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Taurine transporter is expressed in osteoblasts

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Summary. Taurine influences bone metabolism and is taken up by cells via a specific transport system, the taurine transporter (TAUT). We report a link between taurine and bone homeostasis by demonstrating transcription and translation of TAUT in bone-forming cells. TAUT was expressed in human primary osteoblasts, the human osteosarcoma osteoblast-like cell line MG63, and the mouse osteoblastic cell line MC3T3-E1. Immunostaining with polyclonal antibodies also demonstrated the presence of TAUT in both human and murine osteoblasts. TAUT mRNA expression and [^{3}H]taurine uptake increased during differentiation of MG63 cells in culture. Supplementation of culture medium with taurine enhanced alkaline phosphatase activity and osteocalcin secretion. The regulation and detailed function of taurine and TAUT in bone remain unclear, but our findings suggest a functional role for them in bone homeostasis.

Keywords: Taurine – Taurine transport – Osteoblast – Alkaline phosphatase – Osteocalcin

Introduction

Taurine (2-aminoethanesulfonic acid) is a conditionally essential amino acid, which is present in high concentrations in the brain, heart, eye, muscle, liver and bone (Huxtable, 1992). As taurine cannot be incorporated into protein, it is a prevalent intracellular free amino acid in mammalian tissues. Taurine serves a variety of physiological and pharmacological functions, such as stabilizing cell membranes, eliminating oxide free radicals, regulating intracellular osmosis, and maintaining intracellular calcium concentration (Huxtable, 1992; Pasantes-Morales et al., 1998). Thus, changes in the net cellular content of taurine may have a dramatic impact on cell function.

Although taurine does not have a genetic codon and is not incorporated into proteins, the cellular taurine content is a balance between synthesis from methionine/cysteine, active uptake via the saturable taurine transporter (TAUT), and release via a volume-sensitive taurine leak pathway. The level of cysteine sulfonic acid decarboxylase, an enzyme required for the biosynthesis of taurine, is very low in humans and primates. For this reason, taurine has been added to drugs, baby milk, and health foods by pharmaceutical companies. The tissue taurine content is determined by the uptake from plasma, whereas cellular content is determined by uptake via a specific transport system, the Na^+- and Cl^-- dependent TAUT. Intracellular taurine concentrations are often as high as 10 mmol/kg of wet tissue in many mammalian tissues, whereas taurine is found at the concentration of 20–100 nM in plasma (Huxtable, 1992; Wright et al., 1986), suggesting that TAUT plays an important role in maintaining a high concentration of taurine in tissues.

Recently, taurine was found in bone tissue, and evidence suggests it plays an important role in bone metabolism by inhibiting experimental bone resorption, osteoclast formation, and osteoclast survival (Koide et al., 1999) and by regulating osteoblast metabolism via ERK2 activation (Park et al., 2001). These findings indicate that TAUT may be expressed in bone-forming cells. The present work investigated the expression of TAUT in bone-forming cells and the effects of taurine on alkaline phosphatase (ALP) activity and osteocalcin secretion in osteoblasts.

Materials and methods

Cell culture

Primary human osteoblasts (HOB) from the trabecular bone were obtained during surgery for traffic accident victims with informed consent from donors and after approval by the Local Research Ethics Committee, as previously described (Luo et al., 2002). The HOB cells were grown in α-MEM (Sigma Chemical Corp. St. Louis, MO) containing 15% fetal

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bovine serum (FBS, Hyclone Laboratories, Logan, Utah), 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid. Osteoblasts cultured to facilitate mineralization were exposed to hydrocortisone hemisuccinate (200 nM) and β-glycerophosphate (10 mM) (Cambrex) in ambient medium. The phenotype of cells was characterized based on the expression levels of ALP, collagen type 1, and osteocalcin and the formation of mineralization nodules as previously described (Luo et al., 2002; Liao and Luo, 2001).

The mouse osteoblastic cell line MC3T3-E1 was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD), supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin-streptomycin solution. The human osteosarcoma osteoblast-like cell line MG63 was obtained from ATCC. Cells were grown in α-MEM (Sigma, St. Louis, MO, USA), supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin-streptomycin solution. All cells were maintained in a humidified, 95% air, 5% CO_2 atmosphere at 37 °C. The medium was changed twice a week and the cells were subcultured using 0.05% trypsin with 0.01% EDTA.

