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REGULAR ARTICLE

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Isolated primary osteocytes express functional gap junctions in vitro

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Abstract The osteocyte is the most abundant cell type in bone and is embedded in mineralized bone matrix. Osteocytes are still poorly characterized because of their location and the lack of primary osteocyte isolation methods. Data on the cell biology of osteocytes is especially limited. We have isolated primary osteocytes from rat cortical bone by applying repeated enzymatic digestion and decalcification. The isolated osteocytes expressed typical osteocytic morphology with cell-cell contacts via long protrusions after a 1-day culture. These cells were negative or faintly positive for alkaline phosphatase but expressed high levels of osteocalcin, PHEX (phosphate-regulating gene with homology to endopeptidases on the X chromosome), and DMP1 (dentin matrix protein 1). These cells also revealed patchy membrane staining for connexin43. For studying the function of gap junctions in isolated osteocytes, we microinjected rhodamine-labeled dextran (MW: 10,000) and Lucifer yellow (MW: 457) and found that Lucifer yellow was rapidly transmitted to several surrounding cells, whereas dextran remained in the injected cells. Heptanol and 18α-glycyrrhetinic acid inhibited the transfer of Lucifer yellow. This clearly showed the existence of functional gap junctions in cultured osteocytes. Enveloped viruses, such as vesicular stomatitis virus and influenza A virus, were used for studying cell polarity. We were unable to demonstrate plasma membrane polarization with enveloped viruses in isolated primary osteocytes in culture. Our results suggest that osteocytes do not possess apical and basolateral plasma membrane domains as do osteoblasts, which are their precursors.

Keywords Gap junction · Virus infection · Plasma membrane domains · Osteocyte · Microinjection · Rat (Sprague Dawley)

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Introduction

Osteocytes are ubiquitous throughout bone tissue, form gap junctions with adjacent osteocytes and bone-lining cells, and are extremely sensitive to alterations in their local physical environment (Doty 1981; Palumbo et al. 1990a). Several researchers have described the existence of gap junctions between bone cells (Cheng et al. 2001; Civitelli et al. 1993; Doty 1981; Menton et al. 1984). This observation has significant functional implications since gap junctions are known to be involved in intercellular communication and cell synchronization by enabling small molecules to be exchanged between the coupled cells.

Enveloped viruses, such as vesicular stomatitis virus (VSV) and influenza virus, are known to be targeted to specific plasma membrane domains in various polarized cells (Ilvesaro et al. 1999; Lombardi et al. 1985a,b). For instance, in the case of neurons, dendritic processes and the axon represent apical and basolateral membrane domains, respectively (Horton and Ehlers 2003). We have previously shown that other bone cell types including osteoblasts (Ilvesaro et al. 1999) and osteoclasts (Salo et al. 1997) have specific domains at the plasma membrane. At present, no data are available indicating whether osteocytes express specific plasma membrane domains or whether their plasma membrane is completely homogeneous in this respect. In this study, we have approached this open question by infecting isolated and cultured osteocytes with viruses.

Osteocytes are the most abundant cell type in bone, being ten times more abundant than osteoblasts (Jande and Belanger 1973). Osteocytes are regularly spaced throughout the mineralized matrix and communicate with each other and with bone-lining cells via multiple extensions of their plasma membrane that run along the canaliculi (Donahue 2000). Osteocytes are thus ideal cellular candidates for initiating biochemical responses culminating in tissue adaptation during bone growth and remodeling. Osteocytes are generally agreed to play a role in mechanoadaptation (Burger and Klein-Nulend 1999; Donahue 2000; van der Plas et al. 1994). Only a few osteocyte-specific proteins have been described. Recently, dentin matrix protein 1 (DMP1) has been reported to be specifically expressed only in osteocytes (Toyosawa et al. 2001). DMP1 is an acidic non-collagenous protein and is known to be present in the mineralized matrix of both dentin and bone (George et al. 1993; Qin et al. 2001; Toyosawa et al. 2001). The monoclonal antibody (Mab) OB 7.3 against avian osteocytes has recently been shown to recognize PHEX (phosphate-regulating gene with homology to endopeptidases on the X chromosome) protein (Westbroek et al. 2002).

