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Regulation of αVβ3 and αVβ5 Integrins by Dexamethasone in Normal Human Osteoblastic Cells

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Abstract Long-term administration of pharmacological doses of glucocorticoids inhibits bone formation and results in osteoporosis. Since integrin-mediated cell-matrix interactions are essential for osteoblast function, we hypothesized that the detrimental effect of glucocorticoids on bone derived, at least in part, from decreased integrin-matrix interactions. Because αvβ3 and αvβ5 integrins can interact with several bone matrix proteins, we analyzed the effects of dexamethasone (Dex) on the expression of these integrins in normal human osteoblastic cells. We found adhesion of these cells to osteopontin and vitronectin to be dependent on αvβ3 and αvβ5, respectively; this ligand specificity was not altered by Dex. The effects of Dex on the adhesion of human osteoblastic cells to osteopontin and vitronectin were biphasic with an increase after 2 days, followed by a decrease after 8 days of treatment. Consistently, surface αvβ3 and αvβ5 integrins, which were increased after 2 days of Dex treatment, were decreased after 8 days. Similarly, total cellular αv, β3, and β5 proteins, which were increased by Dex early in the culture, were diminished after 8 days. Metabolic labeling studies indicated that Dex exhibited biphasic regulation on the biosynthesis of αvβ5, with stimulation observed during the second day of treatment, followed by inhibition during the 8th day of exposure. By contrast, the biosynthesis of αvβ3 was inhibited by Dex on day 1 and remained inhibited on day 8. Analysis of the mRNA indicated that αv and β5 levels were increased by Dex during early exposure (1–3 days), followed by inhibition after prolonged exposure (≥7 days). By contrast, Dex decreased β3 mRNA level at all the time points analyzed. Consistently, Dex decreased β3 promoter activity after 1 day and persisted over 8-day period. By contrast, Dex stimulated β5 promoter activity after 1 or 2 days but had no effect after 8 days. To further evaluate mechanism(s) leading to the decreased integrin expression after prolonged Dex treatment, mRNA stability was analyzed. Dex was found to accelerate the degradation of αv, β3 and β5 mRNA after an 8-day treatment. Thus, the regulation of αvβ3 was dependent on transcription and posttranscriptional events whereas the expression of αvβ5 was dependent mainly on posttranscriptional events after prolonged Dex treatment. In conclusion, Dex exhibited time-dependent regulation on the expression of αvβ3 and αvβ5 integrins in normal human osteoblastic cells. Short-term exposure to Dex increased the levels of αvβ3 and αvβ5 on the surface and cell adhesion to osteopontin and vitronectin whereas long-term exposure to Dex decreased the expression of both integrins and inhibited the cell adhesion to matrix proteins. J. Cell. Biochem. 77:265–276, 2000.

Key words: integrins; osteoblasts; glucocorticoid; osteopontin; vitronectin

Adhesion of cells to extracellular matrix components is essential for cell migration, proliferation, survival, and differentiation [Adams and Watt, 1993]. The integrins, a family of heterodimeric transmembrane glycoproteins consisting of α and β chains, are the major cell surface receptors responsible for cell adhesion to matrix [Yamada and Miyamoto, 1995; Schwartz et al., 1995; Hynes 1992]. Human osteoblasts (HOB) express a variety of inte-

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grins that bind matrix proteins such as osteopontin, bone sialoprotein, vitronectin, type I collagen, thrombospondin, and fibronectin [Clover et al., 1992; Grzesik and Robey, 1994; Hughes et al., 1993; Saito et al., 1994; Gronthos et al., 1997]. The same integrins are expressed by osteoblasts at various stages of maturation, with levels dependent on the differentiation status of the cells and their local matrix protein environment [Grzesik and Robey, 1994; Hultenby et al., 1993]. Thus, osteoblasts at the bone surface express the highest levels of $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha v\beta5$ integrins, whereas osteocytes and bone marrow stromal preosteoblastic cells express less amounts.

Interactions between bone matrix proteins and osteoblasts have profound effects on osteoblast activities. Cells grown on collagen lattice exhibit accelerated differentiation toward the mature phenotype [Lynch et al., 1995; Ichimura, 1993]. Expression of osteocalcin and matrix mineralization is dependent on the interaction between integrin $\alpha2\beta1$ and collagen [Xiao et al., 1998]. Adhesion of MC3T3-E1 osteoblastic cells to bone sialoprotein results in increased proliferation, differentiation, and matrix mineralization [Zhou et al., 1995]. Similarly, adhesion of UMR106–6 osteoblastic cells to osteopontin increases the expression of alkaline phosphatase [Liu et al., 1997]. Moreover, progressive osteoblast differentiation is inhibited by the presence of RGD peptide or upon disruption of fibronectin-integrin interaction [Moursi et al., 1996; Gronowicz and Derome, 1994].

Osteopontin, one of the abundant noncollagenous matrix proteins, contains the adhesive RGD motif. It has been shown to interact with $\alpha v\beta3$, $\alpha v\beta5$, and $\alpha v\beta1$ integrins in other cell systems [Hu et al., 1995] and is expressed by both osteoclasts and osteoblasts [Termine and Robey, 1996; Dodds et al., 1995; Ingram et al., 1993; Hultenby et al., 1991]. This protein plays an important role in anchoring osteoclasts to remodeling sites and acts as a modulator for osteoclasts [Ross et al., 1993; Hruska et al., 1995; Heinegard et al., 1995]. Osteopontin may also serve as an anchor for newly recruited osteoblasts at the remodeling sites. Indeed, osteopontin has been shown to enhance the attachment of normal human osteoblasts [Grzesik and Robey, 1994]; osteoblasts preferentially adhere to sites of prior bone resorption [Puzas et al., 1997]. Vitronectin is another RGD-containing protein enrich in the mineralized bone matrix [Grzesik and Robey, 1994; Seiffert, 1996; Kumagai et al., 1998]. Human osteoblasts have been shown to adhere to vitronectin in an RGD-dependent manner [Grzesik and Robey, 1994]. Vitronectin interacts with both $\alpha v\beta3$ and $\alpha v\beta5$ integrins in many cell types [Leeuwen et al., 1996; Delannet et al., 1994; Felding-Habermann and Cheresh, 1993]. The migration of several cell types is also reported to be dependent on the interaction between these integrins and vitronectin [Leeuwen et al., 1996; Delannet et al., 1994; Felding-Habermann and Cheresh, 1993; Kim et al., 1994].

We and others have previously demonstrated that glucocorticoids can induce or accelerate the differentiation of human osteoblastic cells in vitro [Cheng et al., 1994; Jaiswal et al., 1997; Wong et al., 1990; Yamanouchi et al., 1997]. Paradoxically, prolonged administration of excessive doses of glucocorticoids in vivo often leads to osteoporosis, with a marked decline in bone mass, total bone volume, and mineral apposition rate, resulting in increased risk of fractures [Canalis, 1996; LoCascio et al., 1990]. Numerous mechanisms have been attributed to mediate the detrimental effects of glucocorticoids on bone, including impaired absorption of calcium, accelerated osteoclast formation, inhibition of the proliferation of osteoprogenitor cells, increased osteoblast apoptosis, and decreased synthesis of bone matrix proteins by osteoblasts [Cheng et al., 1994; Canalis, 1996; LoCascio et al., 1990; Kaji et al., 1997; Weinstein et al., 1997]. In addition to these mechanisms, we hypothesize that the detrimental effects of prolonged glucocorticoid administration derive, in part, from decreased recruitment and adhesion of osteoblasts to the bone surface. In support of this hypothesis, histomorphometric studies indicate that one of the hallmarks of glucocorticoid-induced osteoporosis is a reduction of osteoblast numbers on bone surfaces [Canalis, 1996]. Since integrins govern cell migration and adhesion and several bone matrix proteins interact with $\alpha v\beta3$ and $\alpha\beta5$, we examined the effects of dexamethasone on the expression of these two integrins in normal human osteoblastic cells.