Immunocytochemistry and immunohistochemistry for the TAUT in cells and bone tissue

A TAUT polyclonal affinity-purified IgG was purchased from ADI (Alpha Diagnostic Int., USA). This IgG was raised against a 20 amino acid-peptide near the carboxy-terminal region of TAUT. The TAUT antibody was used for immunocytochemistry, immunohistochemistry, and Western blot analysis as described below.

For the immunocytochemistry, the cells were plated onto 12 mm^2 circular glass cover slips in 24-well dishes. Each coverslip with adherent cells was washed once with phosphate-buffered saline (PBS), fixed with 4% formaldehyde in PBS for 5 min, and washed with PBS again. The cells were permeabilized in 0.2% (v/v) Triton X-100. Fixed cells were treated with 0.3% hydrogen peroxide for 10 min to inhibit endogenous peroxidases. They were then incubated for 1 h with Protein Block Serum-Free Solution and subsequently overnight at 4 °C with the rabbit anti-TAUT antibody (1:100) or casein antibody as a control (1:100). After being washed three times with 0.5% bovine serum albumin in PBS for 10 min each, cells were incubated at room temperature for 1 h with biotinylated goat anti-rabbit IgG. The cells were then washed with PBS three times for 10 min each, and the color reaction was developed with the DAB chromogen kit (Boster Company, WuHan, China) for about 10 min. The color reaction was stopped by washing three times in water.

For the bone immunohistochemistry examination, trabecular bones obtained from surgery were subjected to plastic embedding, according to Erben's method (Erben et al., 1997). The plastic blocks were trimmed to a size of approximately 30 × 20 × 20 mm with a mill. Sections (4 μm thick) were prepared with a HM 360 microtome (Microm, Walldorf, Germany) and were then placed in 2-methoxyethylacetate and acetone for deplasticization. Deplasticized slides were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase and then washed with PBS. Antigen microwave intermittent repair was performed in 0.01 M citric acid buffer for 5, 3, 3, and 2 min, at an interval of 2 min. After the slides were incubated with normal goat serum for 15 min to reduce nonspecific binding, 30 μl of rabbit anti-TAUT antibodies (1:50) or 0.1 M PBS instead of primary antibodies for negative controls were added. The slides were incubated overnight for 18 h at 4 °C. After washing in PBS buffer, biotinylated goat anti-rabbit IgG secondary antibody (1:80) was added, and the slides were incubated for 30 min at room temperature and then washed in PBS. Immunoreactivity in bone sections was visualized with the AEC chromogen kit.

