Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid

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Otsuka, Eri, Akira Yamaguchi, Shige Hisa Hirose, and Hiromi Hagiwara. Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. Am. J. Physiol. 277 (Cell Physiol. 46): C132–C138, 1999.—The stromal cell line ST2, derived from mouse bone marrow, differentiated into osteoblast-like cells in response to ascorbic acid. Ascorbic acid induced alkaline phosphatase (ALPase) activity, the expression of mRNAs for proteins that are markers of osteoblastic differentiation, the deposition of calcium, and the formation of mineralized nodules by ST2 cells. We investigated the mechanism whereby ascorbic acid induced the differentiation of ST2 cells. Inhibitors of the formation of collagen triple helices completely blocked the effects of ascorbic acid on ST2 cells, an indication that matrix formation by type I collagen is essential for the induction of osteoblastic differentiation of ST2 cells by ascorbic acid. We furthermore examined the effects of bone morphogenetic proteins (BMPs) on the differentiation of ST2 cells induced by ascorbic acid. Ascorbic acid had no effect on the expression of mRNAs for BMP-4 and the BMP receptors. However, a soluble form of BMP receptor IA inhibited the induction of ALPase activity by ascorbic acid. These results suggest that ascorbic acid might promote the differentiation of ST2 cells into osteoblast-like cells by inducing the formation of a matrix of type I collagen, with subsequent activation of the signaling pathways that involve BMPs.

ST2 cell; osteoblast; type I collagen; alkaline phosphatase; bone morphogenetic protein

ST2 cells, a clone of stromal cells, were isolated from the bone marrow of BC8 mice and have characteristics typical of preadipocytes (13). They do not exhibit an osteoblastic phenotype in standard cultures. ST2 cells are generally maintained in RPMI 1640 medium, which does not include ascorbic acid. However, when we subcultured ST2 cells in RPMI 1640 medium that had been supplemented with ascorbic acid, they developed an osteoblastic phenotype.

Ascorbic acid is necessary for the expression of osteoblastic markers and for mineralization in a variety of osteoblastic culture systems that include primary cultures of fetal rat calvarial cells (1), chick preosteoblasts (7), and osteoblast-like cell lines (5, 6, 17, 18). In particular, MC3T3-E1 cells, derived from mouse calvaria, have been used to study the role of ascorbic acid in osteoblast differentiation (5, 6, 17–19). The addition of ascorbic acid to cultures of clonal preosteoblastic MC3T3-E1 cells stimulates the deposition of a collagenous extracellular matrix, followed by the induction of specific genes associated with the osteoblastic phenotype, such as genes for alkaline phosphatase (ALPase) (5, 6) and osteocalcin (5, 6, 22).

ST2 cells also differentiate into osteoblast-like cells when bone morphogenetic proteins (BMPs) are added to the culture medium (12, 23). These proteins belong to the transforming growth factor-β superfamily, and they induce the development and differentiation of bone cells and the formation of bone (9, 10, 21, 24). In mesenchymal cells, BMPs are thought to induce stem cell precursors to commit to the osteoblast lineage and to express the osteoblast phenotype. However, there is little evidence for interactions between the effects of ascorbic acid and those of BMPs.

In the present study, we found that ST2 cells that had been cultured with ascorbic acid exhibited characteristics typical of osteoblasts, with induction of the expression of mRNAs for marker proteins of osteoblastic differentiation and the formation of mineralized nodules. We attempted to characterize the mechanism whereby ascorbic acid induces the osteoblastic differentiation of ST2 cells by using the induction of ALPase activity as an index. The effects of ascorbic acid were inhibited by inhibitors of the formation of triple helices of type I collagen. Furthermore, we obtained evidence to suggest that the ascorbic acid-induced differentiation of ST2 cells might involve BMPs and their receptors.

MATERIALS AND METHODS

Materials. L-Ascorbic acid was obtained from Wako Pure Chemical Industries, Osaka, J apan. 3,4-Dehydro-L-proline (DHP) and cis-4-hydroxy-L-proline (CHP) were purchased from Sigma (St. Louis, MO). Recombinant human BMP-2 produced in Chinese hamster ovary cells was provided by Yamanouchi Pharmaceutical (Tsukuba, J apan). Soluble BMP receptor IA (sBMPR-IA) was kindly provided by Drs. N. Ueno and S. Iemura (National Institute for Basic Biology, Okazaki, J apan).32P-labeled nucleotides were obtained from American Pharmacia Biotech (Buckinghamshire, UK). RPMI 1640 medium, fetal bovine serum, and a penicillin-streptomycin antibiotic mixture were obtained from Life Technologies (Grand Island, NY).

