

Differentiation and Cell Surface Expression of Transforming Growth Factor- β Receptors Are Regulated by Interaction with Matrix Collagen in Murine Osteoblastic Cells*

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Although transforming growth factor (TGF)- β enhances bone formation, it inhibits the differentiation of osteoblasts. To clarify the regulatory mechanism of osteoblastic differentiation and TGF- β actions, the relationship among differentiation, TGF- β actions, and matrix protein synthesis was examined using murine osteoblast-like MC3T3-E1 cells. Alkaline phosphatase (ALP) activity continued to increase during long-term cultures, and the increase was closely associated with a reduction in cell surface TGF- β receptors competent to bind TGF- β . Both the stimulation of proteoglycan synthesis and the inhibition of ALP activity by TGF- β were also suppressed. Collagen synthesis inhibitors and an anti- $\alpha 2\beta 1$ integrin blocking antibody blocked the changes in ALP activity and TGF- β receptors, and a DGEA peptide that interferes binding of collagen to $\alpha 2\beta 1$ integrin also blocked the increase in ALP activity. Furthermore, when MC3T3-E1 cells were cultured on extracellular matrix layers obtained from these cells, all the differentiation-associated changes could be observed without collagen production, and the extracellular matrix-induced differentiation was also blocked by an anti- $\alpha 2\beta 1$ integrin antibody. These results demonstrate that the interaction of cell surface $\alpha 2\beta 1$ integrin with matrix collagen synthesized by osteoblasts themselves is involved in the osteoblastic differentiation and the reduction in cell surface receptors and actions of TGF- β . It is suggested that matrix collagen synthesized under the stimulation by TGF- β plays an important role in the regulation of osteoblastic differentiation and TGF- β actions by differentiation-associated down-regulation of TGF- β receptors.

Cells of osteoblast lineage exert various functions to maintain bone formation. After bone resorption, osteoblast precursors migrate and proliferate at the site of bone formation. They, then, synthesize type I collagen and other matrix proteins. Onto the newly formed unmineralized matrices, hydroxyapatite crystals are accumulated, and mineralization of bone is completed (1). In order to form lamellar bones that maintain

structural integrity and physical strength, it appears to be of critical importance for osteoblastic cells to maintain sequentially ordered development of these multiple functions.

Using cultured osteoblastic cells obtained from bone or of clonal origin, it has become clear that these cells express various functional properties in a differentiation-dependent manner from the early proliferation phase, via the matrix formation phase with active matrix protein synthesis, to the mineralization phase (1–3). Various hormones and cytokines are shown to affect the differentiation process and functional properties of these cells. However, the mechanism that controls the differentiation of osteoblastic cells is as yet unclear.

Transforming growth factor- β (TGF- β)¹ is stored in bone matrix as a latent form (4), is thought to be released and activated during osteoclastic bone resorption (5), and to play an important role in the regulation of bone metabolism (6). TGF- β is one of the most potent stimulators of the production of type I collagen in various types of cells (7, 8), and the most pronounced effect of TGF- β in bone is the stimulation of matrix protein synthesis including type I collagen, proteoglycans, and fibronectin (9, 10). Thus, TGF- β stimulates bone formation when given to bone *in vivo* (11, 12). In contrast, TGF- β inhibits the differentiation of many types of mesenchymal cells (13), and is also shown to prevent the development of differentiation-associated phenotypes such as the expression of alkaline phosphatase (ALP) (9) and osteocalcin (14) in cells of osteoblast lineage. Therefore, the actions of TGF- β have to be suppressed when osteoblastic cells differentiate from matrix formation phase into mineralization phase.

Osteoblast-like MC3T3-E1 cells in long-term culture exhibit various phenotypes in a sequential manner, and, after the avid production of matrix proteins, develop the capacity to induce mineralization (15, 16). However, when MC3T3-E1 cells were cultured in the absence of ascorbic acid, these cells cannot differentiate to induce mineralization (16). Because ascorbic acid is essential for collagen synthesis as a cofactor for prolyl-lysyl hydroxylase, the inhibition of differentiation by the removal of ascorbic acid may be due to a suppression of the synthesis of collagen. In addition, the expression of ALP is reported to be dependent upon the production of type I collagen in osteoblastic cell lines (3, 17).

From these previous observations, there is a possibility that the actions of TGF- β on collagen and other matrix protein synthesis and on the differentiation of these cells may be mutually regulated, and that the interaction of osteoblastic cells with matrix proteins including collagen may play an important role in the regulation of osteoblastic differentiation and TGF- β

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¹ The abbreviations used are: TGF- β , transforming growth factor- β ; ALP, alkaline phosphatase; PBS, phosphate-buffered saline; ECM, extracellular matrix; CHAPS, 3-(cyclohexylamino)propanesulfonic acid.

actions. The present studies are undertaken to clarify the relationship among the differentiation, TGF- β actions, and matrix collagen synthesis in osteoblasts using MC3T3-E1 cells in culture.