MATERIALS AND METHODS

Materials

Dexamethasone and vitronectin were obtained from Sigma Chemical Co. (St. Louis, MO). [α-^{32}P]-dCTP, and Megaprime DNA labeling systems were from Amersham (Arlington Heights, IL). [^{125}I]-NaI and Tran^{35}S-Label were from ICN (Costa Mesa, CA). All chemicals for electrophoresis were from Bio-Rad (Richmond, CA). Ultra RiboSep mRNA isolation kit was from Collaborative Biomedical Products (Bedford, MA). Promoter constructs containing either 1.1 or 7 kb of mouse β3 and β5 genomic DNA upstream of their respective transcriptional start site, linked to luciferase, were prepared as previously described [McHugh et al., 1994; Feng et al., 1999]. The β-Galactosidase enzyme assay system with reporter lysis buffer kit was from Promega (Madison, WI). Charcoal-stripped newborn bovine serum was obtained from Gemini Bio-Products (Calabasas, CA). Recombinant Protein A-Sepharose 4B was from Zymed Laboratories (San Francisco, CA). Monoclonal antibody against αv (L230) was purified from the conditioned medium of cultured L230 (M-23, 8448-HB) mouse hybridoma cells obtained from American Type Culture Collection (ATCC) (Rockville, MD). Monoclonal antibody against human αvβ3 (LM609) was either kindly provided by Dr. David A. Cheresh (Scripps Research Institute, La Jolla, CA) or purchased from Chemicon International (Temecula, CA). Monoclonal antibodies against human αv (VNR139) and human αvβ5 (P1F6) were from Life Technologies (Gaithersburg, MD). Monoclonal antibody against human β3 (MAB1974) and polyclonal antibody against human β5 (AB1926) were from Chemicon International.

Cell Cultures

Two normal human osteoblastic cell model systems were used. One was the relatively more mature human osteoblasts (HOB), and the other was the relatively immature preosteoblastic human bone marrow stromal cells (HBMSC). HOB and HBMSC were isolated from ribs according to previously published procedures [Cheng et al., 1994; Kimoto et al., 1994]. Since the results obtained with either HOB or HBMSC were similar, only representative figures are presented.

Preparation of Matrix-Coated Plates

Osteopontin was purified from serum-free conditioned medium harvested from cultured melanoma cells stably transfected with the expression vector pCEP4 carrying full-length human osteopontin cDNA (kindly provided by Dr. Michael C Kiefer, Chiron, Emeryville, CA) as described previously [Liaw et al., 1994]. The conditioned medium was concentrated and applied to DEAE-Sepharose column. Osteopontin was eluted out with 0.1–1.14 M NaCl gradient in PBS and dialyzed extensively against PBS. A total of 200 μl of solutions containing osteopontin (10 μg/ml), vitronectin (5 μg/ml), or heat-inactivated bovine serum albumin (BSA)(10 μg/ml) was added to 48-well plates (Costar), which were incubated overnight at 4°C, followed by blocking with phosphate-buffered saline (PBS) containing 2% BSA for 1 h at 37°C. Plates were then washed three times with PBS and stored at 4°C until use.

Cell Adhesion Assays

Adhesion to osteopontin or vitronectin was performed as previously described [Hu et al., 1995]. Human osteoblastic cells were treated with Dex (10^{-7} M) or control vehicle (ethanol, 0.1%) for 2 or 8 days. Cells were released by treatment with collagenase (1 mg/ml) for 10 min, followed by trypsin/EDTA for 3 min. The reaction was stopped by the addition of soybean trypsin inhibitor to 0.5 mg/ml. Single-cell suspensions were washed three times with serum-free α-MEM supplemented with 1% BSA and allowed to recover for 30 min on a rotating platform at 37°C. 4×10^4 cells in 0.25 ml were seeded to each well of plates precoated as described above. To identify the integrins mediating adhesion to these matrixes, cells were precooled at 4°C for 10 min, followed by incubation with integrin blocking antibodies (LM609, P1F6, or L230) or control ascites (10 μg/ml) at 4°C for 15 min before seeding on the precoated plates. After 1-h incubation at 37°C, wells were washed three times with PBS and adherent cells fixed in 4% paraformaldehyde. Cell number was measured by optical density at 630 nm after staining with 0.5% toluidine blue in 4% paraformaldehyde and dissolving the stain in 1% sodium dodecyl sulfate (SDS). The value of cell adhesion to osteopontin or vitronectin was obtained after subtraction of the BSA background.

Surface and Metabolic Labeling and Immunoprecipitation

Cells in p-150 culture dishes were treated with Dex (10^{-7} M) or control vehicle for 2 or 8 days. Cell layers were washed with PBS and surface-labeled with [^{125}I]-NaI (250 μCi/plate) by lactoperoxidase (20 μg) and glucose oxidase (0.05 U) in 1 ml of 5 mM β-D-glucose in PBS as described previously [Ross et al., 1993]. The reaction was allowed to proceed at room temperature for 30 min with constant swirling of the solution to ensure homogenous labeling of the cell layer. At the end of the labeling period, cells were washed with PBS and extracted with 1 ml of cell lysis buffer consisting of 10 mM Tris-HCl, pH 8.5, 0.15 M NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, 2% Renex 30, and 1 mM AEBSF. Cell lysates were further homogenized by passing 10 times through 18-gauge needles. The solutions were transferred to microfuge tubes and centrifuged for 10 min. Aliquots (~400 μl) of each supernatant containing equal trichloroacetic acid-precipitable radioactivity were transferred to new tubes and precleared by incubating with 100 μl of Protein A-Sepharose for 30 min at 4°C on a Nutator. After centrifugation, supernatants were transferred to new tubes. Monoclonal antibodies against αv, αvβ3, or αvβ5 (5 μl) and 200 μl of Protein A-Sepharose were added to each tube and immunoprecipitation allowed to proceed overnight at 4°C on a Nutator. Tubes were microfuged and pellets were washed twice with 500 μl RIPA buffer containing aprotinin, followed by PT (0.5% Tween 20 and 0.02% NaN_3 in PBS) containing 1 mg/ml ovalbumin twice and finally with PT twice. The pellets were extracted with sample buffer without reducing reagent (80 μl/tube) and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed. The immunoprecipitated integrins were visualized by autoradiography and quantitated by image analysis using ISS SepraScan 2001 (Integrated Separation Systems, Natick, MA). For metabolic labeling, cells were treated with either control vehicle or Dex for the indicated period of time. At 24 h before harvest, cells were incubated in methionine-free and cystine-free medium containing 10% HIFBS and testing agent, together with Tran^{35}S-Label (25 μCi/ml). At the end of incubation, cell layers were harvested and immunoprecipitation performed as described above.

