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Short communication

Dexamethasone induces sodium-dependant vitamin C transporter in a mouse osteoblastic cell line MC3T3-E1

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The regulation of intracellular ascorbic acid (AsA) levels may be under the control of an AsA-specific membrane transporter. The present study investigates AsA uptake and expression of Na-dependent vitamin C transporter (SVCT) mRNA in the mouse osteoblastic cell line, MC3T3-E1. Among eight compounds tested, dexamethasone (Dex) all-*trans* retinoic acid, transforming growth factor β, prostaglandin E_2 and transferrin significantly ($P < 0{\cdot}01$, $P < 0{\cdot}01$, $P < 0{\cdot}05$ and $P < 0{\cdot}01$ respectively) stimulated the update of AsA into MC3T3-E1 cells. Among these five, Dex was the most active, inducing mSVCT2 mRNA and the uptake of AsA in a time- and concentration-dependant manner. Dex did not induce mSVCT1 mRNA. These results suggest that the Dex-induced stimulation of AsA incorporation into osteoblastic cells is mediated by the induction of mSVCT2. Since Dex reduced alkaline phosphatase activity in MC3T3-E1 cells in our culture conditions, Dex-induced stimulation of AsA incorporation might not be the result of differentiation. Hormone-regulated changes of SVCT expression may have an important role in cell functions.

Ascorbic acid: Transporter: Sodium-dependant vitamin C transporter: Osteoblast: MC3T3-E1: Dexamethasone

Ascorbic acid (AsA) is an essential nutrient that has various functions including an essential role in the prolyl and lysyl hydroxylation necessary for the maturation of collagen peptide. It is therefore important in bone formation (Chen & Raisz, 1975) and in the *in vitro* differentiation of mesenchymal cells including osteoblasts (Franceschi *et al.* 1994). To be used for these actions, AsA must traverse the cell membrane from the extracellular space. The incorporation of L-[^{14}C]AsA is saturable and Na^+-dependant in various types of cells and tissues (Rumsey & Levine, 1998; Fujita *et al.* 2000). Tsukaguchi *et al.* (1999) recently cloned two transporters for AsA, called Na-dependant vitamin C transporter (SVCT) 1 and 2, from rat cDNA libraries. However, the relationship between bone formation and SVCT and the significance of SVCT for hormone-regulated change in AsA incorporation remains unknown.

The murine cell line clone MC3T3-E1 maintains osteoblastic characteristics (Kodama *et al.* 1981; Quarles *et al.* 1992; Franceschi *et al.* 1994). We used cultured MC3T3-E1 cells as an *in vitro* model of osteoblasts. Dexamethasone (Dex) stimulates not only osteoblast differentiation (Bellow *et al.* 1987; Boden *et al.* 1996) but also Na^+-dependant L-[^{14}C]AsA uptake (Pandipati *et al.* 1998). However, the relationship between the enhanced uptake and the expression of SVCT had not been investigated. In the present study, we cloned two types of mouse SVCT cDNA and investigated the expression of the SVCT in osteoblastic cells. The data showed that the enhanced transport of AsA by Dex in osteoblastic cells is controlled by SVCT2 expression.

Materials and methods

Cells and cell culture

MC3T3-E cells were originally established by Kodama *et al.*

Abbreviations: AsA, ascorbic acid; Dex, dexamethasone; mYspl3, mouse yoke-sac permease-like molecule-3; SVCT, sodium-dependant vitamin C transporter.

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(1981) from mouse calvaria. MC3T3-E1 (a clone of MC3T3-E) cells were obtained from Dr T. Miyahara (Toyama Medical and Pharmaceutical University, Toyama, Japan) and cultured in α-modification of Eagle's medium with 100 ml fetal calf serum/l in a humidified atmosphere of CO_2–air (5:95, v/v). For uptake assay, cells were seeded on twenty-four-well culture plates at a density of $1{\cdot}0 \times 10^5$ cells/500 μl per well and incubated for 24 h. For RNA preparation, cells were plated on 10 cm culture dishes at a density of $2{\cdot}5 \times 10^6$ cells/10 ml and incubated for 24 h. Cells were then exposed to various compounds under controlled conditions in serum-free medium. Uptake assays or RNA preparation proceeded after exposure for various periods.