EXPERIMENTAL PROCEDURES

Materials— $[^{125}\text{I}]$ TGF- β 1 and $[^{32}\text{P}]\text{dCTP}$ were purchased from Amersham Japan and $[^{35}\text{S}]\text{sulfuric acid}$ from DuPont NEN. Human recombinant TGF- β 1 was obtained from R & D systems (Minneapolis, MN); hamster monoclonal antibody against mouse α 2 β 1 integrin (clone HM α 2) and against mouse α 5 β 1 integrin (clone HM α 5-1) from Sumitomo Denko Co. (Osaka, Japan); hamster non-immune IgG from Cedarlane Laboratories Ltd. (Ontario, Canada); disuccinimidyl suberate from Pierce; ascorbic acid and L-azetidine-2-carboxylic acid from Wako Pure Chemicals (Osaka, Japan); L-proline and sodium deoxycholate from Sigma; and Sephadex G-50 and Q-Sepharose were from Pharmacia Biotech Inc. (Tokyo, Japan). Human type II TGF- β receptor cDNA and rat ALP cDNA were generous gifts from Dr. K. Miyazono (Ludwig Institute for Cancer Research, Uppsala, Sweden) and Dr. G. A. Rodan (Merk, Sharp and Dohme, West Point, PA), respectively. DGEA and KIDGE peptides were synthesized by Drs. M. Hane and N. Tamura of Mitsubishi Chemical Co. (Tokyo, Japan). MC3T3-E1 cells were provided by Dr. H. Kodama (Ohu Dental College, Japan).

Cell Culture—MC3T3-E1 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin and with or without 50 mg/liter L-ascorbic acid. The medium did not contain phenol red, and was changed twice a week. Experiments were performed in the presence of 10% fetal bovine serum unless otherwise indicated. TGF- β 1 of 1 μM was dissolved in 17.5 mM acetic acid as a stock solution. Aliquots of 5 mg/ml ascorbic acid, 0.3 M L-azetidine-2-carboxylic acid, 0.5 M cis-4-hydroxyproline, 0.5 M 3,4-dehydroproline, and 0.3 M L-proline dissolved in distilled water were added to the culture medium as indicated.

Alkaline Phosphatase Activity in Osteoblastic Cell—Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped in 10 mM Tris-HCl containing 2 mM MgCl_2 and 0.05% Triton X-100, pH 8.2. The cell suspension was homogenized using Pellet Pestle (Kontes, Vineland, NJ) on ice following two cycles of freeze and thaw. Aliquots of supernatants were subject to protein assay with a Bio-Rad kit according to Bradford's method and to ALP activity measurement (15). In brief, the assay mixture contained 10 mM *p*-nitrophenyl phosphate in 0.1 M sodium carbonate buffer, pH 10, supplemented with 1 mM MgCl_2 , followed by an incubation at 37 °C for 30 min. After adding 0.1 M NaOH, the amount of *p*-nitrophenol liberated was measured by spectrophotometer.

Affinity Cross-linking Analyses of Cell Surface TGF- β Receptors—Osteoblastic cells in 10-cm culture dishes were washed twice with ice-cold PBS. Binding of 100 pM ^{125}I -TGF- β 1 (total radioactivity of approximately 200,000 cpm) to osteoblastic cells was performed in a buffer containing 128 mM NaCl, 5 mM KCl, 5 mM MgSO_4 , 1.3 mM CaCl_2 , and 25 mM Hepes, pH 7.4, containing 0.3% bovine serum albumin at 4 °C for 4 h as reported previously (18). After washing the cells with ice-cold PBS, bound ^{125}I -TGF- β 1 was chemically cross-linked in 2 ml of PBS containing 0.3 mM disuccinimidyl suberate at 4 °C for 20 min (19). Chemical reaction was terminated by adding 200 μl of quenching buffer (100 mM Tris-HCl, pH 7.5, 200 mM glycine, and 20 mM EDTA), and cells were kept at room temperature for 1 min. After washing twice with PBS, cells were scraped into a tube on ice containing 1 ml of PBS with 1 mM EDTA and a protease inhibitor mixture of 1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, and 10 $\mu\text{g}/\text{ml}$ pepstatin A. Cells were then collected by centrifugation, and solubilized in 40–100 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 1 mM EDTA, and protease inhibitors) for 40 min on ice. Lysates were centrifuged and supernatants were subject to SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel followed by autoradiography. Loading samples were adjusted to contain the same amount of protein. Some experiments were performed at least three times, and representative results from reproducible experiments are shown in the figures.

Northern Blot Analysis—Ten μg of total RNA from each osteoblastic cell culture was loaded on each lane of an agarose gel. Human type II TGF- β receptor cDNA (20) and rat ALP cDNA (21) were used as probes. Probes were radiolabeled with $[^{32}\text{P}]\text{dCTP}$ using the Megaprime DNA labeling system (Amersham) according to the manufacturer's instructions.

Assays for Proteoglycan Synthesis—Synthesis of proteoglycans by osteoblastic cells was determined as described previously (22, 23). Cells

were labeled with 100 $\mu\text{Ci}/\text{ml}$ $[^{35}\text{S}]\text{sulfate}$ for 1 h at 37 °C after treatment with various concentrations of TGF- β 1. Cell layer and media were simultaneously extracted with 4 M guanidine HCl, pH 6.0, containing 0.5% Triton X-100, 50 mM sodium acetate, 50 mM EDTA, and protease inhibitors (4 M guanidine HCl buffer). Unincorporated isotopes were removed by Sephadex G-50 column chromatography eluted with 7 M urea buffer containing 0.25 M NaCl, 50 mM sodium acetate, and 0.5% Triton X-100, pH 6.0. Void fractions containing proteoglycans were collected and applied onto freshly prepared Q-Sepharose column (bed volume of 100 μl). The column was washed with 1 ml of 7 M urea buffer containing 0.3 M NaCl, and then with the same buffer containing 0.5% CHAPS. Proteoglycans bound to Q-Sepharose were eluted with 4 M guanidine HCl buffer containing 0.5% CHAPS. Radioactivity in each eluent was counted.

