Title: Differential Distribution of Microtubules in Immature Osteocytes *in vivo*

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Abstract

Objectives: The transition from osteoblasts to osteocytes is associated with dramatic changes in the cytoskeleton. We previously showed that the formation of osteoblast cell processes in 3D culture is microtubule dependent. However, the distribution of microtubules during the transition from osteoblasts to osteocytes *in vivo* is unknown. In this study, we investigated the distribution of microtubules in osteocytes *in vivo*.

Methods: We observed the microtubules in osteocytes in chick embryonic calvaria via fluorescence staining of microtubules and confocal laser scanning microscopy.

Results: Microtubules were observed throughout the cytoplasm in all examined osteoblasts. In immature osteocytes, several cell processes contained microtubules, whereas in mature osteocytes, microtubules were localized only in the cell body.

Conclusion: These results suggest that the early arrangement of microtubules may be correlated with the initial development of osteocyte processes.

Keywords: osteocyte, osteoblast, microtubule, differential distribution, *in vivo*

1. Introduction

Osteocytes are mature osteoblasts that become embedded within the lacuno-canalicular network of bone during the formation phase of bone remodeling [1-4]. During this process, osteocytes develop cytoplasmic processes that run throughout the canaliculi, forming a communication network that can convert mechanical signals into biochemical signals [5-13]. Therefore, osteocytes are considered to be the major mechanosensory cells in bone tissue that control bone remodeling.

Microtubules are hollow, nanoscale, biopolymer rods that, together with actin filaments and intermediate filaments, form the composite cytoskeleton, which controls cell shape and mechanics [14,18]. Recently, microtubules have been linked to the mechanoresponsive of cultured osteocytes to fluid shear stress [19]. The microtubule network has also been implicated in the regulation of Ca^{2+} signaling and sclerostin production in osteocytes [19] and has been proposed as a target for manipulating the osteocyte response to mechanical cues for therapeutic interventions in bone [19]. Microtubule involvement in the assembly of adhesions has also been well documented [20,21]. In addition, microtubules are used as guides for the localization of cellular structures and act as a highway for the trafficking of organelles [22,23]. Furthermore, microtubules are required for the initial formation of podosomes [24]. We also showed that the formation and integrity of osteoblast cell processes in 3D culture is microtubule dependent [25].

The transition from osteoblasts to osteocytes is associated with dramatic changes in the cytoskeleton [26,27]. Microtubules are present in osteocyte cell bodies and processes *in vitro*, but their distribution in the processes only extends to the proximal regions [27]. However, the differential distribution of microtubules during the transition to osteocytes and where they reside *in vivo* is unknown. We previously developed a method for observing vinculin in osteoblasts and osteocytes in chick calvaria by confocal laser scanning (CLS) and differential interference contrast (DIC) microscopy of cells labeled with a

fluorescent dye [28]. In this study, we used this technique to visualize the differential distribution of microtubules in osteocytes *in vivo*.

2. Materials and methods

2.1. Preparation of bone fragments

Calvaria were obtained from 16-day-old embryonic chickens and washed with PHEM (60 mM piperazine-*N,N′*-bis[2-ethanesulfonic acid], 25 mM *N*-[2-hydroxyethyl] piperazine-*N′*-[2-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid, and 2 mM magnesium chloride; pH 6.9) to remove nonadherent cells. After stripping the periosteum, the calvaria were cut into 3 mm × 3 mm pieces for further use.