Western analysis of TAUT in osteoblast cells

Monolayers of HOB, MC3T3-E1, and MG63 cells in 25 cm^2 culture flasks were rinsed twice with 1 mM EDTA in PBS and lysed with triton lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin, and 1 μg/ml aminoethylbenzenesulfonyl fluoride (Sigma) and then saved after centrifugation. The protein concentrations were determined using the Bradford protein assay. An aliquot of 100 μg protein of each cell lysate was mixed with 2 × SDS gel-loading buffer (100 mM tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) and then heated to 95 °C for 5 min. The samples were loaded onto the gels. After electrophoresis, the SDS-PAGE-separated proteins were transferred to a nitrocellulose membrane (Amersham, Arkington Heights, IL) using a Bio-Rad laboratories, Inc, blotting apparatus. The membrane was blocked with 2.5% nonfat milk in PBS for one night at room temperature and then incubated with 1 μg/ml rabbit anti-TAUT polyclonal antibody in PBS for 3 h at room temperature. After extensive washing with PBS, the membrane was re-probed with goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) at 1:1000 in PBS for 1 h at room temperature. Blots were processed using an ECL kit (Santa Cruz), exposed to the film, and then analyzed by densitometry.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from HOB, MC3T3-E1, and MG63 cells was used for RT-PCR to clone TAUT. Total RNA was prepared with the TRIzol Reagent (GIBCO-BRL). 2 μg of total RNA was reverse-transcribed for the synthesis of single stranded cDNA (Invitrogen, Carsbad, CA, USA). The resultant first-stranded cDNA was used for the PCR procedure. AccuPrime Pfx DNA polymerase (Invitrogen), a high-fidelity DNA polymerase, was used to amplify cDNA. For mouse TAUT, the PCR primers were 5′ GCCA CATACTACCTATTCCA 3′ and 5′ CAGCAGCATACAGTCCCT 3′, yielding a 555 bp fragment. For human TAUT, the PCR primers were 5′ CACTGGGAAGGTCGTCTA 3′ and 5′ CTCAGCCACATCAGCAAT 3′, yielding a 370 bp fragment. For human GAPDH, the PCR primers were 5′-CACCATCTTCCAGGAGCGAG-3′ and 5′-TCACGCCACAGTTTCC CGGA-3′, yielding a 372 bp fragment. The amplification was performed as follows: After an initial denaturation at 94 °C for 5 min, PCR were run for 30 cycles at 94 °C for 45 sec, annealing for 30 sec, and extension at 72 °C for 45 sec (10 min in the last cycle). The annealing temperatures for human and mouse TAUT were 53 °C and 48 °C, respectively, and the annealing temperature for GAPDH was 53 °C. The PCR product for each gene was separated by gel electrophoresis, photographed by the Nikon 300 system. Densitometer analysis of films was performed using a computerized image analysis (NIH IMAGE 1.62, 1999 version) program. Expression was scored by analyzing the TAUT molecule/GAPDH ratio. The PCR product was purified using the Qiagen Plasmid kit (Qiagen Inc., Chatsworth, CA), and sequenced bidirectionally employing the same primer pairs used for PCR by means of an automated sequencer (AB3730, Applied Biosystems, Foster City, CA) in the DNA Sequence Facility of Invitrogen, using dideoxy terminator Taq technology.

To determine the expression changes of TAUT mRNA in differentiating osteoblasts, MG63 cells were culture for 24 days period. RNA isolation and reverse transcription were performed as described above. The amounts of RNA templates (3 ng) and cycle numbers (30 cycles for TAUT gene and 26 cycles for GAPDH gene) for amplification were chosen in quantitative ranges, where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers.

Determination of TAUT function

The MG63 cells (2×10^4 cells/ml) which were grown in six-well polyethylene dishes (9.6 cm^2 per well) were harvested on day 0, 4, 11, 18 or 24, respectively. The cells in five wells were washed twice with 700 μl each of Hanks' balanced salt solution containing 4 mM sodium bicarbonate and 10 mM HEPES, the pH value being adjusted to 7.4 with potassium

hydroxide (the uptake buffer), then were incubated at 37 °C for 10 min with 0.3 μCi of [^{3}H]taurine (Amersham Pharmacia) in 300 μl of the uptake buffer. At the end of the incubation period, the buffer was removed, and monolayer was carefully washed twice for 5 min each with ice-cold phosphate-buffered saline (PBS) containing 0.05% sodium azide. To each well in the culture plate was then added 250 μl of 0.1% Triton X-100, before the dissolved cells were taken into 3 ml of a scintillation cocktail. The tritium content of each monolayer was finally determined with an β-liquid scintillation counter. The sixth well was used for estimation of the average protein content (g protein per well) using a Bradford protein assay.

Analysis of alkaline phosphatase activity and osteocalcin production

MG63 cells were seeded in 6-well plastic plates and grown to 90–95% confluency. Then the cells were washed once with serum-free medium before addition of new medium with or without taurine in the range of 1–20 mM for 24 h. After incubation, they were washed with PBS, scraped into 1 ml of 10 mM Tris-Cl buffer (pH 7.6) containing 0.1% Triton-X-100 on ice, and centrifuged. The lysates were homogenized. Then, ALP activity was assayed by spectrophotometric measurement of p-nitrophenol release at 37 °C. To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay.

Osteocalcin released into the culture media was measured in quintuplicate using a specific human osteocalcin ELISA (Biomedical Technologies Inc.). To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay.

Parallel cultures, as a control for hyperosmolarity induced by 10 mM taurine treatment, were exposed to 10 mM mannitol. ALP and osteocalcin were measured as mentioned above.

Statistical analysis

Data obtained in RT-PCR, [^{3}H]taurine uptake and ELISA passed normality and equal variance test. Statistical comparison between groups and treatments was performed using Student's t test. A probability of less than or equal to 0.05 was considered significant.