Coating of Plastic Culture Plates with Extracellular Matrix Produced by Osteoblastic Cells—MC3T3-E1 cells were cultured on 6-well plastic dishes (Falcon) in the presence and absence of ascorbic acid for 10–14 days. Cells were solubilized with 0.5% sodium deoxycholate in 10 mM Tris-HCl, pH 8.0, at 4 °C for 10 min and washed twice with the same buffer (24). Solubilized materials were extensively wiped away by several washes with ice-cold PBS. Extracellular matrix (ECM) components resistant to the detergent remained on dishes and were stored in 1 ml of PBS on ice. Freshly dispersed cells were inoculated on the coated dishes and cultured at 37 °C in 5% CO_2 atmosphere. Cells were subject to ALP assay after 7 days.

Statistical Analysis—Data were expressed as means \pm S.E. and analyzed by an independent *t* test.

RESULTS

Impaired Osteoblastic Differentiation in the Absence of Collagen Production—Cellular ALP activity in MC3T3-E1 cells continues to increase during long-term culture in the presence of ascorbic acid as reported previously (15), while it is markedly suppressed in the absence of ascorbic acid (17). Ascorbic acid is essential for the synthesis of collagen, and when a collagen synthesis inhibitor, L-azetidine-2-carboxylic acid, was included, the increase in ALP activity after 7 days of culture in the presence of ascorbic acid (1.68 ± 0.22 and 0.23 ± 0.01 nmol/min/ μg of protein in the presence and absence of ascorbic acid, respectively, $p < 0.01$) was almost completely eliminated (0.26 ± 0.01 nmol/min/ μg of protein, $p < 0.01$ versus the group with ascorbic acid alone). L-Azetidine-2-carboxylic acid is a proline analog that replaces prolyl residues in collagen molecules and inhibits prolyl hydroxylation (25). By an addition of a 10 times higher concentration of L-proline (3 mM) together with 0.3 mM L-azetidine-2-carboxylic acid, the inhibitory effect of L-azetidine-2-carboxylic acid on the increase in ALP activity was reversed (1.68 ± 0.32 nmol/min/ μg of protein). Thus, osteoblastic differentiation of MC3T3-E1 cells requires the presence of ascorbic acid, and the effect of ascorbic acid is mediated via the production of collagen in these cells.

Effect of TGF- β 1 on Proteoglycan Synthesis and ALP Activity in MC3T3-E1 Cells—As reported previously (26), treatment with TGF- β 1 for 24 h stimulated proteoglycan synthesis in a dose-dependent manner after 4 days of culture when MC3T3-E1 cells reached confluence, and 100 pM TGF- β 1 stimulated proteoglycan synthesis to 340% of the control (Fig. 1). The stimulation of proteoglycan synthesis by 100 pM TGF- β 1 decreased with the progression of culture period, and was reduced to as low as 141% of the control at 14 days of culture. In contrast, when ascorbic acid was removed from the culture medium, stimulation of proteoglycan synthesis by 100 pM TGF- β 1 was not reduced until day 7, and decreased only slightly at day 14 (Fig. 1).

Consistent with the previous observations of the inhibitory effect of TGF- β on the expression of ALP (9), treatment with 100 pM TGF- β 1 inhibited ALP activity in MC3T3-E1 cells until 7 days of culture in the presence of ascorbic acid (Fig. 2). However, the inhibitory effect of TGF- β 1 on ALP activity disappeared with the increase in ALP activity after 14 days of culture in the presence of ascorbic acid (Fig. 2). Thus, both the

FIG. 1. Effect of TGF- β 1 on proteoglycan synthesis during long-term cultures of MC3T3-E1 cells in the presence and absence of ascorbic acid. MC3T3-E1 cells were cultured in the presence (shaded columns) and absence (open columns) of 50 mg/liter ascorbic acid until 14 days. Cells were treated with various concentrations of TGF- β 1 for 24 h before experiments. Cells were washed with PBS and kept in the medium without any supplements for 1 h at 37 °C. Cells were metabolically radiolabeled with 100 μ Ci/ml [35 S]sulfate for 1 h at 37 °C. Labeled proteoglycans were extracted and isolated as described under "Materials and Methods." The amounts of labeled proteoglycans in each group were calculated as percentages of the control without ascorbic acid or TGF- β 1. Data are expressed as means \pm S.E., $n = 3$.