Western Blot Analysis for Total Cellular αv, β3, and β5 Integrins

Cells treated with either control vehicle or Dex (10^{-7} M) for the indicated period of time were extracted with cell lysis buffer as described above. Aliquots of equal protein concentration (50 μg) were subjected to SDS-PAGE under nonreducing conditions and transblotted to Immobilon-P membranes. The membranes were blocked with Tris (20 mM, pH 7.4)-buffered saline containing 10% dry milk powder (blocking solution) overnight at 4°C. After washing three times with PBS containing 0.05% Tween 20 (PBS-T), membranes were incubated with anti-αv (VNR139), anti-β3 (MAB1974), or anti-β5 (AB1926) antiserum (1:1,000) in PBS-T for 1 h at room temperature. The membranes were then washed three times with PBS-T, 10 min each, and further incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 in PBS-T) for 1 h. After washing five times with PBS-T, the integrin bands were visualized by enhanced chemiluminescence using an ECL kit. Band intensity was measured by using SepraScan image analysis.

Northern Blot Analysis

Poly A^+ mRNA-enriched RNA were extracted using the Ultra RiboSep kit according to protocols provided by the manufacturer. mRNA was separated by electrophoresis on 1.0% agarose-formaldehyde gels, transferred to a nylon membrane, and fixed under ultraviolet (UV) light. mRNA on the membrane was hybridized with Megaprime labeled [^{32}P]-cDNA for human αv, β3, or β5 and reprobed with [^{32}P]-cDNA for human β-actin. mRNA bands were visualized by autoradiography and the intensity of each band was analyzed by image analysis using SepraScan. The relative mRNA levels of αv, β3, and β5 were normalized to β-actin.

Transfection and Luciferase Assay

MC3T3-E1 cells were transfected by the DEAE-dextran method as described [Towler et al., 1994]. Cells were plated at high density (150,000/well) onto 24-well multiwell dishes in Dulbecco's modified Eagle's medium (DMEM)/

F-12 medium containing 10% heat-inactivated newborn bovine serum. After overnight recovery, cells were rinsed twice with serum-free medium; 0.15 ml of DEAE-dextran (0.2 mg/ml serum-free medium) containing 2 μg/ml of β3 or β5 promoter construct and 0.75 μg/ml of cytomegalovirus (CMV) β-galactosidase expression vector was aliquoted into each well. Cells were incubated for 20 min at 37°C, followed by the addition of 0.2 ml of DMEM/F-12 to each well. Incubation was continued for an additional 30 min, at which time cells were shocked for 90 s with 10% dimethylsulfoxide (DMSO) in PBS at room temperature. Cells were rinsed twice and incubated in medium containing charcoal-stripped serum for 24 h. Cells were then treated with ethanol or Dex (10^{-7} M) for the indicated period of time, followed by lysis in reporter lysis buffer. Lysates were analyzed for luciferase activity in Optocomp II Luminometer (MGM Instruments, Hamden, CT). Luciferase activity was normalized to β-galactosidase, measured with β-galactosidase Enzyme Assay System according to the protocol provided by the manufacturer.

Statistical Analysis

Statistical analysis was performed using either Student's *t*-test or analysis of variance (ANOVA) as indicated. Each experiment was performed at least twice. Only the representative data in mean ±SEM are presented.

RESULTS

Adhesion of Human Osteoblastic Cells to Osteopontin and Vitronectin Is Dependent on the Integrins αvβ3 and αvβ5, Respectively

Since both αvβ3 and αvβ5 have been reported to interact with osteopontin and vitronectin in other cell systems, we analyzed the roles of each integrin played in the adhesion of human osteoblastic cells to these two matrix proteins, using function blocking antibodies. The adhesion to osteopontin was found to be dependent on αvβ3, but not on αvβ5, since LM609, a function blocking antibody for αvβ3, decreased the adhesion to approximately 20% of the control level, whereas P1F6, which inhibits αvβ5, had only minor, insignificant effect (Fig. 1A). L230, a function blocking antibody against αv subunit, also inhibited cell adhesion to osteopontin in a manner similar to LM609

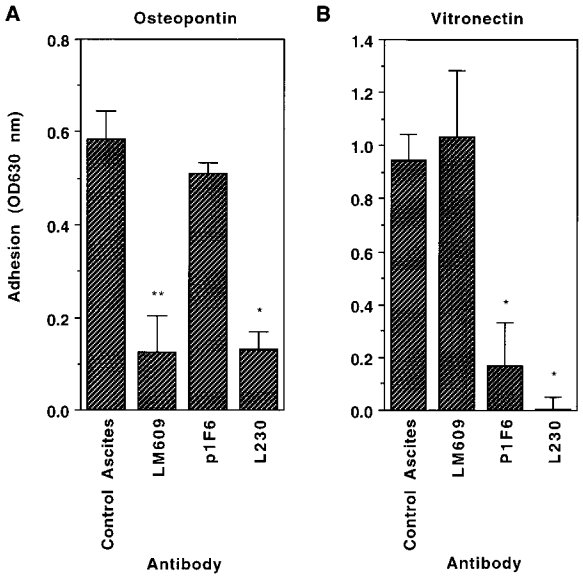

Fig. 1. Adhesion of human osteoblastic cells to osteopontin and vitronectin is dependent on αvβ3 and αvβ5, respectively. Single-cell suspension of human osteoblastic cells was incubated with control ascites, anti-αvβ3 antibody (LM609), anti-αvβ5 antibody (P1F6), or anti-αv antibody (L230) before addition to the culture plates pre-coated with either osteopontin (**A**) or vitronectin (**B**). After 1-h adhesion, nonadherent cells were removed by washing with phosphate-buffered saline (PBS), and the adhered cells were fixed, stained, and enumerated. $*P < 0.001$, $**P < 0.01$, as compared with the corresponding control ascites value by analysis of variance (ANOVA).

(Fig. 1A). Conversely, the adhesion of human osteoblastic cells to vitronectin was dependent on αvβ5, but not on αvβ3, since P1F6 and L230, but not LM609, inhibited this process (Fig. 1B). Thus, human osteoblastic cells bind to osteopontin and vitronectin via αvβ3 and αvβ5, respectively.