Cell culture. ST2 cells were obtained from the RIKEN Cell Bank (Tsukuba, J apan). Cells were maintained in 55-cm2 dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. After reaching 70% confluence, the cells were detached by treatment with 0.05% trypsin, replated in 55-cm2 dishes, 6-well plates (9.4 cm2/well), and 12-well plates (3.8 cm2/well) at a density of 1 × 104 cells/cm2 and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicil-
lin, 50 µg/ml streptomycin, 5 mM β-glycerophosphate, and various concentrations of ascorbic acid. During subculture, the medium was replaced every 3 days, and fresh ascorbic acid was added to the medium just before use. Northern blot analysis. RNA was extracted from ST2 cells by the acid guanidinium-phenol-chloroform method (2). Total RNA (20 µg) was subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde and was then transferred to a MagnaGraph nylon membrane (Micron Separations, Westborough, MA). After the membrane was baked, the RNA on the membrane was allowed to hybridize overnight with cDNA for ALPase, osteocalcin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 42°C in 50% formamide that contained 6x SSPE (1x SSPE is 0.15 M NaCl, 15 mM NaH2PO4, pH 7.0, and 1 mM EDTA), 2x Denhardt's solution [BSA, polyvinylpyrrolidone, and Ficoll (0.1%) each], 1% SDS, and 100 µg/ml herring sperm DNA. Each cDNA probe was radiolabeled with a Ready-to-Go kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was washed twice in 1x SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% SDS at room temperature for 5 min each and twice in 1x SSC that contained 0.1% SDS at 55°C for 1 h each, and then it was exposed to an imaging plate for 4 h. The plate was analyzed with a Bioimage analyzer (BAS 2000; Fuji Film, Tokyo, J apan).

Staining of ALPase and von Kossa staining. ST2 cells were subcultured in RPMI 1640 medium that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and various concentrations of ascorbic acid. The cells were fixed with 10% formalin for 30 min and washed three times with 10 mM Tris-HCl, pH 7.2. Fixed cells were subjected to staining for ALPase and von Kossa staining. ALPase was stained with naphthol AS-MX phosphate and fast blue BB salt (Sigma). The von Kossa staining for calcium was performed as follows. Fixed cells were incubated with 5% silver nitrate for 5 min in daylight, washed twice with H2O, and then treated with 5% sodium thiosulfate. Mineralized nodules were counted under a BH microscope (Olympus, Tokyo, J apan).

Assay of ALPase activity. Cells were subcultured in RPMI 1640 medium that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and various concentrations of ascorbic acid. The cells were fixed with 10% formalin for 30 min and washed three times with 10 mM Tris-HCl, pH 7.2, and sonicated in 1 ml of 50 mM Tris·HCl, pH 7.2, that contained 0.1% Triton X-100 and 2 mM MgCl2 for 15 s with a sonicator (ultrasonic disrupter UD-201; Tomy, Tokyo, J apan). The ALPase activity of the sonicate was determined by an established technique with p-nitrophenyl phosphate as the substrate (8). Concentrations of protein were determined with bichoninic acid protein assay reagent (Pierce Chemical, Rockford, IL), with BSA as the standard.

Quantitation of calcium. Mineralized nodules from a 21-cm2 dish were washed twice with PBS and incubated overnight in 2 ml of 2 N HCl with gentle shaking. The calcium ions in the sample were quantitated by the o-cresolphthalein Complexone method with a Calcium C kit (Wako Pure Chemical Industries) (8).