Investigations of osteocyte functions are impeded by their difficult accessibility. Most of the published studies are based on histological and cytochemical observations of bone sections (Marotti et al. 1998; Palumbo et al. 2004). Wong and Cohn (1974) have developed a sequential enzyme digestion method to isolate various bone cell fractions from calvarial bones. Their main interest is to develop an isolation method for osteoblasts. A number of studies have subsequently been performed that suggest that osteocytes from calvaria can be isolated by a series of enzymatic digestions (Hefley et al. 1981; Mikuni-Takagaki et al. 1995; van der Plas and Nijweide 1992; Wong and Cohn 1974). Nijweide and Mulder (1986) and van der Plas and Nijweide 1992) have applied this method to isolate osteocytes from chicken calvaria and have also developed the osteocyte-specific Mab (OB 7.3). In this study, we have isolated osteocytes from rat cortical bone and developed an in vitro osteocyte culture system in which primary osteocytes form cell-cell contacts within hours, even under conditions in which cells are well spaced. This culture model has allowed us to study cell-cell communication between osteocytes and can be further developed to study, for example, the effect of loading responses on individual osteocytes and their cellular networks.

Materials and methods

Isolation of osteocytes from cortical bone

For the isolation of osteocytes from rat cortical bone, we applied the original methods of Wong and Cohn (1974) and Hefley (1987) for calvaria tissues. Long bones (femora and humeri) were dissected from 3-day-old Sprague–Dawley rats as aseptically as possible and prepared by serial digestion as described in Table 1. Collagenase (type A, 1 mg/ml; Roche, Mannheim, Germany) was dissolved in isolation buffer: 25 mM HEPES, pH 7.4, 70 mM NaCl, 10 mM $NaHCO_3$, 60 mM sorbitol, 30 mM KCl, 3 mM K_2HPO_4, 1 mM $CaCl_2$, 1 mg/ml bovine serum albumin (BSA), 5 mg/ml glucose. EDTA (JT Baker, Deventer, Holland) was dissolved in PBS containing 0.1% BSA to give a final concentration of 5 mM.

Cell culture

Cells from each fraction were washed twice with Hanks' balanced salt solution supplemented with 10% fetal bovine serum (FBS) and cultured either on collagen-coated coverslips or in culture flasks in α-MEM (GIBCO, Lifetechnologies, Paisley, Scotland) supplemented with 5% FBS and 5% calf serum (CS; HyClone Laboratories, Logan, Utah, USA) in a CO_2 incubator. All the experiments were performed within 4 days after isolation. During this time, no significant increase was noted in the number of cells in fraction 8. However, cell number markedly increased in earlier fractions.

Table 1 Procedure for the isolation of primary osteocytes from rat cortical bone

Step	Procedure
1	Soft tissues and periosteum are removed from the long bones of 3-day-old rats.
2	Bones are dipped into 70% ethanol for 15 s to kill most of the cells in the superficial layer.
3	Epiphyses are cut off, shaft is cut longitudinally, and bone marrow is removed by scraping and extensive washing. Bone pieces are washed with PBS (5×3 min each).
4	Pieces of bone are incubated in collagenase solution at 37°C for 20 min, four times (fractions 1–4).
5	Supernatants are collected at each time and centrifuged at 200*g* for 5 min. Pellets are suspended in cell culture medium for cell culture. Bone pieces are rinsed twice with Hanks' balanced salt solution (HBSS), and twice with PBS.
6	Residual bone pieces are treated with 5 mM EDTA in PBS containing 0.1% BSA at 37°C for 20 min. Step 5 is repeated (fraction 5).
7	Bone pieces are treated with collagenase solution at 37°C for 20 min. Step 5 is repeated (fraction 6).
8	Bone pieces are cut into smaller pieces, treated with EDTA at 37°C for 20 min. Step 5 is repeated (fraction 7).
9	Residual bone pieces are washed with HBSS and PBS and treated with collagenase solution at 37°C for 20 min. Step 5 is repeated (fraction 8).

Preparation of coated coverslips

Rat-tail type I collagen (BD Bioscience, Two Oak Park, Bedford, Mass., USA) in 0.02 M acetic acid (0.15 mg/ml) was pipetted onto coverslips so that the surface was totally covered, incubated for 1 h at room temperature, and then stored in sterile PBS in the refrigerator.

Alkaline phosphatase staining

Alkaline phosphatase (ALP) activity was assayed by the method of Kaplow (1955). Isolated primary osteocytes were cultured on collagen-coated glass coverslips for 1–4 days, washed twice with PBS, fixed with 3% paraformaldehyde (PFA) in PBS for 15 min at room temperature, stained for ALP by using a histochemical kit (86-R; Sigma, St. Louis, Mo., USA), and studied under the microscope. Some of the samples were further stained for osteocalcin as described below.