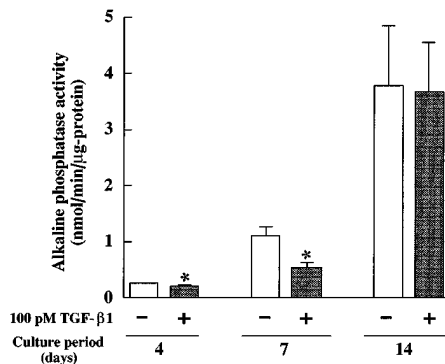
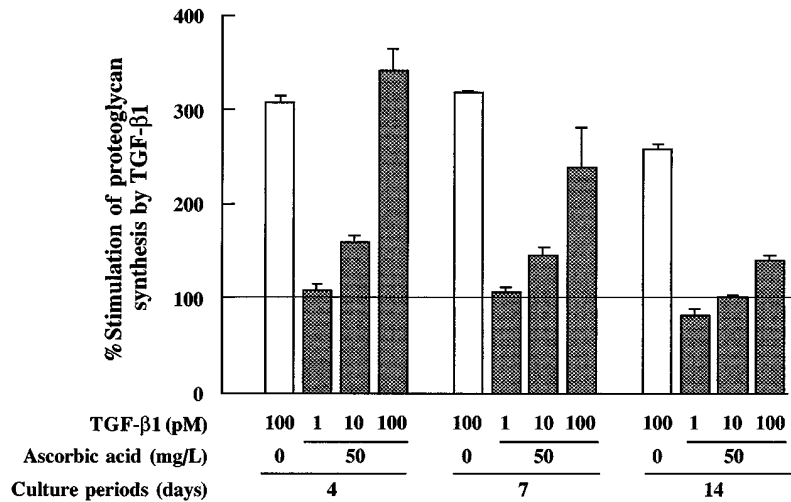


FIG. 2. Effect of TGF- β 1 on alkaline phosphatase activity during long-term cultures of MC3T3-E1 cells in the presence of ascorbic acid. MC3T3-E1 cells were cultured in the presence of 50 mg/liter ascorbic acid until 14 days. At the indicated periods of culture, cells were treated with 100 pM TGF- β 1. Twenty-four hours later, cells were washed twice with ice-cold PBS and were harvested into 50 mM Tris-HCl, 2 mM MgCl₂, 0.05% Triton X-100, pH 8.2. Cells were homogenized on ice and supernatants were collected by centrifugation. Alkaline phosphatase activity and protein content of the supernatants were assayed. Data are expressed as means \pm S.E., $n = 3$. *, significantly different from the control without TGF- β 1 ($p < 0.01$).

stimulation of matrix protein synthesis and the suppression of differentiation-associated phenotypes by TGF- β were lost during the differentiation process of MC3T3-E1 cells when ascorbic acid was present and collagen production was maintained.

Cell Surface Expression of TGF- β Receptors in MC3T3-E1 Cells—In an effort to clarify the mechanism whereby TGF- β actions are reduced after long-term cultures of MC3T3-E1 cells with ascorbic acid, changes in TGF- β binding to its receptors were examined. As shown in Fig. 3, affinity cross-link studies using 125 I-TGF- β 1 revealed that types I and II signal-transducing receptors and beta-glycan were abundantly present in MC3T3-E1 cells at 4 days of culture. The presence of the type II receptor in MC3T3-E1 cells was confirmed by an immunoblot analysis (27). A 72-kDa band bearing 125 I-TGF- β 1 corresponded to the type I receptor because of its molecular size and disappearance after pretreatment of cells with dithiothreitol (28) (data not shown). In the presence of ascorbic acid, the expression of types I and II receptors competent to bind TGF- β 1 was reduced after 7 days, and was almost undetectable after 14 days of culture (Fig. 3). In the absence of ascorbic acid, the abundance of TGF- β receptors competent to bind ligands at 7 days of culture was similar to that at 4 days, and, although it declined thereafter, the abundance of receptors after 14 days of culture was still higher than that in the presence of ascorbic acid at day 7 (Fig. 3). Thus, the changes in the expression of the

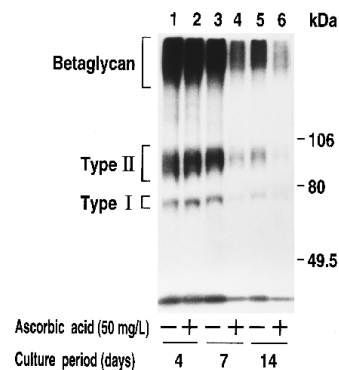


FIG. 3. Cell surface expression of TGF- β receptors during long-term cultures of MC3T3-E1 cells in the presence and absence of ascorbic acid. TGF- β receptors on the cell surface of MC3T3-E1 cells in the presence (lanes 2, 4, and 6) and absence (lanes 1, 3, and 5) of 50 mg/liter ascorbic acid were analyzed by an affinity cross-linking method using 125 I-TGF- β 1. Cells were cultured for 4 days (lanes 1 and 2), 7 days (lanes 3 and 4), and 14 days (lanes 5 and 6). Molecular weight markers are indicated in the right margin.

receptors competent to bind TGF- β were closely correlated with the changes in the actions of TGF- β 1 after long-term cultures of MC3T3-E1 cells.

Changes in mRNA Expression for ALP and Type II TGF- β Receptor—In parallel with the increase in ALP activity after long-term cultures of MC3T3-E1 cells with ascorbic acid, Northern blot analysis revealed that ALP mRNA markedly increased after 14 days of culture (Fig. 4). In contrast, the type II TGF- β receptor mRNA level was not obviously affected after 14 days of culture (Fig. 4).