RT-PCR. We detected mRNAs for BMPs and BMP receptors (BMPRs) in ST2 cells by RT-PCR. Total RNA (5 µg) isolated from ST2 cells was reverse transcribed by Moloney murine leukemia virus RT (200 U; Superscript; Life Technologies), with random primers (50 ng) and a 20-µl reaction mixture. The cDNA was amplified during 30 cycles of PCR in 20 µl of Taq DNA polymerase mixture (Takara, Tokyo, J apan) that contained 250 nM sense primer 5'-CGGGAACAGATA-CAGGAAGC-3' and antisense primer 5'-CCCTTACCATCTCCTGA-3' for mouse BMP-4 (493 bp), 250 nM sense primer 5'-AGAAGAGGTGGCTGGAAT-3' and antisense primer 5'-ACAGTGGTGCCAGG-CAGTAGC-3' for mouse BMP-6 (421 bp), 250 nM sense primer 5'-GGTTCTCTTACCCCTACAA-3' and antisense primer 5'-GTGGTGTGCTGTTGCTTGA-3' for mouse BMP-7 (399 bp), 250 nM sense primer 5'-TAGCACCAGAGGACA TCTCC-3' and antisense primer 5'-AATGCTTACCTGTTCCAAA-3' for mouse BMPR-IA (448 bp), 250 nM sense primer 5'-TGTCGATACCGGACACG-3' and antisense primer 5'-TCCAGCCACATTTCATCA-3' for mouse BMPR-IB (193 bp), or 250 nM sense primer 5'-AACAAAGCCTGTGAT-3' and antisense primer 5'-CGACCTGTTGAGGACTGTTT-3' for mouse BMPR-II (473 bp). The reaction cycle consisted of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. mRNA from mouse embryos (day 14), which express mRNAs for all BMPs and their receptors, was used as a positive control. Products of PCR were subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. DNA markers (molecular weight marker V; Boehringer Mannheim, Tokyo, J apan) were used as size markers.

Statistical analysis. Data are expressed as means ± SE of results from three or four wells for each experiment. Statistical significance was determined by the unpaired Student t-test.

RESULTS

Stimulation of ALPase activity in ST2 cells by ascorbic acid. After 18 days in control culture in RPMI 1640 medium, ST2 cells had very low levels of ALPase activity. However, ascorbic acid (50 µg/ml) stimulated ALPase activity about 5-fold. This effect was more pronounced with concentrations of ascorbic acid ranging from 10 µg/ml to 50 µg/ml. At 50 µg/ml ascorbic acid, the ALPase activity was about 10-fold higher than in control cultures. In a dose-response experiment, ALPase activity was stimulated by concentrations of ascorbic acid ranging from 10 µg/ml to 75 µg/ml. At concentrations of 50 µg/ml and above, the stimulation was significant (p < 0.05). The stimulation of ALPase activity was not accompanied by an increase in the number of progenitor cells, as judged by the number of colonies formed on feeder layers.

The results of this experiment are shown in Figure 1. The figure shows the stimulation of ALPase activity in ST2 cells by ascorbic acid. The stimulation is dose-dependent, with a maximum response at 50 µg/ml ascorbic acid. The stimulation is not accompanied by an increase in the number of progenitor cells, as judged by the number of colonies formed on feeder layers.
activity (Fig. 1A). However, ALPase activity increased markedly when the cells were subcultured in RPMI 1640 medium with 50 µg/ml ascorbic acid (Fig. 1, B and D). β-Glycerophosphate (5 mM) was not involved in the activation of ALPase in ST2 cells (Fig. 1, C and D), even though β-glycerophosphate has been reported to increase the expression of ALPase in osteoblastic cells (11). As shown in Fig. 2, the induction of ALPase activity by ascorbic acid in ST2 cells was time and dose dependent. ALPase activity was markedly induced by ascorbic acid at 10 days after the latter had been added to the culture medium, and the ALPase activity (52.3 ± 1.9 µmol·mg protein⁻¹·30 min⁻¹) in ST2 cells that had been treated with 50 µg/ml ascorbic acid for 18 days was 86-fold higher than the control value (0.61 ± 0.01 µmol·mg protein⁻¹·30 min⁻¹).

Northern blot analysis revealed that ascorbic acid increased the steady-state levels of mRNAs for proteins that are markers of osteoblastic differentiation. As shown in Fig. 3, the addition to the culture medium of 50 µg/ml ascorbic acid increased the expression of mRNA for ALPase 1.6-, 6.9-, and 4.7-fold, and that of mRNA for osteocalcin 1.4-, 5.8-, and 30.6-fold, at days 5, 10, and 20, respectively.

Induction of the deposition of calcium and the formation of mineralized nodules by ascorbic acid. We measured the deposition of calcium by ST2 cells that had been treated for 24 days with ascorbic acid at various concentrations in 21-cm² dishes (Fig. 4). Ascorbic acid enhanced the accumulation of calcium by ST2 cells in a dose-dependent manner at levels above 25 µg/ml. The maximal accumulation, at 75 µg/ml ascorbic acid, was 18.9 ± 3.2 µg calcium/well, and this level was nine times the control level (2.1 ± 1.6 µg calcium/well).