Effects of Dex on Cell Adhesion to Osteopontin and Vitronectin

We then examined the effects of Dex on cell adhesion to these two matrix proteins. Dex regulated human osteoblastic cell adhesion to osteopontin biphasically, with an increase to 4.5-fold of the control level after 2 days, followed by a decrease to 50% of control levels after 8 days (Fig. 2A). In separate experiments, the increased adhesion to osteopontin by Dex was already present after 1 day (data not shown). Similar results were obtained with vitronectin, with the changes manifested as a 2-fold increase after 2 days and a decline to 65% after 8 days (Fig. 2B). Dex treatment for 8 days did not alter the ligand specificity of these two inte-

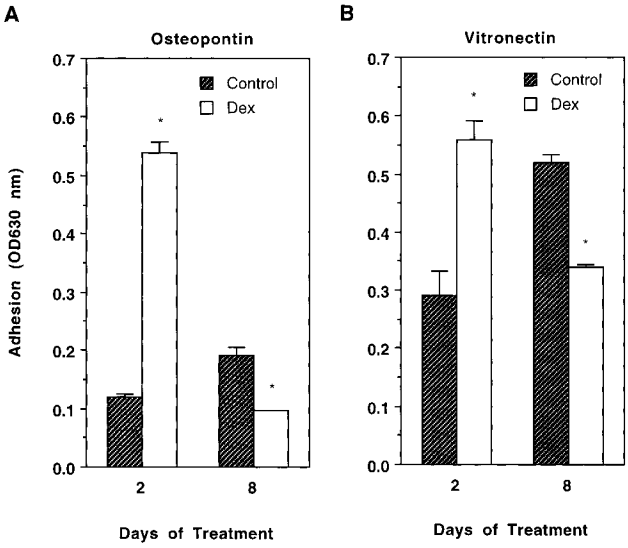

Fig. 2. Effects of dexamethasone (Dex) on the adhesion of human osteoblastic cells to osteopontin and vitronectin. Cells were treated with either 0.1% ethanol (control) or dexamethasone (Dex) (10^{-7} M) for 2 or 8 days. After release from culture dishes, cells were added to wells precoated with osteopontin (**A**) or vitronectin (**B**). The number of cells adhered was determined (mean ± SEM, n = 3). $^{*}P < 0.001$, as compared with the corresponding control value by analysis of variance (ANOVA).

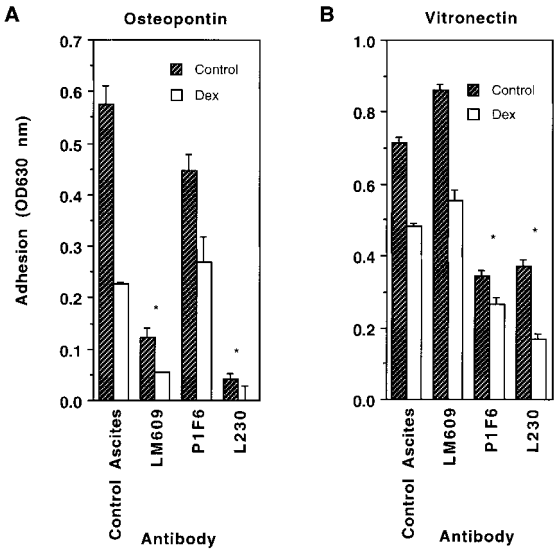

Fig. 3. Prolonged treatment with dexamethasone (Dex) does not alter the ligand specificity of αvβ3 and αvβ5. Cells were treated with either 0.1% ethanol (control) or Dex (10^{-7} M) for 8 days. After release from culture dishes, cells were incubated with control ascites, anti-αvβ3 antibody (LM609), anti-αvβ5 antibody (P1F6), or anti-αv antibody (L230) before addition to culture plates precoated with either osteopontin (**A**) or vitronectin (**B**). After 1-h adhesion, nonadherent cells were removed and the number of adhered cells measured. $^{*}P < 0.001$ for both control and Dex-treated cells, as compared with their corresponding control ascites values by analysis of variance (ANOVA).

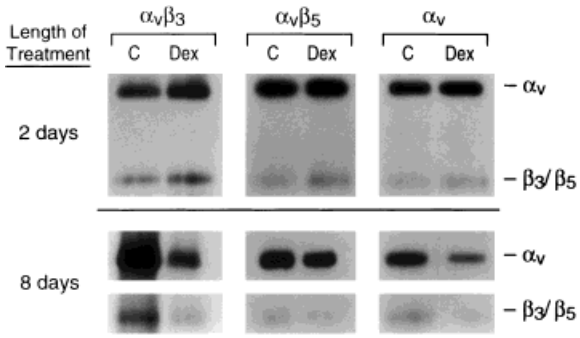

Fig. 4. Effects of dexamethasone (Dex) on the surface protein levels of the integrins αvβ3 and αvβ5. Human osteoblastic cells were treated with either ethanol (C) or Dex (10^{-7} M) for 2 days (top) or 8 days (bottom). Cells were labeled with ^{125}I-NaI, followed by immunoprecipitation with antibodies against αvβ3 (LM609, left), αvβ5 (P1F6, center), or αv (L230, right). The precipitated integrins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

grins because cell adhesion to osteopontin and vitronectin was still dependent on αvβ3 and αvβ5, respectively (Fig. 3A,B). As expected, L230, which blocks both integrins, inhibited cell attachment in all circumstances (Fig. 3A,B).

Effects of Dex on Surface Levels of αvβ3 and αvβ5

Since surface integrin levels govern cell adhesion to matrix proteins, we next examined the effects of Dex on surface expression of αvβ3 and αvβ5. Labeling with ^{125}I-NaI, followed by immunoprecipitation with anti-αvβ3 (LM609) or anti-αvβ5 (P1F6) antibody indicated that Dex treatment for 2 days increased the levels of αvβ3 and αvβ5 on the cell membrane 2-fold and 1.5-fold, respectively (Fig. 4, top, left, and center). However, after 8 days of Dex treatment, the amount of αvβ3 and αvβ5 on the cell membrane had decreased to 25% and 60%, re-

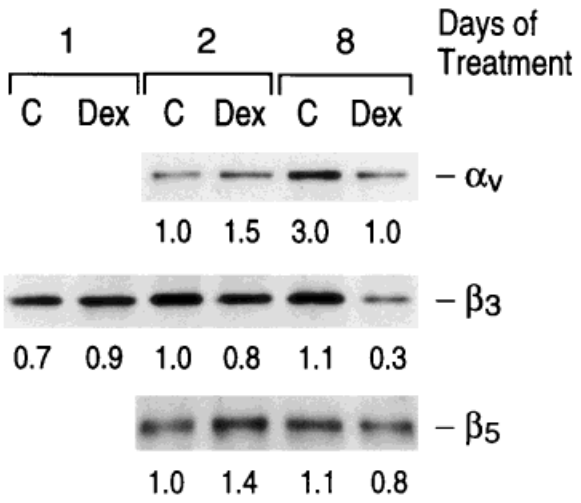

Fig. 5. Effects of dexamethasone (Dex) on total cellular αv, β3, and β5 levels. Human osteoblastic cells were treated with either control (C) or Dex (10^{-7} M) for the indicated period of time. Cell lysates containing equal amount of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and transblotted to Immobilon-P membranes. αv, β3, and β5 levels were measured by Western blotting, using specific antibodies and ECL kit for detection. Band intensities were measured by densitometry analysis, and the day 2 control level of each integrin was defined as 1.0.