Osteocalcin staining

Isolated cells were cultured for 1–4 days on coverslips and fixed as described above. The cells were permeabilized with 0.1% Triton X-100 in PBS for 4 min on ice, blocked with 1% BSA in PBS either overnight at 4°C or for 2 h at room temperature, incubated with goat anti-osteocalcin antibody (1:100, Paesel+Lorei, Frankfurt, Germany) for 1 h at 37°C, washed with PBS three times, incubated with rhodamine-conjugated secondary rabbit anti-goat antibody (1:100, DAKO, Glostrop, Denmark) for 30 min at 37°C, and viewed under the fluorescence microscope.

Connexin43 staining

Osteocytes were cultured, fixed, and blocked as above. The cells were subsequently incubated with mouse anti-connexin43 (Cx43) monoclonal antibody (1:250, Chemicon International, Temecula, Calif., USA) for 30 min at room temperature, washed three times with PBS, incubated with rhodamine-conjugated anti-mouse antibody (1:100, DAKO) for 30 min at room temperature, and viewed under the fluorescence microscope.

RNA isolation and reverse transcription/polymerase chain reaction

RNA was purified from the various collagenase- and EDTA-digested fractions with the Qiagen Rneasy Mini Kit (Qiagen, Hilden, Germany). For cDNA synthesis, 0.6 μg RNA from each fraction was melted at 65°C for 5 min and subsequently transferred to ice. A mixture of 0.6 μg oligo-dT primer, 5 U AMV reverse transcriptase (Promega, Madison, Wis., USA), 20 U RNAse inhibitor (Fermentas, Vilnius, Lithuania), and 0.6 μl dNTP mixture (10 mM each) was added; the final reaction volume was 15 μl. Incubation was carried out at 25°C for 5 min, at 42°C for 60 min, and at 95°C for 5 min. Water (85 μl) was added to each reaction. Polymerase chain reactions (PCRs) were performed with specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rat mRNA, for rat PHEX mRNA, and for rat DMP1 mRNA (GAPDH primers: GAPDHF 5′-GTCGTGGAGTCTACTGGCGTC-3′, GAPDHR 5′-GAA GTCACAGGAGACAACCTGG-3′; PHEX primers: PEXF 5′-TCAGATGTAGCTAACTGCCCTG-3′ and PE XR 5′-GTATGGAGGGACTGGATCAACG-3′; DMP1 primers: DMP1F 5′-GAAGAATCTAAAGGGGATCAC GAG-3′ and DMP1R 5′-CCCAATAGGTTTGTTGTGG TAAGC-3′) in a final reaction volume of 50 μl consisting of 5 μl reaction buffer, 2 μl dNTP mixture (10 mM each), 0.5 μl each primer (50 μM), 2 μl cDNA template, and 0.3 μl DNA polymerase (DyNAzyme II 2 U/il from Finnzymes, Espoo, Finland; RedHot DNA Polymerase 5′U/μl from Abgene, Surrey, UK). PCRs consisted of denaturation at 95°C, annealing at 59°C, and extension at 72°C. Cycling was carried out 16–34 times depending on the primers. Aliquots of 10 μl of the PCR product were analyzed by 1% agarose gel electrophorosis and by Image J to semi-quantify the PCR products. The intensities of PCR products from each sample were compared with GAPDH to standardize the intensity of the PCR products.

VSV and influenza A virus infections

After 1 day in culture, osteocytes were infected with wild-type VSV (Indiana serotype), which was grown in baby hamster kidney cells to give a titer of 5×10^9 pfu/ml, or the influenza virus (A/WSN) strain as previously described (Ilvesaro et al. 1999; Metsikko et al. 1992). Adsorption time was 1 h at 37°C. After a rinse in PBS, cells were placed back into the initial culture medium for 3 h (VSV) or 5 h (WSN) further propagation. Osteocytes were then fixed as above. Antibodies against the gel-purified hemagglutinin (HA) band of the WSN strain to influenza virus HA were used as described earlier (Martin and Helenius 1991). Polyclonal antibody against VSV G protein was employed as previously published (Metsikko et al. 1992). The samples were then washed, incubated with rhodamine-conjugated anti-rabbit antibody (1:400 dilution) for 30 min at 37°C, and viewed under the fluorescence microscope.