We next examined whether ascorbic acid could induce the formation of mineralized nodules. As shown in Fig. 5, mineralized nodules developed in cultures supplemented with ascorbic acid for 24 days. In control cultures, no nodules were formed during the same 24-day period. Ascorbic acid at 50 µg/ml induced the formation of 21.0 ± 1.5 (n = 3) mineralized nodules per 55-cm² dish. We confirmed that nodules formed by ST2 cells contained type I collagen by immunostaining with a specific antibody against type I collagen (results not shown).

Effects of type I collagen on the differentiation of ST2 cells. Ascorbic acid has been shown to induce the formation of collagen triple helices via the hydrox-
ylation and processing of procollagen during the differentiation in vitro of cells to osteoblasts (4). Therefore, we examined the effects of two inhibitors of the formation of such triple helices, DHP and CHP, on the ALPase activity of ST2 cells that had been treated with 50 µg/ml ascorbic acid. As shown in Fig. 6A, DHP and CHP at 0.5 mM reduced ALPase activity by 80 and 40%, respectively, compared with the control level on day 18. DHP inhibited ALPase activity more effectively than did CHP. DHP at 1 mM completely inhibited the ALPase activity of ST2 cells (Fig. 6B).

Type I collagen interacts with various types of cells through the binding of the DGEA motif to α2β1-integrin (17, 18) and facilitates differentiation (17). We examined the effect of a DGEA peptide on the induction of ALPase activity by ascorbic acid. As shown in Fig. 7, the addition of peptide at 5 mM blocked ~60% of the ALPase activity induced by ascorbic acid.

Identification of the BMPs and corresponding BMPRs in ST2 cells and the role of BMPs in the differentiation induced by ascorbic acid. We performed RT-PCR with specific primers for BMP and BMPR mRNAs to identify BMP systems in ST2 cells. Our results showed that the mRNAs for BMP-4 and for BMPR-IA and -II were present in ST2 cells during culture for 20 days (Fig. 8). During this period, ascorbic acid (50 µg/ml) had no effect on the expression of mRNAs for BMP-4 and BMPR-IA and -II. Exogenous BMP-2 dose dependently enhanced ALPase activity in ST2 cells, in the absence of ascorbic acid (Fig. 9). In the presence of 50 µg/ml ascorbic acid, the effects of BMP-2 were enhanced still further and were synergistic. These results indicated that ascorbic acid did not affect the expression of BMPs and their receptors but acted to increase the efficiency of the BMP system. Figure 10 shows the dose-dependent inhibition by a soluble form of BMPR-IA (sBMPR-IA) on the ALPase activity that was induced by ascorbic acid in ST2 cells. sBMPR-IA is an extracellular domain of the type Ia receptor for BMP-2 and BMP-4 and binds to BMP-4 (12). ST2 cells had low ALPase activity in the absence of ascorbic acid (control culture). However, when the cells were cultured in the presence of ascorbic acid (50 µg/ml), the level of ALPase activity increased to four times that in the control culture. sBMPR-IA at 1 µg/ml is enough to inhibit BMP-4 secreted from ST2 cells and inhibited 40% of the ascorbic acid-induced ALPase activity.

DISCUSSION

Osteoblasts, chondrocytes, adipocytes, myoblasts, and fibroblasts that form connective-tissue cells are thought to arise from a common population of mesenchymal stem cells whose progeny become committed to specific
lineages in response to growth factors, hormones, and other signals. Such signals induce the tissue-specific transcription that is necessary for the expression of each differentiated phenotype. In normal cultures, stromal ST2 cells have characteristics of preadipocytes and none of the features typical of the osteoblastic phenotype (23). In this study, we found that ascorbic acid induced the osteoblastic differentiation of ST2 cells. To our knowledge, this is the first report of the ascorbic acid-induced osteoblastic differentiation of stromal ST2 cells. We also demonstrated that the formation of a collagen-containing extracellular matrix in response to ascorbic acid was essential for expression of the osteoblastic phenotype of ST2 cells. Furthermore, we showed that signaling pathways involving BMP might participate in the differentiation of ST2 cells that is induced by ascorbic acid.

Ascorbic acid stimulated the expression of genes for proteins associated with the osteoblast phenotype, such as ALPase and osteocalcin, and it increased ALPase activity.

Fig. 7. Suppression by DGEA peptide of the induction of ALPase activity by ascorbic acid. ST2 cells in 24-well plates (2 cm²/well) were cultured with RPMI 1640 that contained 10% fetal bovine serum and 50 µg/ml ascorbic acid or 5 mM DGEA peptide, as indicated. DGEA peptide was added to the culture medium of ST2 cells on day 3 of culture, and ALPase activity was measured on day 9. Data are means ± SE of results from 3 different wells. *P < 0.001 vs. results obtained in absence of DGEA and ascorbic acid.