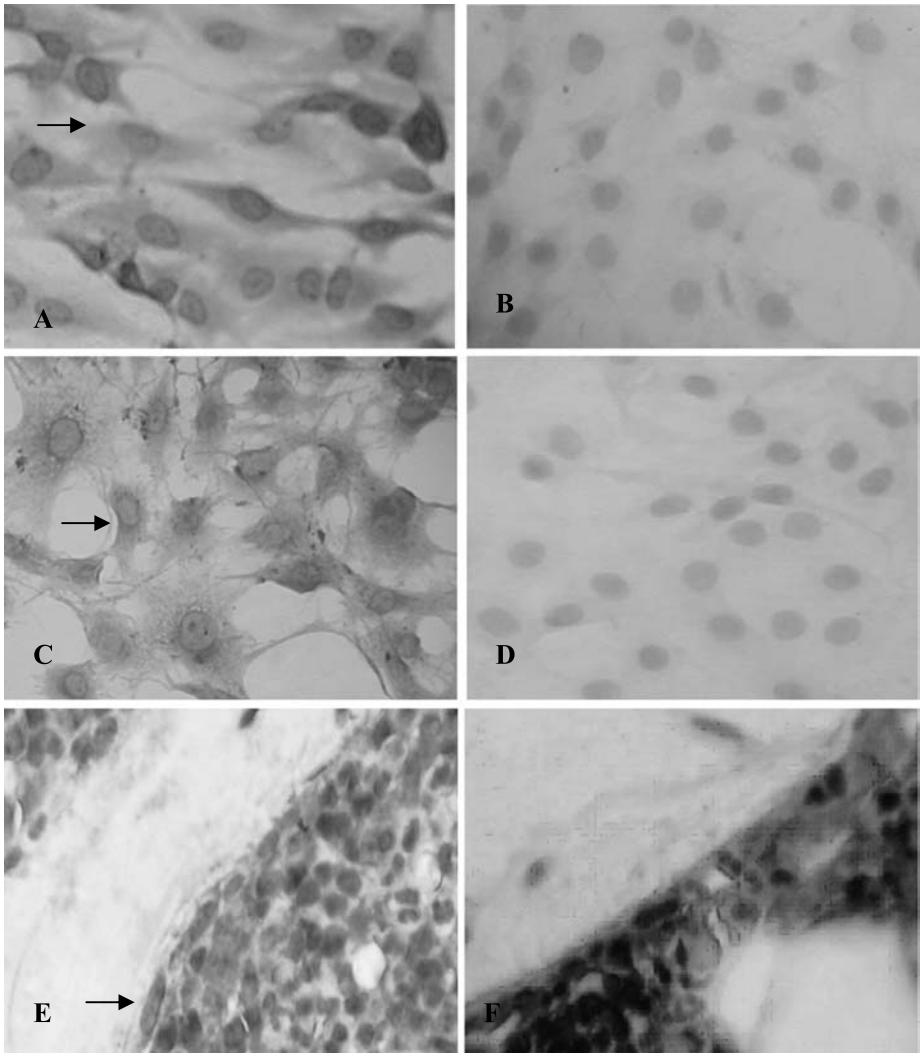

Fig. 1. Immunostaining of TAUT in vitro in human osteoblast-like MG63 cells (**A**), murine osteoblasts MC3T3-E1 cells (**C**), and in vivo in human bone (**E**). No staining was shown for the negative controls (**B**, **D**, **F**). The arrow denotes a positively stained cell. Magnification is the same for all micrographs (×200)

Results

Immunoreactivity of TAUT in HOB, MC3T3-E1 and MG63 cells

As demonstrated in Fig. 1, MG63, MC3T3-E1 cells and human bone tissue sections demonstrated specific labeling for TAUT protein. Thus, immunocytochemistry and immunohistochemistry examination employing a specific anti-TAUT antibody revealed that these osteoblast-like cells expressed TAUT protein in vitro and in vivo. We examined the ability of HOB, MC3T3-E1, and MG63 osteoblasts to express TAUT protein by Western blot analysis with the anti-TAUT pure IgG polyclonal antibody. We detected a 70 kD band from the cell lysate (Fig. 2). These results suggest that both human and mouse osteoblasts can express TAUT.