2.2. Fluorescence staining of actin filaments and microtubules in chick calvaria

The localization of microtubules in chick calvaria was determined by immunostaining using a mouse monoclonal antibody specific for alpha-tubulin (Molecular Probes, Eugene, OR). Actin staining with fluorescently labeled Alexa488-phalloidin (Molecular Probes) was used to delineate the cellular outlines of the osteoblasts and osteocytes. DIC images were used to visualize the outlines of the bone surface, lacunar walls, and canalicular walls. After the 16-day-old chick calvaria were cut into 3 mm × 3 mm pieces, they were rinsed with PHEM and then fixed with 3% paraformaldehyde and 0.05% glutaraldehyde in PHEM for 10 min. The fragments were then washed, stained with an anti-alpha tubulin monoclonal antibody (a 1:200 dilution in PBS containing 1% bovine serum albumin [BSA]) for 24 h at 4 °C and then washed with PBS. After incubation with an Alexa594-conjugated secondary antibody (excitation wavelength, 595 nm; emission wavelength, 615 nm; Molecular Probes) against mouse IgG in PBS containing 1% BSA, the fragments were again washed and incubated overnight with Alexa488-phalloidin (1:200 dilution; excitation wavelength, 495 nm; emission wavelength, 519 nm; Molecular Probes) in PBS containing 1% BSA. After washing with PBS, samples were embedded in fluorescence mounting medium (Dako, Carpentaria, CA) containing 1 mg/ml p-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and then immediately viewed. The bone cells in the chick calvaria as well as the localization of microtubules were visualized with a FLUOVIEW FV500 CLS microscopy system (Olympus, Tokyo,

Japan), with 0.5 µm optical slices of the 60 µm-thick specimen. Images were digitally processed with Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

3. Results

The osteoblast layer **(Fig. 1; a)** and lacunar walls **(Fig. 1; e and i)** were observed by DIC microscopy, and the distribution of microtubules in osteoblasts and osteocytes *in vivo* was examined. In the osteoblasts, microtubules radiated from the perinuclear space **(Fig. 1; b)**, filling the entire cell body, while staining of the actin filaments showed the outline of cells **(Fig. 1; c and d)**. In immature osteocytes, some of cell processes contained microtubules **(Fig. 1; f)**, and the microtubules co-localized with actin along the entire length of the processes **(Fig. 1; g and h)**. However, in mature osteocytes, microtubules were localized only in the cell body **(Fig. 1; j)** and did not co-localize with actin in the processes **(Fig. 1; k and l)**.

4. Discussion

The transition from osteoblasts to osteocytes is accompanied by dramatic changes in the distribution of cytoskeletal components [26,27]. We previously developed a method for observing vinculin in osteoblasts and osteocytes in chick calvaria in fluorescently labeled cells by both CLS and DIC microscopy [28]. In this study, we used this technique to visualize the differential distribution of microtubules in chick calvaria.

In this study, we observed the distribution of microtubules *in vivo.* Our results demonstrate that, during the transition from osteoblasts to osteocytes, the distribution of microtubules undergoes three distinct changes. In osteoblasts, microtubules radiate from the perinuclear space to fill the entire cell body. In immature osteocytes, several cell processes contain microtubules along their entire length. Finally, in mature osteocytes, microtubules are only localized in the cell body and not in the processes. These changes were compared to the distribution of microtubules in osteoblasts and osteocytes observed *in vitro* **(Fig. 2),** which are presented in a schematic in **Fig. 3**. In osteoblasts, microtubules radiated from the perinuclear space to fill the entire cell body. In osteocytes, microtubules were only localized to the cell body and did not co-localize with actin in the processes. *In vivo*, microtubules were only present in the cell processes of immature osteocytes, when the processes initially formed, at a time when the microtubules may transport actin subunits and other molecules [29] to the cell processes in the initial formation of these processes. Once the processes formed, the microtubules were pushed back to the base of the process. At this time point, actin is a major component of the stable osteocyte process. In isolated osteocytes, there were well-formed, relaxed, stable processes covering the cell body, and these processes could only reform after seeding on a glass support [30]. During the initial step of process formation, the microtubules may transport actin and other molecules to the processes from within the cell body; however, these processes are likely to be unstable in culture. The results of this study suggest a correlation between the early arrangement of microtubules and the initial development of osteocyte

processes. Although the evidence presented in this study supporting the existence of interactions between microtubules and the initial development of osteocyte processes is limited, it is sufficient to show that microtubules exist in some processes of immature osteocytes.

