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1[alpha],25-dihydroxyvitamin D_3 Enhances Annexin II Dependent Proliferation of Osteoblasts

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Abstract Cells experience a variety of physiological and non-physiological stresses and consequently have appropriate mechanisms to deal with such deviations from homeostasis. Particularly subject to mechanical stress and shear forces are the cells that make up the bones. Osteoblastic cells can interpret this stress as a stimulus for proliferation; however, the molecular mechanisms underlying this phenomenon are poorly understood. We have identified annexin II as being specifically upregulated in mechanically stressed osteoblasts and found that increased levels of this protein are necessary for 1[alpha],25-dihydroxyvitamin D_3 mediated augmentation of the proliferative response of osteoblasts after mechanical stress. Our data demonstrate a novel interaction between 1[alpha],25-dihydroxyvitamin D_3 and annexin II in the proliferative response of osteoblasts as well as a novel function for annexin II in the stress response. These findings may offer new therapeutic opportunities for conditions that require regenerative osteoblastic activity such as osteoporosis. J. Cell. Biochem. 100: 679–692, 2007. © 2006 Wiley-Liss, Inc.

Key words: Annexin II; osteoblast; 1[alpha],25-dihydroxyvitamin D_3; mechanical stress

Under proper mechanical conditions, the assembly of the bone matrix is accomplished by an increase in cell number and/or an enhancement of the matrix synthesis rate, which results in higher bone mass. Increases in bone loading lead to increased bone mass [Aloia et al., 1978; Eisman et al., 1990; Bassey and Ramsdale, 1994], whereas reduction in loading, caused by bed rest or weightlessness, is associated with bone loss [Aloia et al., 1978]. It has been established that mechanical forces are essential for the maintenance of the structural integrity of bone tissue, and bone cells from osteoporotic patients may be impaired in their

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long-term response to mechanical stress. Therefore, mechanical loading is considered to be a crucial factor for both the prevention and therapy of osteopenia [Aloia et al., 1978; Kaspar et al., 2000], improvement of retarded bone healing [Neidlinger-Wilke et al., 1994] as well as the tissue adaptation of the healthy bone.

A number of candidate mechano-transducers have been proposed. These include the integrin-cytoskeletal structure [Ingber, 1991], mechano-sensitive cation channels within the cell membrane [Davidson et al., 1990; Klein-Nulend et al., 1995a], G-protein-dependent pathways [Reich et al., 1997], phospholipase C [Brophy et al., 1993], protein kinase C [Reich and Frangos, 1993], prostaglandins, insulin-like growth factor-I (IGF-I) and transforming growth factor-α (TGF-α) [Ajubi et al., 1996; Klein-Nulend et al., 1995b] c-fos and osteocalcin [Inaoka et al., 1995]. One of the earliest responses detected in mechanically stimulated bone cells is a rapid rise in intracellular calcium concentration. Various types of mechanical stimuli elicit an increase of calcium concentration in osteoblasts following second messenger response. Parathyroid hormone (PTH) has been shown to be required for mechanically induced bone formation in rats [Chow et al., 1998]. PTH and mechanical loading activate many of the same second messenger pathways in osteoblasts, including an increase in intracellular calcium, therefore PTH can potentiate the effects of mechanical stimulation. The expression of an inducible isoform of cyclooxygenase (COX-2) was increased through the addition of PTH to mechanically loaded cells. COX-2 appears to be key to the osteogenic response of bone to exogenous loading. However, the mechanisms by which bone process mechanical input are poorly understood. We show that annexin II is upregulated after mechanical stimulation and addressed its relevance for the proliferative response of osteoblasts.

The annexin family belongs to the Ca^{2+}- and membrane-binding proteins, and includes 12 annexin subfamilies (A1–A11 and A13). The membrane binding occurs in a reversible manner, in contrast to most of the other members of Ca^{2+} binding proteins. Across the sub-classification, all annexins are expressed in most eukaryotic cell types. Annexins display a wide spectrum of functions, such as apoptosis, calcium signaling, vesicle trafficking, or anti-inflammatory function. Annexin II forms with S100A10 a tetramer and is secreted onto the cell surface of monocytes and macrophages. S100A10 itself has been shown to bind directly to several ion channels at the plasma membrane. However, annexin II is shown to be involved in fibrinolysis and releasing plasmin from the cell surface [Brownstein et al., 2004]. Therefore, it activates the fibrinolytic cascade and overexpression of annexin II in leukemic cells leads to increased bleeding [reviewed in Rescher and Gerke, 2004].