Fig. 8. Detection of subtypes of bone morphogenetic protein (BMP) and BMP receptor (BMPR) in ST2 cells and effects of ascorbic acid on expression of BMPs and BMPRs. Total RNA was isolated from ST2 cells after they had been cultured with 50 µg/ml ascorbic acid for 5, 10, and 20 days, and products of RT-PCR amplification were analyzed. Reaction cycles and sequences of specific primers are described in MATERIALS AND METHODS. RNA from mouse embryos on day 14 was used as the standard sample.

Fig. 9. Stimulation of the expression of ALPase by BMP-2 and/or ascorbic acid in ST2 cells. ST2 cells in 12-well plates (3.8 cm²/well) were cultured for 6 days with RPMI 1640 that contained 10% fetal bovine serum and BMP-2 at various concentrations in absence and presence of 50 µg/ml ascorbic acid. ALPase activity was determined as described in MATERIALS AND METHODS. Data are means ± SE of results from 3 different wells. *P < 0.005 vs. control; **P < 0.0001 vs. control.

Fig. 10. Inhibitory effects of soluble BMPR-IA (sBMPR-IA) on ALPase activity in ST2 cells cultured with ascorbic acid. ST2 cells in 24-well plates (2 cm²/well) were cultured in absence and presence of 50 µg/ml ascorbic acid. sBMPR-IA at indicated concentrations was included in culture medium of ST2 cells for 9 days, and ALPase activity was determined as described in MATERIALS AND METHODS. Data are means ± SE of results from 3 different wells. *P < 0.05 vs. control; **P < 0.01 vs. control.
activity and the deposition of calcium by ST2 cells. These events are the hallmarks of cells that are committed to the osteoblast lineage. We showed that ascorbic acid has the potential to induce these events in ST2 cells at concentrations from 5 to 25 µg/ml for the activation of ALPase and from 25 to 75 µg/ml for the deposition of calcium. The normal concentration of ascorbic acid in human plasma ranges from 5 to 10 µg/ml. Our results suggest that the effect of ascorbic acid on ST2 cells might be physiological and that ascorbic acid might be necessary for the differentiation of stromal cells into osteoblast-like cells.

Ascorbic acid has been shown to stimulate the formation of a collagen matrix at multiple levels that include gene transcription, hydroxylation, and the processing of procollagen. Type I collagen in the extracellular matrix mediates the differentiation in vitro of preosteoblast MC3T3-E1 cells (5, 6, 17, 18) and of osteoprogenitor ROB-C26 cells (16). Franceschi and Iyer (5) and Franceschi et al. (6) showed that ascorbic acid increases the levels of mRNAs for ALPase and osteocalcin through the production of collagen in MC3T3-E1 cells. Takeuchi et al. (17, 18) also demonstrated that collagen formed in response to ascorbic acid causes the differentiation of MC3T3-E1 cells and downregulation of the expression of the receptor for transforming growth factor-β in MC3T3-E1 cells. We showed, in this study, that inhibitors of the formation of a collagen matrix completely blocked the induction of ALPase activity by ascorbic acid (Fig. 6). These observations strongly suggested that type I collagen itself and/or the type I collagen-containing extracellular matrix formed in response to ascorbic acid might be essential for the osteoblastic differentiation of ST2 cells. Therefore, we attempted to identify the signaling pathways that are operative after the formation of the type I collagen-containing extracellular matrix that occurs in response to ascorbic acid.

The interaction of integrins with matrix proteins has various effects on the proliferation and differentiation of cells via the activation of a variety of signal transduction pathways (3). The α2β1-integrin serves as a collagen-specific receptor, and matrix collagen interacts with a variety of cells through the specific binding of the DGEA motif to α2β1-integrin on cells (17). It has been reported that the interaction between collagen and integrin induces the differentiation of preosteoblastic MC3T3-E1 cells that are committed to the osteoblastic phenotype (18). We also examined the effect of a DGEA peptide on the induction of ALPase activity by ascorbic acid. The addition of the peptide at 5 mM blocked ~60% of the ALPase activity induced by ascorbic acid. Our results suggest that the matrix of type I collagen formed in response to ascorbic acid directly promotes osteoblastic differentiation of ST2 cells. Takeuchi et al. (18) reported that, after the attachment of MC3T3-E1 cells to collagen, integrin activates the focal adhesion kinase and the mitogen-activated protein kinase pathway. We next tried to determine whether pathways involving BMPs are operative in the ascorbic acid-induced osteoblastic differentiation of ST2 cells.