In summary, this study showed for the first time the distribution of microtubules during the transition from osteoblasts to osteocytes, which may be related to changes in cell shape and function. The establishment of osteocyte processes may depend, in part, on the function of microtubules. Studying the cytoskeleton of bone cells will facilitate a greater understanding of the mechanism of mechanotransduction within bone cells and the physiological regulation of bone remodeling.

Ethical approval

All animal experiments were approved by the Institutional Ethics Review Board for animal and human experiments and were conducted in accordance with the guidelines for animal care.

Acknowledgements

This study was partially supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan as well as by Research Fellowships for Young Scientists from the Japan Society for the Promotion of Science.

Conflict of interest

The authors declare that they do not have any potential conflicts of interest.

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Figure legends

Figure (1) DIC and fluorescence images of osteoblasts, immature osteocytes, and mature osteocytes in chicken calvaria. Dual fluorescence staining with anti-alpha-tubulin (b, f, j) and Alexa448-conjugated phalloidin (c, g, k) as well as merged images (d, h, l). The anti-alpha-tubulin staining shows microtubules filling the entire cell body of the osteoblasts (compare b and c). In immature osteocytes, several cell processes contained microtubules (Arrows in f) and actin (compare f and g). In mature osteocytes, microtubules were localized only in the cell body, while the cell processes contained actin (compare j and k). The large inset in (d) is a merged image of the small inset in (d). The large inset in (h) is a merged image of the small inset in (h). The large inset in (l) is a merged imaged of the small inset in (l). Bars, 10 µm.

Figure (2) Images of osteoblasts and osteocytes cultured *in vitro*. Dual fluorescence staining with Alexa488-conjugated phalloidin (a, d) and anti-alpha-tubulin (b, e), and merged images showing dual fluorescence staining (c, f). Anti-alpha-tubulin staining shows that in osteoblasts, the microtubules filled the entire cell body (compare a and b). In osteocytes, the microtubules were localized only in the cell body, while the cell processes contained actin (compare d and e); Bars, 10 µm.

Figure (3) Schematic diagram showing two possibilities for the eventual fate of the microtubules observed in some of the processes in immature osteocytes (b) during the differentiation from osteoblasts (a) to mature osteocytes (c, d) in chicken calvaria. In osteoblasts, microtubules filled the entire cell body. In immature osteocytes, several cell processes contained microtubules. In mature osteocytes, microtubules were localized only in the cell body, which may have been due to disappearance of processes that contained microtubules (c) or the disappearance of microtubules from the processes (d).

Figures

Figure (1)