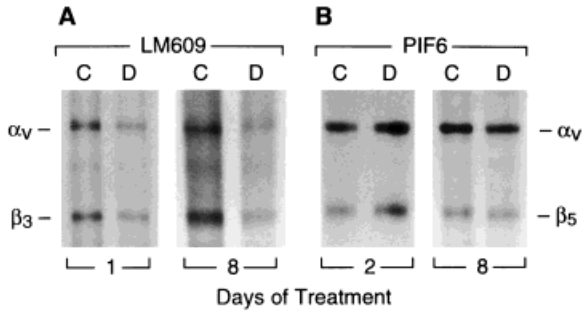

Fig. 6. Effects of dexamethasone (Dex) on the biosynthesis of αvβ3 and αvβ5 in human osteoblastic cells. Cells were treated with either ethanol (C) or Dex (D, 10^{-7} M) for the indicated period of time. At 24 h before harvest, cells were incubated in medium containing Tran^{35}S-Label as described under Materials and Methods. Samples containing equal amount of trichloroacetic acid (TCA)-precipitable counts were immunoprecipitated with either anti-αvβ3 (LM609) or anti-αvβ5 (P1F6) antibody. Precipitated integrins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

spectively (Fig. 4, bottom, left, and center). The time-dependent biphasic regulation of αvβ3 and αvβ5 by Dex was further confirmed by experiments in which both integrins were coimmunoprecipitated with anti-αv (L230) antibody (Fig. 4, right, top, and bottom). Dex increased αv and β3/β5 to 166% of the control values after 2 days but inhibited their expression on the cell membrane to 30% of the control levels after 8 days. These data indicated that Dex effects on cell adhesion derive from the biphasic changes in surface αvβ3 and αvβ5 protein levels.

Effects of Dex on the Total Cellular Levels of αv, β3, and β5 in Human Osteoblastic Cells

To analyze further the Dex effects on integrin αvβ3 and αvβ5 expression, we measured total cellular αv, β3, and β5 content by Western blot analysis. These integrin subunits were also regulated biphasically by Dex, with an initial increase followed by inhibition (Fig. 5). While Dex treatment led to a 1.3-fold increase in β3 by day 1, enhancement of αv and β5, to the extent of 1.5- and 1.4-fold, respectively, was seen after 2 days, by which time β3 had declined to 80% of the control level (Fig. 5). After 8 days, the total concentrations of αv, β3, and β5 were decreased to 33%, 27%, and 73%, respectively, of their corresponding control levels in Dex treated cultures (Fig. 5).

Effects of Dex on the Biosynthesis of αvβ3 and αvβ5 Proteins

Employing metabolic labeling methods, we next asked whether the effects of Dex on αvβ3 and αvβ5 integrin levels were derived, at least in part, via protein synthesis. As shown in Figure 6A, Dex inhibited the biosynthesis of αvβ3 during the first day of treatment to 35% of the control level and this inhibition persisted on day 8 (25% of control levels). By contrast, Dex stimulated the biosynthesis of αvβ5 on day 2 (210% of control level) but the same parameter was inhibited by day 8 (65% of control level) (Fig. 6B). These accumulated data suggested that the short-term stimulatory effect of Dex on total cellular αvβ3 protein levels derives from the inhibition of protein degradation, since synthesis was inhibited. By contrast, the long-term inhibitory effect by Dex on this integrin was derived, at least in part, as a result of decreased synthesis. Finally, both the short-term stimulatory effect and the long-term inhibitory effect of Dex on αvβ5 appeared to result from the regulation of synthesis by Dex.

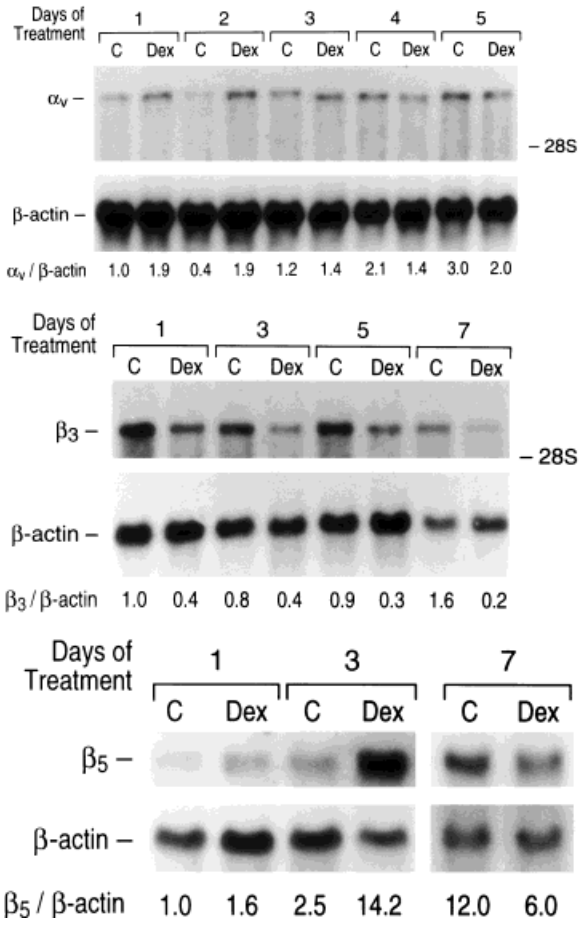

Fig. 7. Effects of dexamthasone (Dex) on the steady-state mRNA level of αv (top), β3 (middle), and β5 (bottom) in human osteoblastic cells. Cells were treated with either ethanol (C) or Dex (10^{-7} M) for the indicated period of time. mRNA was isolated and Northern blot analysis performed as described under Materials and Methods. The ratio of each subunit to β-actin after 1-day treatment was defined as 1.0 for the control culture.

Effects of Dex on Steady-State mRNA Levels of αv, β3, and β5

To analyze further the mechanism mediating the regulation of Dex on αvβ3 and αvβ5 integrins, we examined the steady-state mRNA levels of each subunit. Dex exhibited a biphasic regulation on the steady-state αv mRNA levels in human osteoblastic cells. After 1-day treatment, Dex increased the αv mRNA level to 1.9-fold of the control level (Fig. 7, top). The stimulation of αv mRNA by Dex was maximum (4.75-fold of the corresponding control level) after 2 days. By the third day, however, the stimulation almost disappeared. By days 4 and 5, Dex-treated cultures expressed only 67% of the corresponding control levels (Fig. 7, top). In a separate experiment, Dex was found to decrease the αv mRNA level to approximately 30% of the corresponding control level by day 7 (data not shown). Unlike αv, however, Dex decreased β3 steady-state mRNA level in human osteoblastic cells at all time points (1–7 days) studied (Fig., middle). After 1 day, Dex decreased β3 mRNA to 40% of the control level and the inhibition persisted with maximal suppression observed after 7 days (12% of the corresponding control level). Similar to αv, Dex stimulated β5 mRNA level 1.6-fold after 1 day and the stimulation was further enhanced after 3 days to 5.7-fold of the corresponding control level (Fig. 7, bottom). After 7 days, however, Dex decreased the β5 mRNA level to 50% of the corresponding control level (Fig. 7, bottom). The inhibition of αv, β3, and β5 mRNA after 7 days exposure to Dex was found to be effective at a dose as low as 10^{-10} M with maximal inhibition observed at 10^{-7} M (data not shown). Thus, the regulation patterns of αvβ3 and αvβ5 mRNA levels by Dex were consistent with the effects on integrin protein biosynthesis. Furthermore, the data also confirmed earlier report that β subunit is the rate-limiting protein in the heterodimer formation of integrins [Inoue et al., 1998], since the inhibition of αvβ3 biosynthesis correlated well with the decreased β3 mRNA level despite the increase in αv mRNA during the early phase of Dex treatment.