BMPs have been reported to induce the differentiation of mesenchymal cells into osteoblastic cells (10, 15, 23, 24). Clonal preosteoblast MC3T3-E1 cells express the osteoblastic phenotype, for example, they have elevated ALPase activity when they respond to BMPs that they produce themselves (12). By contrast, ST2 cells express the osteoblastic phenotype when BMPs are added to the culture medium (12, 23, 25). BMPs stimulate the expression of mRNAs for proteins that are markers of osteoblastic differentiation, such as ALPase and osteocalcin, in ST2 cells. Moreover, ST2 cells generated mineralized bone after transplantation with BMP into the peritoneal cavities of athymic mice (23). These results indicate that the signal transduction pathways distal to BMPRs are operative in ST2 cells. However, the mechanism of induction of differentiation of ST2 cells by BMPs has not been fully clarified. We examined whether BMPs might be involved in the osteoblastic differentiation of ST2 cells that is induced by ascorbic acid. We first identified the subtypes of BMP and BMPR in ST2 cells because their expression differs from cell to cell. ST2 cells expressed mRNAs for BMP-4 and for BMPR-IA and -II (Fig. 8). Ascorbic acid had little effect on the levels of mRNAs for BMP-4 and the receptors during the differentiation of ST2 cells. However, as shown in Fig. 9, ALPase activity was synergistically elevated in ST2 cells when ascorbic acid was added with BMP-2 to the culture medium. These results suggest that ascorbic acid can potentiate the actions of BMPs on ALPase activity. To investigate whether BMPs might actually be involved in the ascorbic acid-induced differentiation of ST2 cells, we added sBMPR-IA, the extracellular domain of the type I receptor for BMP-2 and -4 (12), with ascorbic acid to the culture medium. The added soluble BMPR is considered to bind BMP-4 that is secreted by ST2 cells into the culture medium. sBMPR-IA caused the dose-dependent inhibition of the induction of ALPase activity by ascorbic acid (Fig. 10). Although sBMPR-IA at 1 µg/ml was enough to completely inhibit the ALPase activity induced by exogenous BMP-4 (100 ng/ml) in ST2 cells (12), it inhibited only 40% of the ALPase activity induced by ascorbic acid. Our results suggest that part of the action of ascorbic acid might be due to BMP-4 produced by ST2 cells, even though ascorbic acid acts predominantly through the collagen-integrin pathway for the differentiation of ST2 cells. BMPs are stored in the extracellular matrix (19). Therefore, it is possible that BMP-4 might function more effectively when stored in the collagen-containing extracellular matrix that forms in response to ascorbic acid. The facts that the collagen matrix formed in response to ascorbic acid was essential for the differentiation of ST2 cells (Fig. 6B) and that ascorbic acid accelerated the effects of BMP (Fig. 9) strongly support this hypothesis.

In this report, we have demonstrated that ascorbic acid induces the osteoblastic differentiation of ST2 cells. This process requires the formation of a collagen-containing extracellular matrix in response to ascorbic acid. Furthermore, we obtained evidence that suggests
that the ascorbic acid-induced differentiation of ST2 cells might be due to the BMP system in addition to the interaction between collagen and \(\alpha_2\beta_1\)-integrin. Commercial medium may or may not contain ascorbic acid. Similar situations might occur with other components and with other types of cells, and therefore such possibilities should be kept in mind when cultured cell systems are used. ST2 cells retain the potential to support osteoclast differentiation (20). A very recent study showed that ascorbic acid is necessary for osteoclast differentiation in cocultures of bone marrow cells and ST2 cells (14). Our present results suggest that ascorbic acid might induce ST2 cells at a particular stage of differentiation to support osteoclast differentiation.

We thank Drs. N. Ueno and S. Iemura (National Institute for Basic Biology, Okazaki, Japan) for the generous gift of the sBMPR-IA used in this study. A. Torii (Tokyo Institute of Technology, Yokohama, Japan) for helpful discussions.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and by grants from the Smoking Research Foundation and Ground Research Announcement for Space Utilization promoted by NASDA and the Japan Space Forum. Address for reprint requests and other correspondence: H. Hagiwar, Research Center for Experimental Biology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japam (E-mail: hhagiwar@bio.titech.ac.jp).

Received 21 September 1998; accepted in final form 2 April 1999.

CITED REFERENCES