Effects of Collagen Synthesis Inhibitors on Cell Surface TGF- β Receptors—In order to find out if the effect of ascorbic acid on the abundance of TGF- β receptors was also mediated by its effect on collagen synthesis, the influence of collagen synthesis inhibitors on TGF- β 1 binding to its receptors was examined. When MC3T3-E1 cells were cultured in the presence of ascorbic acid and treated with 0.3 mM L-azetidine-2-carboxylic acid, the reduction in the binding of TGF- β 1 to its receptors after 7 days of culture was blocked, whereas a simultaneous addition of 3 mM L-proline eliminated the effect of 0.3 mM L-azetidine-2-carboxylic acid (Fig. 5). Similar results could be obtained when MC3T3-E1 cells were treated with other inhibitors of collagen synthesis, cis-4-hydroxyproline and 3,4-dehydropyrrolidine, in the presence of ascorbic acid (data not shown). None of these collagen synthesis inhibitors affected the binding of TGF- β 1 to its receptors in the absence of ascorbic acid. These results demonstrate that the synthesis of collagen is required

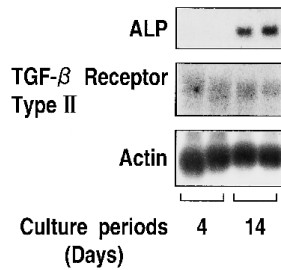


FIG. 4. Expression of mRNAs for alkaline phosphatase and type II TGF- β receptor in MC3T3-E1 cells during long-term cultures. MC3T3-E1 cells were cultured in the presence of 50 mg/liter ascorbic acid until 14 days. Total RNA was extracted at 4 and 14 days of culture. Northern blot analyses were performed using cDNAs for rat ALP and human type II TGF- β receptor as probes. Ten μ g of total RNA was electrophoresed in 1% agarose gel and was transferred onto a nylon membrane followed by hybridization with the radiolabeled cDNA probes. mRNA expression of β -actin was indicated as an internal reference of a housekeeping gene.

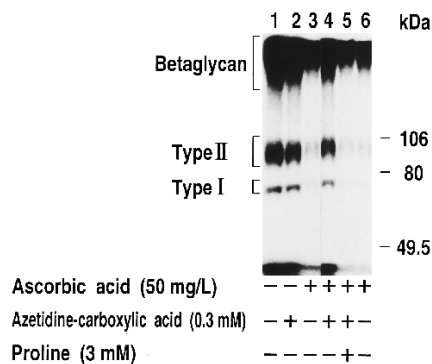


FIG. 5. Effects of a collagen synthesis inhibitor, L-azetidine-2-carboxylic acid, on cell surface TGF- β receptors in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the absence of ascorbic acid for 4 days. Cells were then treated with (lanes 2, 4, and 5) or without (lanes 1, 3, and 6) 0.3 mM L-azetidine-2-carboxylic acid in the presence (lanes 3–6) or absence (lanes 1 and 2) of 50 mg/liter ascorbic acid for 3 days. Ten times higher concentration of L-proline (3 mM) was added with 0.3 mM L-azetidine-2-carboxylic acid and ascorbic acid in lane 5. Cell surface TGF- β receptors were demonstrated by a procedure described in the legend to Fig. 3. Molecular weight markers are indicated in the right margin of the figure.

for the down-regulation of TGF- β receptors competent to bind ligands as well as for the increase in ALP activity after long-term cultures of MC3T3-E1 cells.

Effect of Anti- α 2 β 1 Integrin Blocking Antibody and DGEA Peptide on the Differentiation and TGF- β Receptors—Type I collagen interacts with various types of cells via the specific binding of DGEA motif on α 1 chain to cell surface α 2 β 1 integrin (29, 30). In order to clarify if the effect of ascorbic acid on the differentiation of MC3T3-E1 cells was in fact mediated via the interaction of collagen with α 2 β 1 integrin, the influence of anti- α 2 β 1 integrin blocking antibody and DGEA peptide on ALP activity was examined. As shown in Table I, 10 mg/liter anti- α 2 β 1 integrin blocking antibody significantly inhibited the effect of ascorbic acid on ALP activity. The same concentration of an anti- α 5 β 1 integrin antibody that interferes with the binding of α 5 β 1 integrin to fibronectin did not affect ALP activity. The addition of 5 mM DGEA peptide also blocked the increase in ALP activity by ascorbic acid, but a related peptide, KDGE, without binding affinity for α 2 β 1 integrin had no effect (Table I). Furthermore, the decrease in TGF- β 1 binding to its receptors induced by ascorbic acid was also blocked by the anti- α 2 β 1 integrin antibody (Fig. 6). The antibody had no effect on TGF- β receptors in the absence of ascorbic acid. The addition of neither anti- α 2 β 1 integrin blocking antibody nor DGEA peptide

visibly disrupted the collagen matrix organization or dislodged osteoblastic cells from culture dishes.