Effects of Dex on the Activity of the β3 and β5 Integrin Promoters

Since Dex altered steady-state mRNA levels of the rate-limiting β3 and β5 subunits, we next examined whether these changes arose as a result of alterations in gene transcription. To this end, we needed to transfect cells with reporter constructs for these two integrin promoters and evaluate the consequences of Dex treatment. Because human osteoblastic cells are difficult to transfect, we employed murine MC3T3-E1 osteoblast-like cells, which exhibit similar effects by Dex on β3 and β5 integrin mRNA levels (data not shown) and are easily transfected [Towler et al., 1994]. As shown in Figure 8 (top), Dex inhibited β3 promoter activity to 78% of the control level after 1 day and to 62% after 2 days. The inhibition persisted after 8 days (69% of the control level, Fig. 8, top). By contrast, Dex increased the β5 pro-

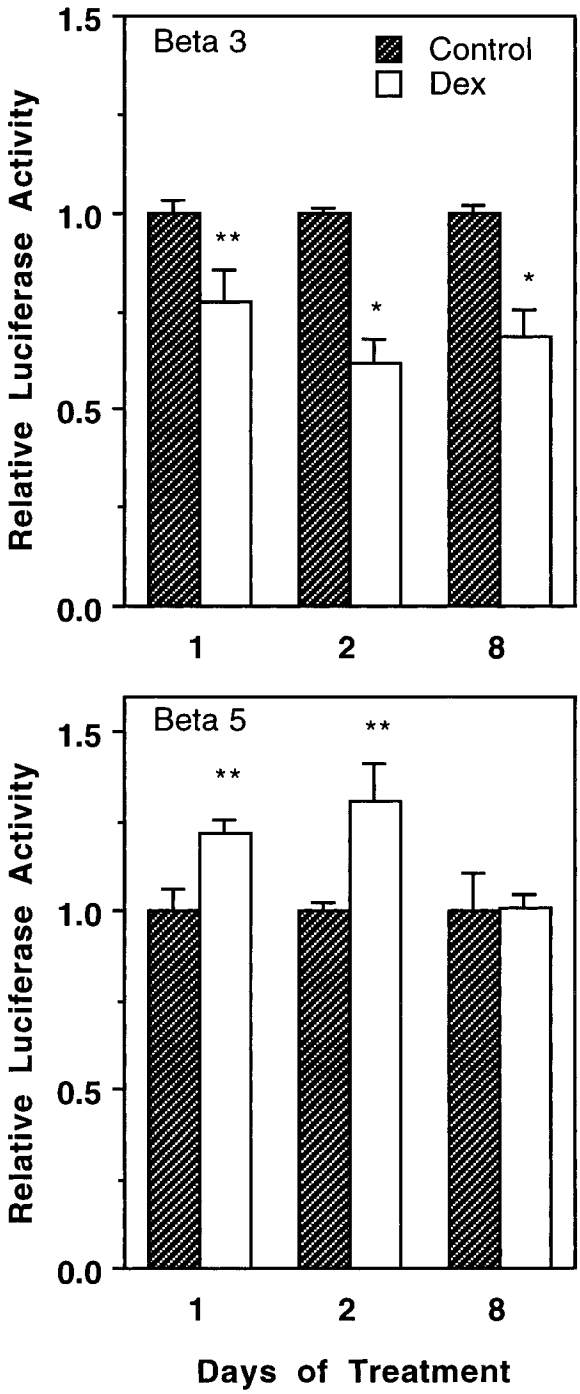

Fig. 8. Effects of Dex on β3 and β5 promoter activities. MC3T3-E1 cells were transfected with β3 (top) or β5 (bottom) promoter construct carrying luciferase reporter gene as described under Materials and Methods. After overnight recovery, cells were treated with either ethanol (control) or Dex (10^{-7}M) for the indicated period of time. Cells were then lysed and luciferase and β-galactosidase activities measured with the latter used for normalization (mean ±SEM, n = 3). *$P < 0.01$, **$P < 0.05$ compared with the corresponding control value using Student's *t*-test.

TABLE I. Effects of Dex on the Integrin mRNA Stability†

	Half-life of mRNA (h)		
Treatment	αν	β3	β5
Control	27.2 ± 2.9	27.9 ± 4.5	43.2 ± 1.7
Dex	16.2 ± 1.0*	9.7 ± 0.7*	34.1 ± 2.8*

†Human osteoblastic cells were treated with ethanol (control) or Dex (10^{-7} M) for 8 days. Actinomycin D (5 μg/ml) was then added to the medium and cells were harvested 0, 4, 8, 24, and 48 h later. mRNAs were isolated and Northern blot analysis performed.
*$P < 0.05$ as compared with the corresponding control value by Student's *t*-test.

moter activity to 122% and 131% of the control level after 1 and 2 days, respectively, whereas no effect was observed after 8 days (Fig. 8, bottom). Thus, the inhibition of β3 promoter activity since the beginning of Dex treatment appeared to contribute to the reduction of β3 mRNA level. The early stimulation of β5 promoter activity by Dex was also consistent with the initial increase on β5 mRNA level. The lack of effect on β5 and the moderate inhibition on β3 promoter activities by Dex after 8 days of treatment, however, could not explain fully the alterations in β3 and β5 mRNA levels, which accompanied prolonged Dex treatment.

Effects of Dex on β3 and β5 mRNA Stability

Since β3 and β5 gene transcription fail to fully explain Dex effects on the steady-state mRNA levels after long-term treatment, we then asked whether the changes arose from alteration in mRNA stabilization. As shown in Table I, Dex decreased the half-life of αv (27.2 h to 16.2 h), β3 (27.9 h to 9.7 h), and β5 (43.2 h to 34.1 h) mRNAs after 8 days of treatment in human osteoblastic cells. These data, taken with the earlier findings on gene transcription, suggested that the reduction of αvβ3 and αvβ5 integrin mRNA levels after prolonged Dex treatment derived from a combination of transcriptional and mRNA stabilization processes. The significance of each event for establishing net steady-state levels differs for the two integrins.

DISCUSSION

The detrimental effects of prolonged high-dose glucocorticoid administration on bone have been well documented with numerous

mechanisms implicated to mediate this deleterious consequence [Cheng et al., 1994; Canalis, 1996; LoCascio et al., 1990; Kaji et al., 1997; Weinstein et al., 1997]. In this report, we show that prolonged (≥7-day) exposure of normal human osteoblastic cells to Dex results in decreased expression of the integrins αvβ3 and αvβ5, with a consequent diminution of osteoblast adhesion to osteopontin and vitronectin. These data, combined with earlier reports documenting the inhibition of osteoblastic β1 integrins (major integrins for binding to type I collagen and fibronectin) by glucocorticoids [Gronowicz and McCarthy, 1995; Doherty et al., 1995], suggest that integrins play a critical role in the skeletal response to glucocorticoids. The decreased capacity for osteoblastic attachment to bone matrix should result in fewer osteoblasts on the bone surface, a hallmark of glucocorticoid-induced osteoporosis. With integrin-matrix interaction implicated as essential for cell survival [Malik, 1997; Frisch and Ruoslahti, 1997], decreased adhesion of osteoblastic cells to bone matrix may also contribute to glucocorticoid-induced apoptosis in osteoblasts [Weinstein et al., 1997].