Ascorbic acid incorporation

MC3T3-E1 cells in twenty-four-well culture plates were rinsed with incubation medium composed of 10 mM-2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid, 137 mM-NaCl, 5·4 mM-KCl, 0·3 mM-Na_2HPO_3, 0·4 mM-KH_2PO_4, 0·4 mM-$MgSO_4$, 1·3 mM-$CaCl_2$, 0·5 mM-$MgCl_2$ and 0·5 mM-thiourea, pH 7·4. Cells were then incubated with the same medium containing various concentrations of L-[^{14}C]AsA (0·295 MBq/μmol; NEN-DuPont, Boston, MA; USA) at 37°C for 60 min in air. The incorporation radioactivity was measured using a liquid scintillation counter. Non-specific incorporation was determined using an excess amount of cold AsA at a concentration of 10 mM. Specific incorporation was calculated as total incorporation minus non-specific incorporation. Values are normalised with protein contents and are expressed as relative units unless otherwise indicated.

cDNA cloning

A search of the sequence database revealed that mouse yolk-sac permease-like molecule-3 (mYspl3; GenBank ac. no. AF058320) cDNA (Faaland *et al.* 1998) has high homology to rSVCT1 (93 %). On the basis of this sequence similarity, we assumed that mYspl3 is the mouse counterpart of rSVCT1. On the other hand, a mouse cDNA similar to rSVCT2 was not found in the database. Type 2 SVCT have been cloned only from rats and human subjects (Rajan *et al.* 1999; Tsukaguchi *et al.* 1999; Daruwala *et al.* 1999). We designed polymerase chain reaction primers based on the 5′- and 3′-non-coding region conserved in rSVCT2 and hSVCT2 cDNA. The cDNA for mYspl3/mSVCT1 and mSVCT2 cDNA were generated by polymerase chain reaction from a mouse kidney and from a mouse brain cDNA pool respectively. The primers were ATGAAAACTCCGG-AGGACCCC and TCAGACCTTGGTACACACAGACCC for mYspl3/mSVCT1, ATGATGGGTGTCGGCAAGAA and ACAGATGCATGCCGTACT-GT for mSVCT2. SVCT cDNA fragments generated by polymerase chain reaction were subcloned into pBluescript II and sequenced. The sequence of the mYspl3 cDNA fragment matched that from the database. The sequence from the predicted mSVCT2 cDNA fragment had high homology to rSVCT2 (96 %).

Northern blots

Cellular RNA was isolated, and 6 μg total RNA was fractionated on a 1 % formaldehyde–3-(*N*-morpholino)propanesulfonic acid agarose gel. RNA in the gel was capillary transferred to Hybond-N membranes (Amersham Pharmacia, Amersham, Bucks., UK) then immobilised by u.v. crosslinking. mYspl3/mSVCT1 and mSVCT2 cDNA fragments were labelled with [α-^{32}P]dCTP and hybridised with the blot. The mRNA signals were detected and analysed using a BAS1500 Mac radio imaging system (FUJI FILM, Tokyo, Japan). All membranes were reprobed with a ^{32}P-labelled DNA probe for mouse 18S rRNA as an internal control.

Statistical analysis

The data were analysed by ANOVA and the Dunnett multiple comparisons test.

Results

The uptake of AsA into MC3T3-E1 cells was originally reported by Franceschi *et al.* (1994). We confirmed AsA-specific and Na^+-dependant transport under our experimental conditions. MC3T3-E1 cells were incubated with 25 μM-L-[^{14}C]AsA for various periods up to 60 min. The incorporated radioactivity increased linearly with the incubation until 60 min. Total incorporation as well as specific incorporation of L-[^{14}C]AsA increased in a concentration-dependent manner. The apparent K_m values and the V_{max} for AsA in the presence of 137 mM-Na^+ were estimated as 50·5 μM and 462 pmol/10^5 cells per/h, respectively. Na dependence was also confirmed in our experimental conditions.