Effect of Extracellular Matrix on the Differentiation and TGF- β Receptors—If a direct interaction of extracellular collagen with α 2 β 1 integrin is involved in the differentiation and the reduction in cell surface TGF- β receptors of osteoblastic cells, contact of MC3T3-E1 cells with collagen fibrils should cause similar changes even when collagen synthesis is inhibited. In order to test this possibility, ECM layer was first produced by culturing MC3T3-E1 cells for 10 days in the presence of ascorbic acid, and then cells were removed by a treatment with nonionic detergent. On these ECM layers, MC3T3-E1 cells were cultured with or without ascorbic acid. As shown in Table II, when cells were cultured on ECM layers, ALP activity was elevated even in the absence of ascorbic acid to a similar level to that in cells cultured in the presence of ascorbic acid without ECM layers. Although ECM layers contained a small amount of ALP, ALP activities associated with ECM layers were less than 0.1 nmol/min/ μ g of protein, and could not account for the increase observed in MC3T3-E1 cell cultures with ECM. The addition of ascorbic acid to cultures on ECM layers did not further increase ALP activity. The stimulation of proteoglycan synthesis in response to TGF- β 1 was also inhibited when cells were cultured on the ECM (Table II). Furthermore, the addition of 10 mg/liter anti- α 2 β 1 integrin blocking antibody at the seeding of cells onto the ECM layer inhibited the increase in ALP activity, and partially restored the responsiveness in proteoglycan synthesis to TGF- β 1 (Table II). The attachment of cells to ECM layers was not apparently inhibited by the antibody. In parallel with these changes, TGF- β receptors competent to bind ligands decreased without ascorbic acid if cells were cultured on the ECM (Fig. 7). The ECM layer without MC3T3-E1 cells did not appreciably bind TGF- β 1, indicating that the reduction in the binding of labeled TGF- β 1 to its receptors was not due to sequestration of labeled TGF- β 1 by binding to the ECM layers (Fig. 7, lane 4).

DISCUSSION

Production of type I collagen is one of the early events associated with osteoblastic differentiation. Following the synthesis of type I collagen, sequential expression of ALP and osteocalcin, markers of osteoblastic differentiation, is observed (31–33). The present studies demonstrate that the differentiation of osteoblastic MC3T3-E1 cells, as indicated by the elevation of ALP activity, is markedly suppressed when collagen synthesis is inhibited by either eliminating ascorbic acid or adding a collagen synthesis inhibitor. Thus, the synthesis and the accumulation of matrix collagen appear to be involved in the osteoblastic differentiation of these cells. These results are consistent with the previous observations that the production of collagen is required for the development of ALP activity in osteoblastic cells (3, 17).

TGF- β is thought to play an important role in bone formation (6). The most pronounced effect of TGF- β in osteoblasts is the stimulation of the synthesis of bone matrix proteins including type I collagen, proteoglycans, and fibronectin. In contrast, it strongly inhibits the expression of differentiation-associated phenotypes of osteoblasts such as ALP and osteocalcin (9, 14, 33). Therefore, if TGF- β actions persist, osteoblasts continue to accumulate matrix proteins but cannot further differentiate to mineralize the extracellular matrix, and bone formation cannot be promoted. However, when applied *in vivo*, TGF- β markedly stimulates bone formation (11, 12). In the present studies, the development of ALP activity is associated with a suppression of the actions of TGF- β 1 on both the stimulation of proteoglycan synthesis and the inhibition of ALP activity (Figs. 1 and 2). The changes in these actions of TGF- β 1 are closely correlated with

TABLE I
Suppression of alkaline phosphatase activity induced by ascorbic acid by an inhibition of interaction between collagen and $\alpha 2\beta 1$ integrin in MC3T3-E1 cells

MC3T3-E1 cells were cultured in the absence of ascorbic acid for 4 days followed by treatment with 50 mg/liter ascorbic acid for three more days. Some cultures were also treated with 10 mg/liter monoclonal anti-integrin blocking antibodies or with indicated concentrations of synthetic $\alpha 1(I)$ collagen peptides, DGEA or KDGE. Alkaline phosphatase activity in cell layers were determined. The baseline values and the fold stimulation of alkaline phosphatase activity varied depending upon the culture passage of cells used in each experiment. Data are expressed as means \pm S.E., $n = 3$.

Ascorbic acid mg/liter	Anti-integrin antibody	Peptides	Alkaline phosphatase activity nmol/min/ μ g protein
Experiment 1			
0	Non-immune IgG	0	0.64 \pm 0.01
50	Non-immune IgG	0	1.55 \pm 0.05 ^a
50	10 mg/liter anti- $\alpha 2\beta 1$	0	0.92 \pm 0.05 ^b
50	10 mg/liter anti- $\alpha 5\beta 1$	0	1.57 \pm 0.17 ^a
Experiment 2			
0	0	0	0.98 \pm 0.10
50	0	0	4.38 \pm 0.33 ^a
50	0	1 mM DGEA	4.00 \pm 0.24 ^a
50	0	5 mM DGEA	0.86 \pm 0.03 ^b
50	0	1 mM KDGE	5.20 \pm 0.26 ^a
50	0	5 mM KDGE	4.25 \pm 0.17 ^a

^a Significantly higher than the control with no treatment ($p < 0.01$).

^b Significantly less than the cultures with 50 mg/liter ascorbic acid alone ($p < 0.01$).