The ability of osteoblast-residing αvβ3 and αvβ5 to exclusively bind one or the other of the two RGD-containing bone matrix proteins, osteopontin and vitronectin, is a unique property of normal human osteoblastic cells, since these two integrins interact with both osteopontin and vitronectin in other cell types [Hu et al. 1995; Leeuwen et al., 1996; Delannet et al., 1994; Liaw et al., 1995]. The dependence on αvβ5 for adhesion to vitronectin has been reported in osteogenic sarcoma cells [Freed et al., 1989]. However, the lack of participation of αvβ5 in the adhesion of human osteoblastic cells to osteopontin was unexpected. It was reported earlier that αvβ3 is not involved in the adhesion of normal human osteoblastic cells to vitronectin, using different αvβ3 function blocking antibody [Gronthos et al., 1997]. Our data confirm this observation and further implicate that αvβ5 is the integrin mediating human osteoblast adhesion to vitronectin. While at this time we have no plausible explanation for the discriminatory ability of these two integrins, it is possible that subtle changes in the extracellular domains of the two heterodimeric complexes account for our findings. Since the cytoplasmic tails of the two integrins are quite distinct, different combinations of cytoplasmic and/or cytoskeletal proteins may bind to each tail, leading to separate "inside-out" signals [Schwartz et al., 1995]. In such an instance, the extracellular domains of the two integrins may adopt differing conformations, each capable of recognizing the RGD sequence and its flanking amino acids in one, but not the other matrix protein. Irrespective of the reasons, the differential interactions toward these two bone matrix proteins suggest that αvβ3 and αvβ5 play distinctive roles in osteoblast function, a finding not without precedent. Thus, αvβ5, not αvβ3, mediates the uptake of vitronectin by fibroblast [Panetti and McKeown-Longo, 1993]. αvβ3 is involved in migration on vitronectin whereas αvβ5 plays a role in spreading on the same substrate in human monocytes [De Nichilo and Burns 1993]. Moreover, αvβ3 and αvβ5 have been shown to distribute differentially with αvβ3 at the focal adhesion sites whereas αvβ5 more diffusely distributed when human osteoblasts are cultured in serum, presumably on vitronectin [Pistone et al., 1996].

Although prolonged (≥7 days) exposure of human osteoblastic cells to Dex results in decreased αvβ3 andαvβ5 expression, short-term treatment with Dex (2 days) actually increases the surface levels of αvβ3 and αvβ5. Consistently, cell adhesion to osteopontin and vitronectin also exhibits early rise followed by later inhibition in Dex-treated cells. Thus, the adhesion properties of human osteoblastic cells to these two matrix proteins reflect the surface αvβ3 and αvβ5 integrin levels. The total cellular αv, β3, and β5 levels are also regulated in the same fashion by Dex. Since the total cellular β3 level is increased after 1 day, but is decreased after 2 days and beyond, maintenance of the increased αvβ3 level on the cell membrane after 2 days suggests that the turnover of surface αvβ3 lags behind the decline of total cellular β3 level in the early phase of Dex treatment. The biphasic regulation on αvβ5 by Dex is also observed at protein synthesis and mRNA levels. The increase in the mRNA levels of αvβ5 in the early phase of Dex treatment derives, in part, from increased transcription. Since the increase in the β5 promoter activity is far short of the increments observed in protein and mRNA levels, other mechanisms must be involved. After long-term treatment, the decrease in β5 mRNA level appears to depend primarily on the destabilization of β5 mRNA. By contrast, the transcription and the mRNA

level of β3 are inhibited by Dex since the beginning of treatment. Similarly, the protein synthesis of αvβ3 is inhibited within 1 day of Dex exposure. These data suggest that the early rise in β3 total and surface protein levels is derived from posttranscriptional events, i.e. increased protein stability and trafficking to the membrane. Whereas Dex induces early rise in αvβ3 and αvβ5 integrin levels on the cell membrane, these increases may not be clinically significant since they occur only transiently.

Although the expression of both αvβ3 and αvβ5 are inhibited after long-term Dex exposure, the degree of inhibition differs. The inhibition of αvβ3 expression by Dex is greater than that of αvβ5 at all the levels examined. Thus, the expression of αvβ3 is more sensitive to Dex treatment than that of αvβ5. The severe suppression of αvβ3 by Dex may have physiological significance, since the decline in osteoblast-osteopontin interaction may lead to inhibition of osteoblast differentiation [Liu et al., 1997].

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REFERENCES

Adams JC, Watt FM. 1993. Regulation of development and differentiation by the extracellular matrix. Development 117:1183–1198.

Canalis E. 1996. Clinical review 83 mechanisms of glucocorticoid action in bone: implications to glucocorticoid-induced osteoporosis. J Clin Endocrinol Metab 81:3441–3447.

Cheng S-L, Yang JW, Rifas L, Zhang S-F, Avioli LV. 1994. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Clover J, Dodds RA, Gowen M. 1992. Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. J Cell Sci 103:267–271.

Delannet M, Martin F, Bossy B, Cheresh DA, Reichardt LF, Duband JL. 1994. Specific roles of the αvβ1, αvβ3 and αvβ5 integrins in avian neural crest cell adhesion and migration on vitronectin. Development 120:2687–2702.

De Nichilo MO, Burns GF. 1993. Granulocyte-macrophage and macrophage colony-stimulating factors differentially regulate αv integrin expression on cultured human macrophages. Proc Natl Acad Sci USA 90:2517–2521.

Dodds RA, Connor JR, James IE, Rykaczewski EL, Appelbaum E, Dul E, Gowen M. 1995. Human osteoclasts, not osteoblasts, deposit osteopontin onto resorption surfaces: an in vitro and ex vivo study of remodeling bone. J Bone Miner Res 10:1666–1680.

Doherty WJ, DeRome ME, McCarthy MB, Gronowicz GA. 1995. The effect of glucocorticoids on osteoblast function. The effect of corticosterone on osteoblast expression of β1 integrins. J Bone Joint Surg Am 77:396–404.

Felding-Habermann B, Cheresh DA. 1993. Vitronectin and its receptors. Curr Opin Cell Biol 5:864–868.

Feng X, Teitelbaum SL, Quiroz ME, Towler DA, Ross FP. 1999. Cloning of the murine β5 integrin subunit promoter. Identification of a novel sequence mediating granulocyte-macrophage colony-stimulating factor-dependent repression of β5 integrin gene transcription. J Biol Chem 274: 1366–1374.

Freed E, Gailit J, Van Der Geer P, Rouslahti E, Hunter T. 1989. A novel integrin β is associated with the vitronectin receptor α subunit (αv) in a human osteosarcoma cell line and is a substrate for protein kinase C. EMBO J 8:2955–2965.

Frisch SM, Ruoslahti E. 1997. Integrins and anoikis. Curr Opin Cell Biol 9:701–706.

Gronowicz GA, Derome ME. 1994. Synthetic peptide containing Arg-Gly-Asp inhibits bone formation and resorption in a mineralizing organ culture system of fetal rat parietal bones. J Bone Miner Res 9:193–201.

Gronowicz GA, McCarthy MB. 1995. Glucocorticoids inhibit the attachment of osteoblasts to bone extracellular matrix proteins and decrease β1-integrin levels. Endocrinology 136:598–608.

Gronthos S, Stewart K, Graves SE, Hay S, Simmons PJ. 1997. Integrin expression and function on human osteoblast-like cells. J Bone Miner Res 12:1189–1197.