Detection of TAUT mRNA in HOB, MC3T3-E1, and MG63 cells by RT-PCR

TAUT-specific intron-spanning primers were utilized to ensure that the TAUT products were not the result of amplification of contaminating genomic DNA segments. RT-PCR with these TAUT-specific primers amplified products of the sizes expected for cDNA synthesized from TAUT transcripts in HOB (370 bp), MC3T3-E1 (555 bp) and MG63 cells (370 bp) (Fig. 3, lanes 1–3). No products were observed when the RT was omitted during synthesis of cDNA and H_2O substituted the template. DNA sequence analysis of each PCR product revealed 100% homologies with the Mus musculus retina and human placental TAUT cDNA sequences, respectively. These results indicate that the RT-PCR products corresponded to an authentic TAUT sequence, suggesting the presence of TAUT transcripts in these cells. During a differentiation period of 24 days, the expression of TAUT mRNA in MG63 increased 3.67-fold when normalized to the expression of GAPDH (Fig. 4).

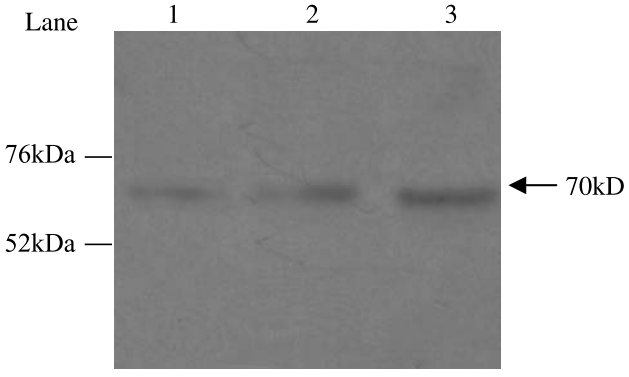

Fig. 2. Total cellular protein was subjected to immunoblot analysis using an anti-TAUT antibody. The anti-TAUT antibody identified a band at 70 kDa. *1* Primary human osteoblast lysate; *2* osteosarcoma osteoblast-like cell line MG63 lysate; *3* mouse osteoblastic cell line MC3T3-E1 lysate. Left margin indicates wt standards (kD)

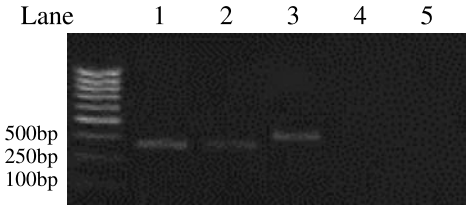

Fig. 3. Total RNA from primary human osteoblasts (*1*), human osteosarcoma osteoblast-like cell lines MG63 (*2*), and mouse osteoblastic cell line MC3T3-E1 (*3*) was subjected to RT-PCR. The PCR products (370 bp for human TAUT and 555 bp for murine TAUT) were visualized in a 1.2% agarose gel stained with ethidium bromide. Omission of RT (*4*) and H_2O substituted for the template (*5*) were used as negative control

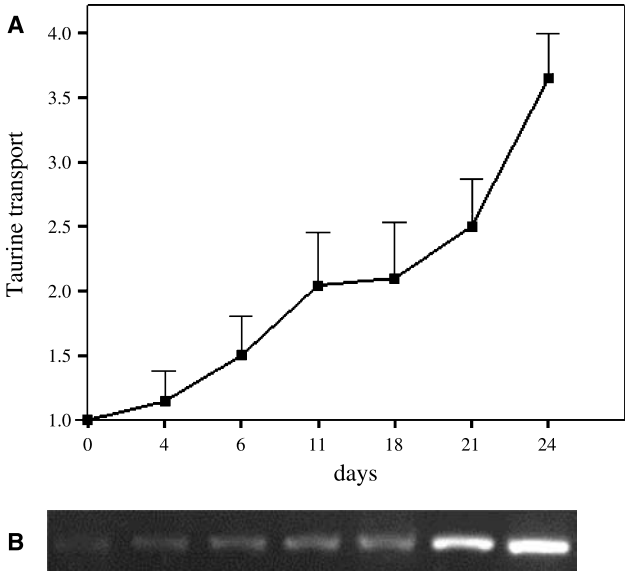

Fig. 4. **A** The expression was calculated relative to the level at 24 h mRNA expression which was normalized to GAPDH. The data are presented as means ± SD of three similar experiments with duplicate samples. **B** A RT-PCR result from one representative experiment