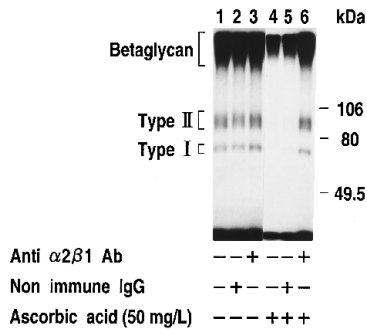


FIG. 6. Effects of anti- $\alpha 2\beta 1$ integrin blocking antibody on TGF- β receptors in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the absence of ascorbic acid. After 3 days of culture, cells were treated with 50 mg/liter ascorbic acid, and 10 mg/liter monoclonal anti-mouse $\alpha 2\beta 1$ integrin antibody was added to cultures. TGF- β receptors on the cell surface were determined as described in the legend to Fig. 3. Cell surface TGF- β receptors in MC3T3-E1 cells cultured with (lanes 4–6) or without (lanes 1–3) ascorbic acid. Anti- $\alpha 2\beta 1$ integrin antibody (lane 3 and 6) or non-immune IgG (lanes 2 and 5) was added along with ascorbic acid. Migration positions of type I and type II TGF- β receptors and beta-glycan are indicated. Molecular weight markers are indicated in the right margin.

a reduction in TGF- $\beta 1$ binding to its receptors (Fig. 3). In addition, when collagen synthesis is inhibited, all the differentiation-associated changes described above are blocked. From these results, it is plausible to assume that the accumulation of matrix collagen enhances the differentiation of osteoblastic cells and suppresses the actions of TGF- β by reducing the receptors competent to bind TGF- β , and that the change in the responsiveness to TGF- β allows these cells to escape from the inhibitory effect of TGF- β on osteoblastic differentiation and to further differentiate into cells with mineralization capacity. Thus, it is suggested that type I collagen in bone matrix plays an important role in the regulation of osteoblastic differentiation, and that bone matrix collagen synthesized under the stimulatory effect of TGF- β regulates the actions of TGF- β by differentiation-associated down-regulation of its receptors.

A decrease in TGF- β receptors has also been reported during retinoic acid-induced osteoblastic differentiation of pluripotent mesenchymal cells (34). In addition, myogenic differentiation of myoblasts to myotubes is reported to be inhibited by TGF- β (35, 36), and myocytes lose TGF- β receptors after the terminal differentiation (37). Stimulation of monocytes by cytokines (38)

and suspension of lipocytes obtained from rat liver (39) have also been shown to result in down-regulation of TGF- β receptors on the cell surface. Taken together with our results, these observations suggest that a decrease in TGF- β receptors is one of the mechanisms by which cells escape from the control of TGF- β . The decrease in TGF- β receptors competent to bind ligands does not appear to be caused by a reduction in the synthesis of receptor proteins, because the abundance of the transcript for type II TGF- β receptor is not reduced to a similar extent (Fig. 4) (39). Because matrix proteins can bind TGF- β , and because matrix layers surrounding these cells can hinder TGF- β receptors from binding to TGF- β , the reduction in TGF- β binding to its receptors may be caused by a reduction in the accessibility of labeled TGF- $\beta 1$ to its receptors. However, labeled TGF- $\beta 1$ does not appreciably bind to ECM layers without MC3T3-E1 cells (Fig. 7). In addition, when cells were pre-incubated at 4 °C for 16 h before the TGF- $\beta 1$ binding studies, a procedure known to inhibit endocytosis, ascorbic acid-induced down-regulation of TGF- $\beta 1$ binding to its receptors was inhibited.² These observations are consistent with the assumption that the decrease in the binding of TGF- β to its cell surface receptors is not due to a reduction in the synthesis of receptors, or entrapment of TGF- β or sequestration of TGF- β receptors by collagen matrix, but may be due to a change in the intracellular trafficking of receptor proteins. However, further investigation is required on the precise mechanism whereby TGF- β binding to its receptors is reduced with osteoblastic differentiation.

As to the mechanism of the regulation of the differentiation and the down-regulation of TGF- β receptors by matrix collagen, there are possibilities that the synthesis of collagen *per se* or the interaction of osteoblastic cells with the accumulated collagen affects the differentiation of osteoblastic cells. Matrix collagen interacts with various cells through a specific binding of DGEA motif to cell surface $\alpha 2\beta 1$ integrin (40, 41). The present results demonstrate that an anti- $\alpha 2\beta 1$ integrin antibody that specifically blocks the interaction of $\alpha 2\beta 1$ integrin with its ligands inhibits not only the differentiation but also the decrease in TGF- $\beta 1$ binding to its receptors of MC3T3-E1 cells (Table I, Fig. 6). The differentiation of these cells is also blocked when a peptide containing DGEA motif is added to the culture (Table I). Furthermore, when these cells are cultured on dishes

² Y. Takeuchi and T. Matsumoto, unpublished observations.