Grzesik WJ, Robey PG. 1994. Bone matrix RGD glycoproteins: immunolocalization and interaction with human primary osteoblastic bone cells in vitro. J Bone Miner Res 9:487–496.

Heinegard D, Andersson G, Reinholt. 1995. Roles of osteopontin in bone remodeling. Ann Acad NY Acad Sci 760:213–222.

Hruska KA, Rolnick F, Huskey M, Alvarez U, Cheresh D. 1995. Engagement of the osteoclast integrin αvβ3 by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity. Endocrinology 136:2984–2992.

Hu DD, Lin EC, Kovach NL, Hoyer JR, Smith JW. 1995. A biochemical characterization of the binding of osteopontin to integrins αvβ1 and αvβ5. J Biol Chem 270:26232–26238.

Hughes DE, Salter DM, Dedhar S, Simpson R. 1993. Integrin expression in human bone. J Bone Miner Res 8:527–533.

Hultenby K, Reinholt FP, Oldberg A, Heinegard D. 1991. Ultrastructural immunolocalization of osteopontin in metaphyseal and cortical bone. Matrix 11:206–213.

Hultenby K, Reinholt FP, Heinegard D. 1993. Distribution of integrin subunits on rat metaphyseal osteoclasts and osteoblasts. Eur J Cell Biol 62:86–93.

Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.

Ichimura S. 1993. Cell mediated calcification in three-dimensional collagen gel culture of the osteoblast-like cells derived from rat calvaria. J Jpn Orthop Assoc 67: 963–976.

Ingram RT, Clarke BL, Fisher LW, Fitzpatrick LA. 1993. Distribution of noncollagenous proteins in the matrix of adult human bone: evidence of anatomic and functional heterogeneity. J Bone Miner Res 8:1019–1029.

Inoue M, Namba N, Chappel J, Teitelbaum SL, Ross FP. 1998. Granulocyte macrophage-colony stimulating factor reciprocally regulates αv-associated integrins on murine osteoclast precursors. Mol Endocrinol 12:1955–1962.

Jaiswal N, Haynesworth SE, Caplan AI, Brudder SP. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312.

Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Chihara K. 1997. Dexamethasone stimulates osteoclast-like cell formation by directly acting on hematopoietic blast cells and enhances osteoclast-like cell formation stimulated by parathyroid hormone and prostaglandin E_2. J Bone Miner Res 12:734–741.

Kim JP, Zhang K, Chen JD, Kramer RH, Woodley DT. 1994. Vitronectin-driven human keratinocyte locomotion is mediated by the αvβ5 integrin receptor. J Biol Chem 269:26926–26932.

Kimoto S, Cheng S-L, Zhang S-F, Avioli LV. 1994. The effect of glucocorticoid on the synthesis of biglycan and decorin in human osteoblasts and bone marrow stromal cells. Endocrinology 135:2423–2431.

Kumagai T, Lee I, Ono Y, Maeno M, Takagi M. 1998. Ultrastructural localization and biochemical characterization of vitronectin in developing rat bone. Histochem J 30:111–119.

Leeuwen RL, Yoshinaga IG, Akasaka T, Dekker SK, Vermeer BJ, Byers HR. 1996. Attachment, spreading and migration of melanoma cells on vitronectin. The role of αvβ3 and αvβ5 integrins. Exp Dermatol 5:308–315.

Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM. 1994. Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circ Res 74:214–224.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM. 1995. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of αvβ3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest 95:713–724.

Liu YK, Uemura T, Nemoto A, Yabe T, Fujii N, Ushida T, Tateishi T. 1997. Osteopontin involvement in integrin-mediated cell signaling and regulation of expression of alkaline phosphatase during early differentiation of UMR cells. FEBS Lett 420:112–116.

LoCascio V, Bonucci E, Imbimbo B, Ballanti P, Adami S, Milani S, Tartarotti D, DellaRocca C. 1990. Bone loss in response to long-term glucocorticoid therapy. Bone Miner 8:39–51.

Lynch MP, Stein JL, Stein GS, Lian JB. 1995. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp Cell Res 216:35–45.

Malik RK. 1997. Regulation of apoptosis by integrin receptors. J Pediatr Hematol Oncol 19:541–545.

McHugh K, Teitelbaum S, Kitazawa S, Ross FP. 1994. Cloning and characterization of the murine integrin β3 gene promoter. J Bone Miner Res 9(suppl 1):S248.

Moursi AM, Damsky CH, Lull J, Zimmerman D, Doty SB, Aota S-I, Globus RK. 1996. Fibronectin regulates calvarial osteoblast differentiation. J Cell Sci 109:1369–1380.

Panetti TS, McKeown-Longo PJ. 1993. The αvβ5 integrin receptor regulates receptor-mediated endocytosis of vitronectin. J Biol Chem 268:11492–11495.

Pistone M, Sanguineti C, Federici A, Sanguineti F, Defilippi P, Santolini F, Querze G, Marchisio PC, Manduca P. 1996. Integrin synthesis and utilization in cultured human osteoblasts. Cell Biol Int 20:471–479.

Puzas JE, Lewis G, Hsu J, Reynolds PR, Rosier RN, O'Keefe RJ, Hicks DG, Cushing J. 1997. Osteoblasts preferentially adhere to sites of prior bone resorption. J Bone Miner Res 12(suppl 1):S413.

Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA. 1993. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin αvβ3 potentiate bone resorption. J Biol Chem 268:9901–9907.

Saito T, Albelda SM, Brighton CT. 1994. Identification of integrin receptors on cultured human bone cells. J Orthop Res 12:384–394.

Schwartz MA, Schaller MD, Ginsberg MH. 1995. Integrins: emerging paradigms of signal transduction. Annu Rev Cell Dev Biol 11:549–599.

Seiffert D. 1996. Detection of vitronectin in mineralized bone matrix. J Histochem Cytochem 44:275–280.

Termine JD, Robey PG. 1996. Bone matrix proteins and the mineralization process. In: Favus MJ, editor. Primer on the metabolic bone diseases and disorders of mineral metabolism. Philadelphia: Lippincott-Raven. p 24–28.

Towler DA, Bennett CD, Rodan GA. 1994. Activity of the rat osteocalcin basal promoter in osteoblastic cells is dependent upon homeodomain and CP1 binding motifs. Mol Endocrinol 8:614–624.

Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. 1997. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102:274–282.

Wong M-M, Rao LG, Ly H, Hamilton L, Tong J, Sturtridge W, McBroom R, Aubin JE, Murray TM. 1990. Long-term effects of physiologic concentrations of dexamethasone on human bone-derived cells. J Bone Miner Res 5:803–813.

Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. 1998. Role of the α2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem 273:32988–32994.

Yamada KM, Miyamoto S. 1995. Integrin transmembrane signaling and cytoskeletal control. Curr Opin Cell Biol 7:681–689.

Yamanouchi K, Gotoh Y, Nagayama M. 1997. Dexamethasone enhances differentiation of human osteoblastic cells in vitro. J Bone Miner Metab 15:23–29.

Zhou H-Y, Fujisawa TR, Mizuno M, Kuboki Y. 1995. Stimulation by bone sialoprotein of calcification in osteoblast-like MC3T3-E1 cells. Calcif Tissue Int 56:403–407.