Microinjection of dyes

Isolated primary osteocytes were seeded on coverslips (approximately 1,000 cells/coverslip). Most of them were able to form cell-cell contacts by gap junctions within 24 h. Lucifer yellow (MW: 457) and dextran-tetramethylrhodamine (MW: 10,000) (Molecular Probes, Eugene, USA) were mixed 3:1 and microinjected into the cells. The cells were viewed under the fluorescence microscope 5–30 min

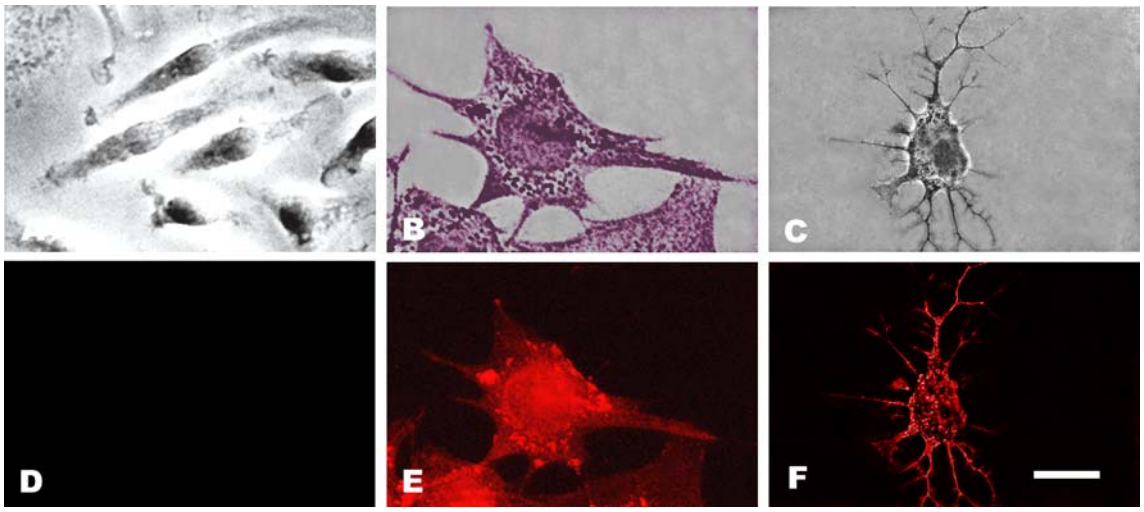

Fig. 1 Characterization of isolated bone cells from rat cortical bone. Isolated bone cells from fractions 1, 3, and 8 (see Table 1) were cultured on glass coverslips for 24 h. After fixation, the cells were stained for ALP (**a**–**c**; phase contrast) and with anti-osteocalcin antibody (**d**–**f**). The elongated spindle-shaped fibroblast-like cells (**a**, **d**) were negative for ALP and osteocalcin. The osteoblast-like cuboidal cells (**b**, **e**) were positive for ALP and osteocalcin. The osteocyte-like stellate cells (**c**, **f**) with extensive dendritic protrusions were negative or only weakly positive for ALP, but they expressed osteocalcin (**f**). *Bar* 20 μm

after microinjection. Two inhibitors, viz., heptanol (JT Baker, Holland; 3 mM) and 18α-glycyrrhetinic acid (Sigma, Steinheim, Germany; 3 μM), were used to study their effects on dye transfer between osteocytes and were added to osteocyte cultures 18–20 h before the microinjection of dyes. Equal amounts of vehicle (ethanol) were added to the controls.

Statistical analysis

Each experiment was performed with 12 3-day-old Sprague–Dawley rats. The percentage of each cell type was obtained by counting all cells from five different fields (20× objective) in the middle of the coverslip after the cells had been stained for ALP. In each case, the total number of cells counted was more than 100. Values were expressed as the mean ratio of all the kinds of cells in each fraction. In dye transfer experiments, the numbers of cells containing Lucifer yellow and the number of connected cells for each independent injection were recorded. The percentage of cells containing transmitted Lucifer yellow was calculated. Differences between groups were tested by Student's *t* test. A value of $P<0.05$ was considered to be significant.

Results

Characterization of cell fractions from cortical bone

On the basis of cell morphology, three main cell types were observed in isolated fractions. These three cell types were: (1) elongated spindle-shaped cells, (2) cuboidal cells, and (3) stellate cells with long protrusions (Fig. 1). In order to identify the observed cell types further, we performed enzyme cytochemical staining for ALP and immunocytochemical staining for osteocalcin. As expected, cuboidal cells were strongly positive for ALP, whereas stellate cells with long protrusions were negative or only weakly positive, and all spindle-shaped cells were completely negative. Osteocalcin staining was strongly positive in cuboidal cells and also in stellate cells with long protrusions, whereas spindle-shaped cells were negative. We concluded that spindle-shaped cells, cuboidal cells, and stellate cells represented fibroblasts (or pre-osteoblasts), osteoblasts, and osteocytes, respectively. The distribution of cells into these three groups in the various cell fractions is presented in Table 2. The percentage of fibroblast-like cells peaked in earlier fractions, whereas osteoblast-like cells and osteocyte-like cells were mainly found in the middle and late fractions, respectively. This result was also supported by the histology of residual bone tissue studied after each digestion step (data not shown). Tissue sections of residual bone demonstrated that superficial cells were lost during these sequential digestion steps, and only a small fragment of bone matrix could be observed.