Little has been published regarding the stimulation of AsA transport by endogenous factors in osteoblastic cells (Wilson & Dixon, 1995; Pandipati *et al.* 1998). MC3T3-E1 cells were incubated for 24 h with eight compounds indicated in Fig. 1, all of which are supposed to promote osteoblast differentiation and/or proliferation. The maximum stimulative concentrations were based on the results of differentiation- and proliferation-inducing experiments (Kumegawa *et al.* 1983; Tsunoi *et al.* 1984; Hakeda *et al.* 1985; Katagiri *et al.* 1990; Amarnani *et al.* 1993; Kanatani *et al.* 1993; Kaji *et al.* 1995; Kasperk *et al.* 1995). The uptake of L-[^{14}C]AsA was significantly increased by Dex (10^{-7} M), all-*trans* retinoic acid (10^{-7} M), transforming growth factor β (10 ng/ml), transferrin (10^{-7} M) and prostaglandin E_2 (10^{-7} M) (Fig. 1). The greatest response was elicited by Dex. In addition to Dex and transforming growth factor β (Wilson & Dixon, 1995; Pandipati *et al.* 1998), we found three more factors that stimulate AsA uptake in osteoblastic cells.

To examine whether the stimulative effect of Dex on AsA uptake is mediated by the induction of mYspl3/mSVCT1 and/or mSVCT2, we examined mSVCT mRNA expression in MC3T3-E1 cells. MC3T3-E1 cells cultured with Dex for 24 h showed a dose-dependent increase of mSVCT2 mRNA (Fig. 2(A)). On the other hand, mYspl3/mSVCT1 mRNA was not detected in MC3T3-E1 cells. The Dex-induced

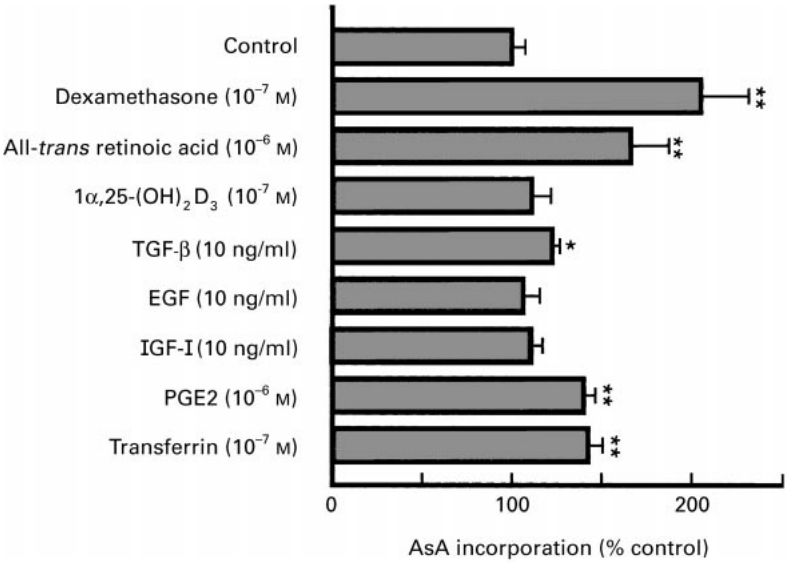

Fig. 1. Effect of hormones on ascorbic acid (AsA) uptake of MC3T3-E1 cells. MC3T3-E1 cells were incubated with indicated concentrations of dexamethasone, all-*trans* retinoic acid, 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2D_3$), transforming growth factor (TGF) β, epidermal growth factor (EGF), insulin-like growth factor (IGF)-I, prostaglandin E_2 (PGE2), and transferrin for 24 h. Thereafter, cells were incubated with 25 μM-L-[^{14}C] AsA for 1 h and levels of incorporated radioactivity were determined. Specific incorporation in control cells was $1{\cdot}11 \times 10^3$ (SD $0{\cdot}04 \times 10^3$) Bq/well per h. Values are expressed relative to the control and are the mean values and standard deviations of at least three cultures. Mean values were significantly different from those of the control culture: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