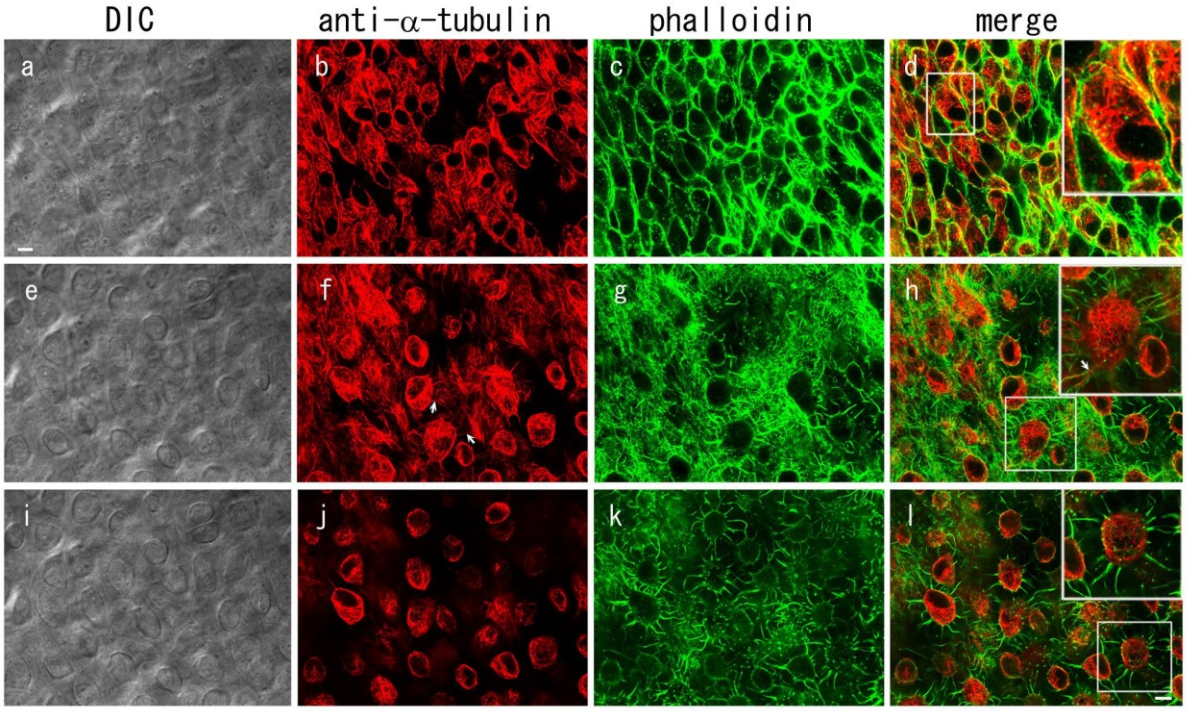

Figure (2)

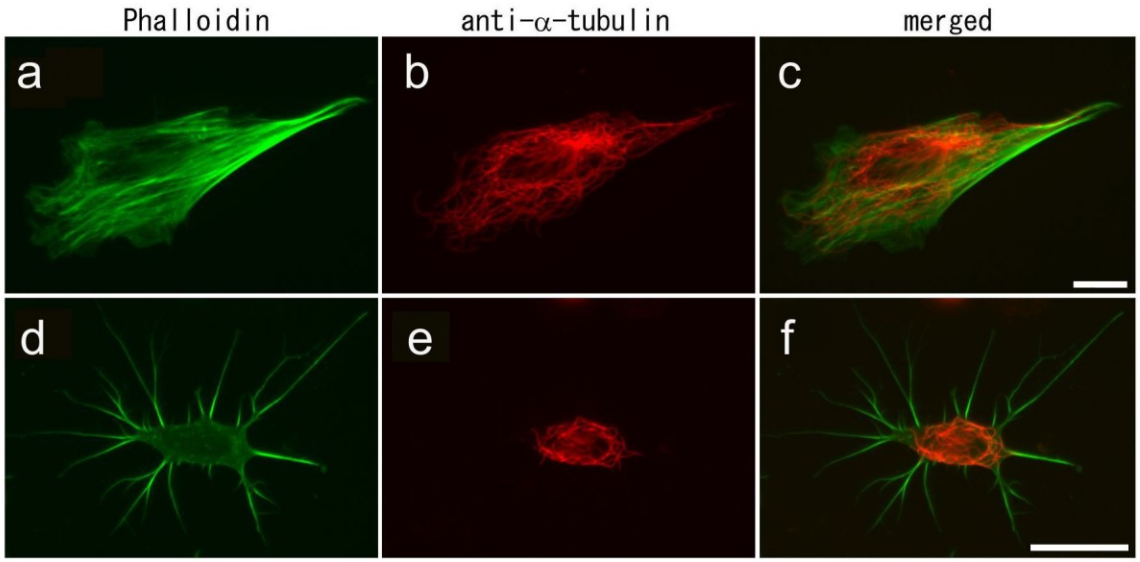

Figure (3)