Table 2 Cell types in the various cell fractions

Fraction	Fibroblast-like cells[a]	Osteoblast-like cells[b]	Osteocyte-like cells[c]
1+2	52.5%	36.8%	10.7%
3+4	28.3%	50.3%	21.4%
5	26.9%	29.1%	44.0%
6	22.6%	18.9%	58.5%
7	10.7%	12.8%	76.5%
8	10.6%	11.5%	77.9%

[a]ALP-negative elongated spindle-shaped cells
[b]ALP-positive and cuboidal in shape
[c]ALP-negative (or only weakly positive) stellate cells

In order to clarify further that the last fractions were enriched with osteocytes, we studied the mRNA levels of osteocyte markers DMP1 and PHEX in these fractions. The distribution of mRNA for DMP1 is shown in Fig. 2a, which demonstrates a clear enrichment in the last three fractions. RT-PCR of PHEX from various fractions gave similar results. GAPDH as a control was evenly expressed in all the samples. We analyzed the result with Image J to semi-quantitate the intensity of PCR products (Fig. 2b). This data agreed with the morphological data in Table 1, and thus we used the last two fractions in further experiments.

Functional studies of isolated osteocytes

Phase-contrast microscopy of cultured cells revealed potential cell-cell contacts, especially in the three last fractions. Immunocytochemical staining for connexin43 (Cx43) revealed immunoreactivity at several locations of the plasma membrane and especially at the tips of long protrusions where cells were in contact with each other (Fig. 3). Cells in the last fractions were able to form cell–cell contacts within a few hours after being seeded onto glass coverslips, even at a low cell density (10^4 cells/ml). Each cell usually made contact with 4–8 adjacent cells. In order to demonstrate that Cx43-positive cell-cell contacts were functional gap junctions, we injected these cells with both dextran and Lucifer yellow.

Within 5 min of microinjection of a single cell with both dyes, we observed Lucifer yellow in several adjacent cells, whereas rhodamine-labeled dextran was observed only in the injected cell. After 15 min following the injection, the Lucifer yellow in the adjacent cells became brighter, and the stain had also spread further to the neighboring cells (Fig. 4a,c).

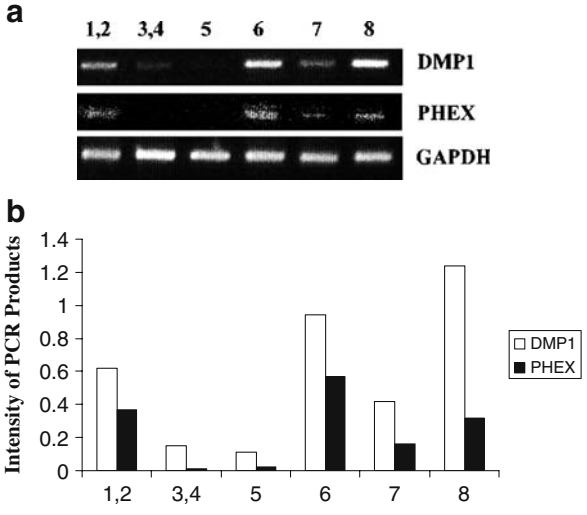

Fig. 2 Expression of DMP1 and PHEX in isolated cell fractions. **a** DMP1 mRNA with the size of 789 bp and PHEX mRNA with the size of 216 bp were amplified by RT-PCR. Agarose gel electrophoresis of RT-PCR products from various fractions (*top*). GAPDH was used as a loading control (576 bp). **b** RT-PCR products were analyzed by image analysis. The intensities of DMP1 and PHEX were compared with that of GAPDH from each fraction

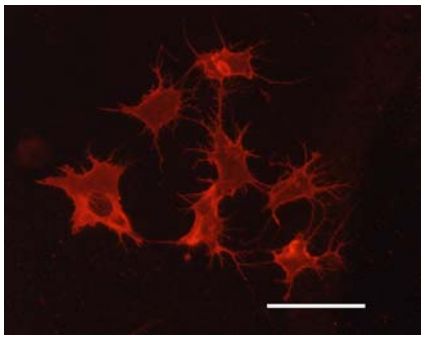

Fig. 3 Immunofluorescence staining of connexin 43 (Cx43). Cells from fraction 8 were cultured for 4 days and immunostained for Cx43. Strong positive immunostaining was observed in all osteocyte-like cells. Four to eight cells usually formed a connected network. *Bar* 50 μm

To study further if functional gap junctions were present, we added gap junction inhibitors, viz., either heptanol or 18α-glycyrrhetinic acid, to the culture medium 18–20 h before microinjection. In the presence of an inhibitor, Lucifer yellow was observed only in the injected cells after 15 min of injection. During the whole period of observation (1 h after microinjection), no Lucifer yellow transmission was observed in the cells connected to the injected cell, which was identified on the basis of its rhodamine-labeled dextran. These results showed that heptanol (3 mM) and 18α-glycyrrhetinic acid (3 μM) were able to block gap-junctional intercellular communication between osteocytes (Fig. 4d,f).