induction of mSVCT2 mRNA was consistent with the results of AsA-uptake into 48 h-treated cells in the same concentration range. We also examined the time course of the upregulated mSVCT2 mRNA expression and AsA uptake induced by Dex in MC3T3-E1 cells. The mSVCT2 mRNA level was upregulated in MC3T3-E1 cell incubated for 4–8 h with 10^{-6} M-Dex and the expression of mSVCT2 mRNA was time-dependant (Fig. 2(B)). The AsA uptake also increased a few hours after the induction of mSVCT2 mRNA. We did not detect mYspl3/mSVCT1 mRNA in the present study. Alkaline phosphatase activity, a differentiation marker of osteoblasts, in PEG-*p*-isooctylphenyl ethyl (NP-40) extract was determined in the Dex-treated cells. Dex (0·1 μM) reduced alkaline phosphatase activity time-dependently (75 % and 58 % of control at 24 and 48 h, respectively).

Discussion

Two cDNA encoding the transporters rSVCT1 and rSVCT2, have been isolated from a rat kidney cDNA library (Tsukaguchi *et al.* 1999). These transporters possess high affinity and specificity to AsA and are driven by a Na^+ gradient. The cDNA sequences of hSVCT1 and hSVCT2 were later identified as the human homologues of rSVCT1 and rSVCT2 respectively (Rajan *et al.* 1999; Wang *et al.* 2000). We isolated cDNA for the mouse homologues of rSVCT, mYspl3/mSVCT1 and mSVCT2. The sequences of mSVCT showed high similarity to the rat and human clones. Tsukaguchi *et al.* (1999) have shown that not SVCT1 but SVCT2 is predominantly expressed in osteoblasts. Our result from control culture supports their observation (Fig. 2). The cells in which Dex enhanced the uptake of AsA did not appear to express mSVCT1 mRNA. These findings show that the transport of AsA in MC3T3-E1 cells is driven by the Na^+ gradient-dependant AsA-specific transporter, SVCT2. The apparent K_m value of rSVCT2 for AsA is 30 μM (Tsukaguchi *et al.* 1999) and the estimated value of murine MC3T3-E1 cells was 50 μM in the present study. The reported concentration of AsA in plasma and extracellular fluid is about 50 μM (Hornig, 1975). Therefore, SVCT2 would act as a transporter *in vivo*, and the expressional change of SVCT2 would influence the cellular concentration of AsA *in vivo*.

Osteoblastic cells can synthesise extracellular matrix and cause it to differentiate (Gerstenfeld *et al.* 1987). Ascorbic acid relates not only to the formation of collagen but also to the differentiation of osteoblasts (Franceschi *et al.* 1994). The activity of the osteoblast-specific transcription factor, which is essential for osteoblast function, is stimulated by AsA (Ducy *et al.* 1997). Therefore, changes in the AsA transport should have profound effects on osteoblast function.

Several factors such as hormones and growth factors also affect osteoblastic cell function. For example, Dex or transforming growth factor β can stimulate not only the synthesis of extracellular matrix but also Na^+-dependant L-[^{14}C]AsA uptake (Wilson & Dixon, 1995; Pandipati *et al.* 1998). However, the relationship between these actions and the expression of AsA transporters was unknown. The