TABLE II
Effects of extracellular matrix (ECM) produced by MC3T3-E1 cells on alkaline phosphatase activity and proteoglycan synthesis after TGF- β 1 treatment in MC3T3-E1 cells

MC3T3-E1 cells were cultured in the presence of 50 mg/liter ascorbic acid for 10 days. Cells were solubilized with 0.5% sodium deoxycholate in 10 mM Tris-HCl, pH 8.0, at 4 °C for 10 min, and soluble materials were removed. Freshly subcultured MC3T3-E1 cells were plated on the ECM layer in the presence and absence of ascorbic acid for 7 days. As controls, cells were cultured on plastic culture plates without ECM layer. Alkaline phosphatase activity and proteoglycan synthesis were determined as described under "Materials and Methods." Stimulation of proteoglycan synthesis by 100 pM TGF- β 1 was expressed as percentages of the control cultures without TGF- β 1. The baseline values and the fold stimulation of alkaline phosphatase activity and proteoglycan synthesis varied depending upon the culture passage of cells used in each experiment. Data are expressed as means \pm S.E., $n = 3$.

Ascorbic acid	ECM	Anti- α 2 β 1 antibody	Alkaline phosphatase activity	Stimulation of proteoglycan synthesis
mg/liter		mg/liter	nmol/min/ μ g protein	% of the control
Experiment 1				
0	—	0	0.26 \pm 0.01	185 \pm 12
0	+	0	0.58 \pm 0.12 ^a	146 \pm 8 ^a
50	—	0	0.46 \pm 0.02 ^a	133 \pm 21 ^b
50	+	0	0.54 \pm 0.06 ^a	NE ^c
Experiment 2				
0	—	0	0.24 \pm 0.01	150 \pm 11
0	+	0	1.38 \pm 0.17 ^a	116 \pm 5 ^b
0	+	10	0.44 \pm 0.11 ^d	138 \pm 2 ^e

^a Significantly different from cultures on plastic dishes without ascorbic acid ($p < 0.01$).

^b Significantly different from cultures on plastic dishes without ascorbic acid ($p < 0.05$).

^c NE, not examined.

^d Significantly less than cultures on ECM-coated dishes without the antibody ($p < 0.01$).

^e Significantly higher than cultures on ECM-coated dishes without the antibody ($p < 0.01$).

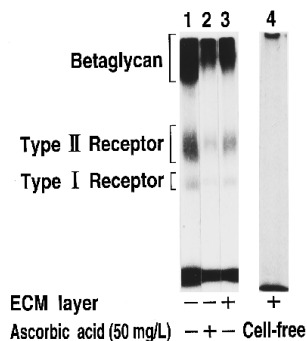


FIG. 7. Effects of ECM produced by MC3T3-E1 cells after long-term culture on TGF- β receptors in MC3T3-E1 cells. Cell surface TGF- β receptors were demonstrated by a procedure described in the legend to Fig. 3 using ¹²⁵I-TGF- β 1 after 7 days of culture on plastic plates without (lanes 1 and 2) and with ECM layer in the absence (lanes 3 and 4) and presence of ascorbic acid (lane 2). A control sample was prepared from ECM-coated plates without cells (lane 4).

coated with ECM produced by MC3T3-E1 cells themselves, these cells are able to differentiate in association with the decrease in the TGF- β action through the reduction in TGF- β binding to its receptors even in the absence of ascorbic acid (Table II, Fig. 7). The ECM-induced differentiation of these cells along with the decrease in the responsiveness to TGF- β are also inhibited by a blocking antibody against α 2 β 1 integrin (Table II). These observations are consistent with the assumption that the interaction of osteoblastic cells with matrix collagen via α 2 β 1 integrin plays an important role in the differentiation and associated down-regulation of TGF- β receptors and actions in these cells. Interestingly, when these cells were cultured on dishes coated with denatured type I collagen, none of these changes could be observed.² Because maintenance of the conformation of collagen fibrils is shown to be important for the specific binding of DGEA motif on collagen molecule to α 2 β 1 integrin (42), contact of osteoblastic cells with native collagen may be required to cause α 2 β 1 integrin-collagen interaction. However, the possibility cannot be ruled out that some ECM-associated factors may also be involved in the osteoblastic differentiation.

In conclusion, the present studies demonstrate that the syn-

thesis and the accumulation of matrix collagen are involved in the differentiation of osteoblastic cells, and that osteoblastic differentiation is associated with a reduction in TGF- β binding to its receptors and a suppression of the actions of TGF- β on both the stimulation of matrix protein synthesis and the inhibition of differentiation. Furthermore, the differentiation-associated changes are suppressed by a blocking antibody against α 2 β 1 integrin and a DGEA peptide that interfere with the binding of cells to type I collagen. The osteoblastic differentiation is also promoted by culturing cells on ECM, which is again inhibited by an anti- α 2 β 1 integrin antibody. These results suggest that bone matrix collagen synthesized under the stimulatory effect of TGF- β interacts with osteoblasts via α 2 β 1 integrin and plays an important role in the regulation of osteoblastic differentiation and TGF- β actions by differentiation-associated down-regulation of TGF- β receptors. Recent studies demonstrate that the interaction of integrins with matrix proteins provokes various changes in cellular proliferation, differentiation, and functions via the activation of intracellular signal transduction pathways. These include non-receptor tyrosine kinase cascades such as Src and focal adhesion kinase, and the Ras-mitogen-activated protein kinase pathway (43). The present observations warrant further investigation into the mechanism of the control of osteoblastic differentiation and TGF- β receptors by the interaction between matrix collagen and cell surface integrin.

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