In order to demonstrate that the inhibitors used were not toxic, we incubated cells with these compounds for 18 h and then removed the inhibitors by several gentle washings just before microinjection with Lucifer yellow and dextran. These experiments demonstrated that inhibition was reversible, since after the washing out of the inhibitors, Lucifer yellow again spread to the neighboring cells, although not as effectively as in the control (Fig. 5).

In order to study whether osteocytes possessed plasma membrane polarity as demonstrated, for example, in neurons and osteoblasts, we infected the cells with two viruses, one being targeted to the apical membrane and the other to the basolateral plasma membrane. Immunofluorescence staining with the antibody against the VSV G protein revealed that it was localized throughout the plasma membrane with some condensed staining near dendritic protrusions (Fig. 6a). Immunostaining of influenza HA mainly gave similar results (Fig. 6b). Almost all fine cell protrusions were stained, clearly showing the connections between cultured osteocytes. We were unable to demonstrate any clear difference between the distributions of VSV G protein and influenza HA on the plasma membrane, suggesting that the osteocytes in culture did not possess typical apical and basolateral membrane domains.

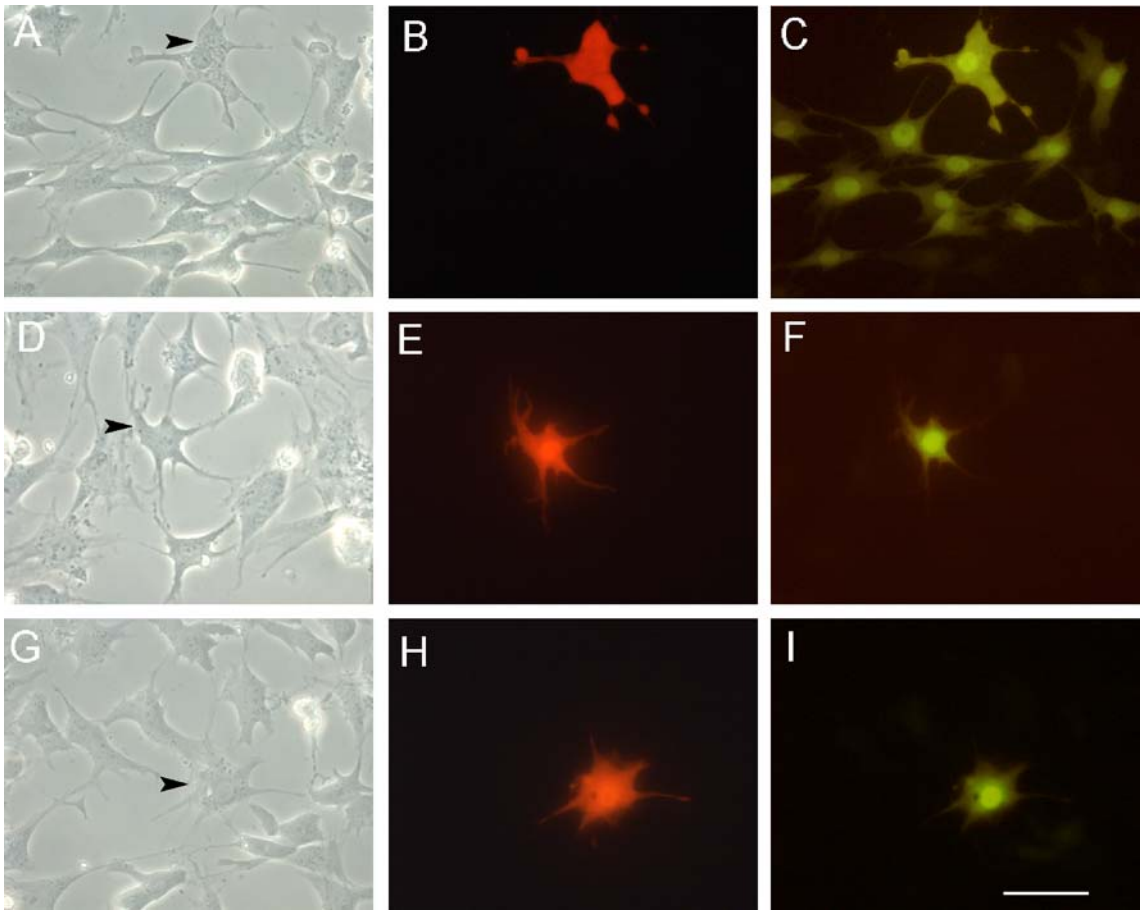

Fig. 4 Isolated osteocytes form functional gap junctions. Microinjection of a single cell (*arrow*) with Lucifer yellow (MW: 457; *yellow*) and rhodamine-labeled dextran (MW: 10; *red*). By 15 min after the microinjection, rhodamine-labeled dextran was still restricted to the injected cell, whereas Lucifer yellow could be seen in more than ten neighboring cells. They were either directly or indirectly connected to the injected cell (**a**–**c**). When isolated cells were incubated with either 3 μM 18α-glycyrrhetinic acid (**d**–**f**) or 3 mM heptanol (**g**–**i**) before and during the microinjection, the transfer of Lucifer yellow to neighboring cells was prevented, and only the injected cell was labeled. *Bar* 50 μm

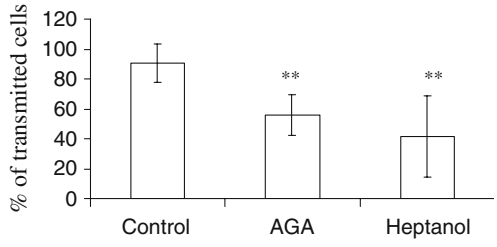