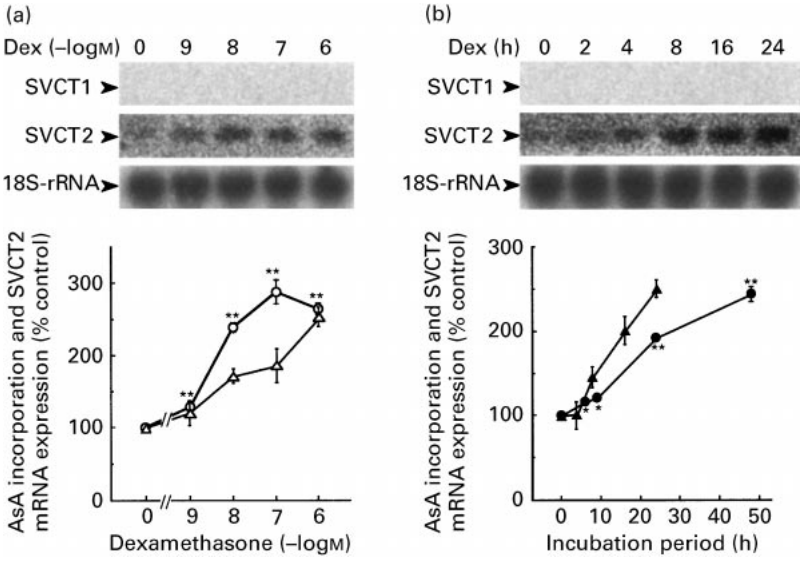

Fig. 2. Time- and dose-dependent induction of ascorbic acid (AsA) uptake and sodium-dependant vitamin C transporter (SVCT) mRNA expression by dexamethasone (Dex). (a) MC3T3-E1 cells were incubated with indicated concentrations of Dex for 24 h (Northern blotting) and 48 h (uptake assay). Thereafter, Northern blotting (upper panel) and L-[^{14}C] AsA uptake assays (lower panel, ○) were performed. Probes were ^{32}P-labelled cDNA fragments of mouse yoke-sac permease-like molecule-3 (mYspl3)/mSVCT1 and mSVCT2. Quantification of SVCT bands was performed and the results were shown as fold increase in the lower panel (△). Incorporated radioactivity was normalized with protein contents per well. Control cells specifically incorporated $6{\cdot}67 \times 10^4$ (SD $0{\cdot}03 \times 10^4$) Bq/mg protein per h. (b) MC3T3-E1 cells were incubated with 10^{-6} M-Dex for indicated periods. Thereafter, Northern blotting (upper panel) and L-[^{14}C]AsA uptake assays (lower panel, ●) were performed. The results of quantified SVCT bands are shown in the lower panel (▲). Control cells specifically incorporated $8{\cdot}10 \times 10^4$ (SD $0{\cdot}19 \times 10^4$) Bq/mg protein per h. Values are expressed relative to the controls, and are the mean values and standard deviations of three cultures. Mean values were significantly different from those of the control culture: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

present study showed that Dex-induced stimulation of AsA uptake is due to the enhanced expression of mSVCT2. Other than Dex and transforming growth factor β, we found that all-*trans* retinoic acid, prostaglandin E_2 and transferrin also induce the AsA-transport activity of osteoblastic cell (Fig. 1). It is important to define the SVCT-inducing activity of these AsA-transport inducers.

Glucocorticoid at high concentrations has potent long-term antiproliferative effects on mesenchymal cells, including bone cells (Kasperk *et al.* 1995; Ishida *et al.* 1996). In the present study, 1 μM Dex inhibited about 30 % of the protein content compared with control *in vitro* (results not shown). At low concentrations, short-term experiments have shown that glucocorticoids stimulate osteoblast proliferation (Kasperk *et al.* 1995). Thus, the effects of glucocorticoid may be related not only to dose and treatment duration but also to the degree of cellular differentiation. At physiological concentrations, glucocorticoid provides a basal stimulus for differentiation, ensuring not only a continuous stream of mesenchymal stem cells maturing into proliferating osteoblastic precursor cells, but also their differentiation into osteoblasts. Although a promoter sequence of mSVCT2 has not yet been identified, we speculated that glucocorticoid-responsive element is located in the promoter region of the mSVCT2 gene. We also speculate that another mechanism is responsible for the Dex-induced expression of mSVCT2. Levels of the differentiation-related transcription factor, osteoblastic-specific transcription factor, are increased by Dex (Chang *et al.* 1998) and this factor may regulate mSVCT2 mRNA expression.

In our experimental conditions, alkaline phosphatase activity in MC3T3-E1 cells was reduced by Dex. Therefore, Dex-induced expression of SVCT2 is not a result of osteoblastic differentiation. The role of SVCT in osteoblast differentiation and the molecular mechanism of the glucocorticoid-induced expression of SVCT2 remain to be elucidated. AsA must be involved in the growth and function of various cells, rather than only those of osteoblastic lineage. Regulation of the expression of the SVCT in other cells should also be investigated.

Acknowledgements

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