins. In our opinion, it is likely that a molecule closely homologous to vertebrate GFAP is present in molluscs, although this requires confirmation by sequencing. It would be interesting from the evolutionary point of view to determine whether heterotypic intermediate filaments are present in molluscs, just as they are in vertebrates (Herrmann and Aebi, 1999).

Microtubules and tubulin

The immunocytochemical findings of the present study provide further evidence for a close homology between cephalopod and vertebrate tubulins. Like actin, tubulins are ubiquitous components of eukaryotic cells whose structure has been highly conserved during evolution. However, cartilage cells do not usually contain the abundance of microtubules observed in Sepia chondrocytes, and it seems evident that this abundance is related to the ability of these cells to form extensive ramifications of cytoplasmic processes. In fact, we suggest that tubulin expression may be the principal differentiation characteristic of cephalopod chondrocytes which enables them to perform their characteristic

structural, dynamic and metabolic functions in cartilage (Bairati et al., 1998).

Comparative aspects

Our previous light microscope (Bairati et al., 1987) and ultrastructural studies (Bairati et al., 1998) indicated that cephalopod chondrocytes possess structural characteristics typical of both the chondrocytes and osteocytes of vertebrates. The chondrocytes of vertebrates express vimentin, cytokeratin and GFAP as main cytoskeletal components (Benjamin et al., 1994), while actin and tubulin are also present in the cytosol. Vertebrate osteocytes not only express vimentin (Shapiro et al., 1995) but also actin concentrated in the cytoplasmic processes (Jones and Taylor, 1990; Aarden et al., 1994). The present immunocytochemical study provides evidence that tubulin, vimentin, GFAP and actin are present in cephalopod chondrocytes; however, microtubules were perticularly abundant in relation to the extensive development of cytoplasmic processes that caharacterise these cells and permit them to form a cartilagenous tissue in many respects similar to the bone of vertebrates (Bairati et al., 1987) although it lacks mineralisation.

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