Fig. 5 Function of gap junctions is recovered after removal of gap junction inhibitors before microinjection. The isolated osteocytes were incubated with a gap-junction inhibitor for 18 h. Before microinjection of rhodamine-labeled dextran and Lucifer yellow, the cells were removed to high HEPES medium without inhibitors. After 15 min, Lucifer yellow was observed in most connected cells, but not in all. The numbers of cells with Lucifer yellow were recorded, as were the number of connected cells in each independent injection. The percentage of cells with transmitted Lucifer yellow was calculated. In controls, this value was almost 100%, but in both inhibitor-treated groups (data collected from eight different injections/group), this value was much lower. Differences between the control group and the inhibitor-containing groups were significant (**P<0.05)

Discussion

The diversity of cell types present in bone tissue makes it difficult to assess the functional role of each cell type separately. Osteocytes in particular are barely accessible because they are confined in the calcified matrix and their isolation is problematic for in vitro studies, since they are terminally differentiated and only limited information can be obtained by using transformed cell lines. Some reports are available giving methods for the enrichment of osteocytes from calvarial bones and their culture (Hefley 1987; van der Plas and Nijweide 1992), but here we report, for the first time, that primary osteocytes can also be isolated from cortical bone for functional in vitro studies.

Whether osteocytes derived from long bone and calvaria are different from each other is unclear but is likely since differences are apparent both in the structure and in the development of these bones. Although rat cortical bone does not reveal clear-cut Haversian osteons (Singh et al. 1974), we propose that this bone can be used as a relevant source for primary cortical osteocytes. Thus, there are good

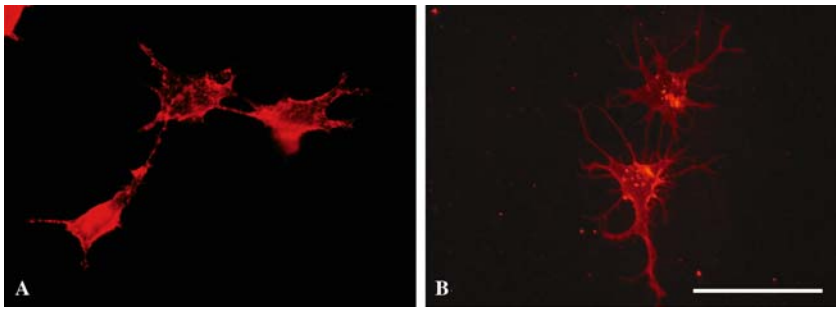

Fig. 6 Immunostaining of VSV G protein (**a**) and influenza A hemagglutinin (HA) protein (**b**) in cells infected with corresponding viruses. The samples were stained either with anti-VSV G protein antibody or influenza HA antibody to demonstrate the distribution of viral proteins in osteocytes. The cells were nonpermeabilized so that only the surface stain is shown. *Bar* 50 μm

reasons to believe that the osteocytes from long bones and from calvaria may differ from each other, and the isolation of primary osteocytes from long bones should provide us with a useful in vitro model for studying specifically the function of cortical bone osteocytes.