Uptake of [^{3}H]taurine during the osteoblastic differentiation

To further study the TAUT function during the osteoblastic differentiateon, MG63 cells were cultured on six-well dishes for 0, 4, 11, 18 or 24 days. At each of these

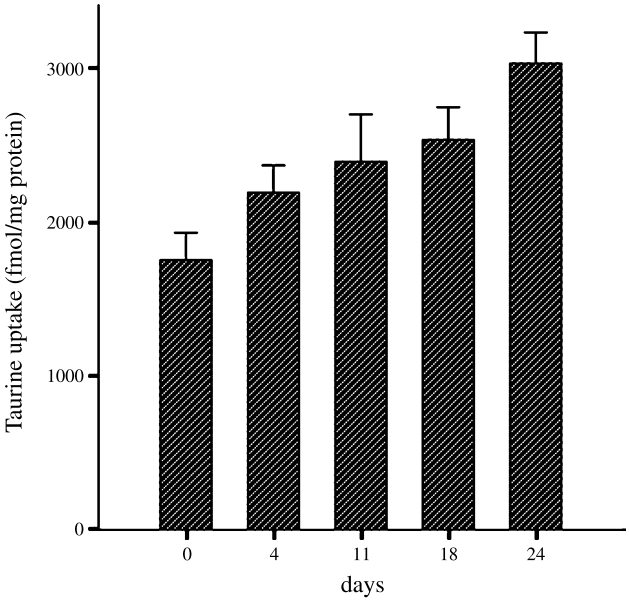

Fig. 5. [^{3}H]Taurine uptake into osteoblast during a 24-day differentiation period. [^{3}H]Taurine uptake was normalized to the quantity of protein (mg) in the well. At the 24th day, [^{3}H]taurine incorporation was significantly increased about 1.74-fold compared with the first day uptake. The data are presented as means ± SEM (n = 5)

time-points, [^{3}H]taurine uptake was examined. Figure 5 shows that [^{3}H]taurine uptake was significantly increased about 1.74-fold during MG63 cells differentiation.

Effects of taurine on osteoblastic differentiation

ALP and osteocalcin secretion

ALP and osteocalcin secretion are regarded as indices of osteoblastic differentiation and play an important role in bone mineralization. Therefore, we investigated whether taurine influences ALP activity and osteocalcin in MG63 cells. Taurine significantly increased ALP activity at the 10 and 20 mM concentrations and promoted osteocalcin secretion at 10 mM (Table 1). A similar change in osmolarity of 10 mM taurine obtained with use of 10 mM mannitol failed to reproduce the effects of taurine.

Table 1. Effect of taurine on ALP activity and osteocalcin secretion in culture MG-63 cells

	ALP activity (nmol/min mg protein)	Osteocalcin (ng/mg protein)
Control	2.53 ± 0.43	2.20 ± 0.45
10 mM Mannitol	2.64 ± 0.36	2.13 ± 0.41
Taurine		
1 mM	2.61 ± 0.34	2.38 ± 0.47
5 mM	2.82 ± 0.42	2.35 ± 0.33
10 mM	3.21 ± 0.39*	3.28 ± 0.53*
20 mM	3.14 ± 0.32*	2.81 ± 0.29

Cells were exposed to 1–20 mM taurine for 24 h. The cells were homogenized for the ALP activity assay. Cell culture media were collected for osteocalcin secretion assay. ALP activity and osteocalcin secretion were determined as described in Material and methods. Values are mean ± SD obtained from the three cultures and normalized to total cellular protein. (*$p < 0.05$ vs. control). 10 mM Mannitol was used as a control of hyperosmolarity which is equal to 10 mM taurine treatment

Discussion

The present findings indicate that the murine osteoblast cell line MC3T3-E1, human osteosarcoma osteoblast cell line MG63, and primary human osteoblasts expressed TAUT protein, as assessed by Western blot analysis and immunocytochemistry with a TAUT-specific pure IgG in vitro. Furthermore, immunohistochemistry on human bone tissue sections indicate that the TAUT protein was expressed in vivo. Moreover, RT-PCR performed on total RNA isolated from the three cell lines followed by sequence analysis of the PCR products, demonstrated the presence of TAUT transcripts (Fig. 3). Thus, the present study suggests that these osteoblast-like cell lines express both TAUT protein and mRNA.

TAUT is a member of the neurotransmitter transporter family that includes the transporters for serotonin (SEROT) and (gamma)-amino butyric acid (GAT1-3), as well as the creatine transporter (CREAT) (Lill and Nelson, 1998). All members of this family span the membrane 12 times, with the N- and C-terminal ends exposed to the cytosolic compartment. TAUT-cDNA has been cloned from a great variety of tissues or cells from various mammalian species: rat brain (Smith et al., 1992), mouse brain (Liu et al., 1992), dog renal MDCK cell line (Uchida et al., 1992), human thyroid (Jhiang et al., 1993), human placenta (Ramamoorthy et al., 1994), human retinal pigment epithelial cell line (Ramamoorthy et al., 1994; Miyamoto et al., 1996), mouse retina (Vinnakota et al., 1997), and bovine endothelial cells (Qian et al., 2000). They all encode an approximately 620 amino acid protein of a molecular weight approximately 70 kDa. Mammalian TAUTs show a greater-than-90% amino acid sequence identity. Western blotting experiments using the anti-TAUT antibody revealed one distinct band in both human and mouse osteoblasts. Three TAUT protein bands in the range of 50–70 kDa have been previously demonstrated by immunoblotting in Ehrlich ascites tumor cells (Poulsen et al., 2002), basal plasma membrane (Roos et al., 2004) and mouse epididymis (Xu et al., 2003). The variance in the molecular mass of TAUT reflects differences in

post-transcriptional modifications such as glycosylation and/or phosphorylation or expression of different TAUT isoforms that need to be investigated further. It is generally assumed that TAUT is just a membrane protein, which uptake taurine from extracelluar environment. However, a recent study showed TAUT localized to a region of NIH3T3 fibroblasts cell line, which appears to be within the nucleus, as well as in the cytosol and at the plasma membrane using confocal laser scanning microscopy and Western blotting (Voss et al., 2004). Voss et al. considered the presence of an intracellular pool of transporters that can be mobilized to the plasma membrane upon specific stimuli. And a fraction of the TAUT proteins in the cytoplasm may also represent immature TAUT present in the ER/Golgi compartments (Voss et al., 2004). With regard to the nucleus localization of TAUT, the finding reported by Terauchi et al. (1998), which showed the present of taurine in nucleus, may support the expression and function of TAUT on the nucleus, but more studies are needed to clarify the potential role of TAUT in nucleus. Our immunocytochemistry studies demonstrated that the cytoplasmic, plasma membrane, and nucleus fractions could be positively stained using the TAUT-specific antibody, which was consistent with the TAUT protein expression in NIH3T3 cells (Voss et al., 2004). In addition, the RT-PCR and sequence analysis confirmed that transcription of TAUT mRNA occurs in both human and murine osteoblasts. During osteoblast differentiation, the expression of TAUT mRNA was augmented about 3.67-fold and [^{3}H]taurine uptake was increased about 1.74-fold. These findings demonstrate that transcription and translation of TAUT occurs in bone-forming cells.

Previous reports investigated the effect of taurine on stimulated ALP activity and collagen synthesis in osteoblast-like UMR-106 cells. Yasutomi et al. (2002) suggested taurine promotes osteoblast mineralization by activating the intracellular MEK-ERK-Cbfa1 signaling system. The present results indicate that administration of taurine can enhance ALP activity and osteocalcin secretion in MG63 cells, but not a comparable increase in osmolality obtained by mannitol, indicating a functional role for taurine in bone remodeling.

Taurine may directly influence the metabolism of cells in bone, as has been described earlier in muscle and retinal pigment epithelium cells (Hillenkamp et al., 2004). The mechanisms of taurine influences on the regulation of bone metabolism have not been elucidated. The presently observed expression of TAUT in bone-forming cells provides fundamental information for this intriguing issue.

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