The morphology of cells in the various cell fractions revealed that three main types of cells were obtained from cortical bone after careful removal of the periosteum and bone marrow elements. On the basis of their morphology, our assumption was that they represented fibroblasts (or pre-osteoblasts), osteoblasts, and osteocytes. This hypothesis was supported by their staining characteristics for ALP and osteocalcin (Liu et al. 1997; Reilly et al. 1998). Osteoblasts have high ALP activity and express osteocalcin (McCarthy et al. 1988). On the other hand, osteocytes express low levels of ALP or are negative for ALP but are positive for osteocalcin (van der Plas and Nijweide 1992), whereas fibroblasts are negative for both. We concluded that the cells that we obtained in the final fractions were osteocytes. This assumption was further supported by the observations that fibroblast-like cells showed a high proliferative capacity (data not shown), whereas osteocyte-like cells did not proliferate at all.

DMP1, a member of the SIBLING family of acid phosphoproteins, is expressed in teeth and bone. DMP1 mRNA and protein are highly and selectively expressed in osteocytes in which the protein is localized along dendritic processes (Toyosawa et al. 2001). DMP1 can thus be considered as a specific marker for osteocytes. Our results with respect to DMP1 mRNA expression in the last fractions was in accordance with our morphological data and confirmed the enrichment of osteocytes.

PHEX belongs to the M13 family of metalloendopeptidases (Du et al. 1996). Previous studies have shown that PHEX mRNA is expressed in human and mouse bone and teeth (Beck et al. 1997). PHEX mRNA expression is upregulated during osteoblast differentiation in vitro (Guo and Quarles 1997). PHEX mRNA is expressed to a similar extent in osteocyte and fibroblast populations population but not in osteoblasts (Westbroek et al. 2002). In our experiments, PHEX mRNA expression had a similar pattern to DMP1, supporting the idea that the isolated cells in the last fractions were mainly osteocytes and not osteoblastic cells.

As our purpose was to obtain a pure osteocyte fraction, we developed a method to delete as many fibroblasts as possible early in the preparation of the initial material. Hence, bone pieces were dipped into 70% ethanol, not only for sterilization, but also for deleting the majority of superficial fibroblasts. This may be the reason that the distribution of isolated cells was different from the results obtained in a previous work with calvaria (van der Plas and Nijweide 1992). We also found that, by switching from normal serum-containing medium to serum-free medium after 1 day in culture, the overgrowth of fibroblasts was completely prevented, even in long-term cultures (3 weeks).

Marotti and coworkers have elegantly shown, by electron microscopy, that the arborization of osteocytes is asymmetrical with regard to both the numbers and length of cytoplasmic processes, and that they might be polarized cells (Palumbo et al. 1990a,b). Using a well-characterized viral glycoprotein transport model (Ilvesaro et al. 1999; Metsikko et al. 1992; Rahkila et al. 1998), we have studied the membrane polarization of isolated osteocytes. Influenza virus HA protein is distributed similarly to VSV G protein, although some condensed staining has been found in osteocyte processes connecting them with other cell processes. This suggests that classical apical and basolateral domains are not present in osteocytes, although their precursors, the osteoblasts, express these domains. Our preliminary experiments using viral infections of osteocytes in cultured bone pieces seem to support the observation obtained from tissue culture (Ilvesaro et al. 1999) that osteocytes do not target these virus proteins to distinct domains (data not shown). We conclude that osteocytes do not possess typical apical and basolateral plasma membrane polarization.

The existence of gap junctions between bone cells has previously been described by several authors (Davidson et al. 1986; Doty 1981; Palumbo et al. 1990a; Schiller et al. 1992). These studies suggest that gap junctions play an important role in cell–cell communication and cell synchronization by enabling small molecules to be exchanged between the coupled cells. Most of the studies are morphological (Doty 1981; Palumbo et al. 1990b) and have mainly been performed with osteoblasts. Palumbo et al. (1990a,b) have proposed that osteocytes and osteoblasts

are functionally coupled during osteogenesis and probably also in newly formed bone. Recently, osteocytes have been reported to control bone formation via sclerostin (Winkler et al. 2003). This report also emphasizes the importance of communication between osteocytes and osteoblasts.

In conclusion, our data show that osteocytes can be isolated from cortical bone and maintained in vitro cultures for several days or even weeks. We have also demonstrated that they rapidly form small syncytial networks in which cells are connected to each other via functional gap junctions. Although there are apparently no classical apical and basolateral domains in the plasma membrane of osteocytes in vitro, another type of membrane specialization, e.g. in gap-junction-containing projections, may well be present. Our isolation procedure and culture system offers an easy model for studying several new aspects of the cell biology of osteocytes in vitro.

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