The role of 1[alpha],25-dihydroxyvitamin D_3 in bone metabolism is well established. 1[alpha],25-dihydroxyvitamin D_3 deficiency is shown to contribute to accelerated bone loss and increasing fragility, but also to neuromuscular impairment that can increase the risk of falls [reviewed in Raisz, 2005]. However, contradictory results were obtained in clinical trials. There is evidence which indicates that supplementation of 1[alpha],25-dihydroxyvitamin D_3 and calcium can decrease bone resorption, increase bone mass, decrease fracture rates, and even decrease the frequency of falling [Raisz, 2005]. Moreover, clinical trials proofed the effect of 1[alpha],25-dihydroxyvitamin D_3 in prevention of osteoporosis [Vieth, 2005]. However, on the other hand studies showed that supplementation of 1[alpha],25-dihydroxyvitamin D_3 and calcium did not reduce fracture risk [Grant et al., 2005]. One reason might be polymorphisms in the 1[alpha],25-dihydroxyvitamin D_3 receptor (VDR). Differences are associated with variabilities in the calcitriol response or the interaction with calcium.

MATERIALS AND METHODS

Each Experiment was Repeated at Least Three Times

Primary osteoblasts. Osteoblast cultures were established form cortical bone samples taken from the tibia or femur of patients between the ages of 25–70. All samples were taken from healthy donors, who underwent surgery for fracture repair. The bone cell cultures were established as described by Robezy and Termine [1985]. Phosphate buffered saline (PBS), calcium and magnesium free, pH 7.5, was used for all washes. The osteoblast cells were sub-cultured by trypsin/EDTA treatment and cells were used up to the fourth cell passage. Usually the second or third passage was used for the experiments. To

determine the osteoblast phenotype, AP and osteocalcin activity was measured by a colorimetric assay (Roche, Germany). At least 1 week before the cells were seeded, the silicon dishes were treated with DMEM supplemented with 20% FCS to establish a protein phase on the dishes. The cells were seeded on the conditioned dishes and maintained for 3 days until subconfluent cell density was obtained.

MC3T3-E1. The MC3T3-E1 cell line was established from the calvaria of a mouse embryo. Cells were plated and grown to confluency in MEM containing 10% FCS, antibiotics (100 U penicillin G/ml, 0.01 mg streptomycin/ml) and 20 mM L-glutamine.

RobC26. RobC26 is an osteoblast cell line derived from rats, cultured in DMEM containing 10% FCS, 100 U penicillin G/ml, 0.01 mg streptomycin/ml.

HaCaT. HaCaT cells are human keratinocytes, which were used as a control. HaCaT cells were cultured in DMEM, supplemented with 10% FCS and 100 U penicillin G/ml, 0.01 mg streptomycin/ml. HaCaT cells were equally treated.

Cell stretching experiments. The cells were seeded on conditioned deformable silicone dishes. The dishes were cyclically stretched in an electro-mechanical cell stretching apparatus that applied uniform strain by simultaneous stretching of six silicone dishes in their long axis as described by Neidlinger-Wilke et al. [1994]. Sub-confluent cultures were stimulated by cyclic biaxial strain whereas strain magnitude was 1% strain (10,000 μstrain) and the frequency was 1 Hz. In all experiments osteoblasts grown in the same elastic silicone dishes but without mechanical stimulation served as controls.

Centrifugation. Sub-confluent cultures of MC3T3-E1, HaCaT, and RobC26 cells were centrifuged for 5 min at 287*g*, and returned to the incubator for 24 h. Additionally primary human osteoblast cells, obtained from Promocell (Heidelberg, Germany) were used in the same experiments.

Ultrasound. The ultrasound experiments were done in a Sonifier 250, Branson. Cells were cultured as described, and transferred to a microtube, treated 3 times for 5 s at 2 kHz, 2 W. After this procedure, the cells were seeded in culture dishes.

1[alpha],25-dihydroxyvitamin D_3 treatment. Cells were seeded and 1[alpha],25-dihydroxyvitamin D_3 (Sigma) was added at different time points after the stress application. The most efficient concentrations were 100 pM and 10 nM. The measurement was done 24 h after stress application.

2-D gel electrophoresis and Western blotting. For 2-D PAGE analysis, cells were grown to 70–80% confluency. After washing three times with PBS, cells were harvested by scraping with a plastic scraper (Costar). The cell suspension was centrifuged at 4,000 rpm, 4°C for 4 min. The pellet was redissolved in 9 M Urea (Merck), 4% CHAPS (Sigma), 0.8% IPG-Buffer (Pharmacia), and 15 mM DTT (Sigma). Cells were disrupted by two freezing-thawing cycles in liquid nitrogen and ultrasonic treatment at 4°C. The whole sample was then cleared by centrifugation through a 0.1 μm filter Unit (Millipore). Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) according to the supplier's manual. Equal amounts of protein from the treated and untreated cells were used for the 2-D electrophoresis. The protein samples were diluted in 8 M Urea (Merck), 2% CHAPS (Sigma), 15 mM DTT (Sigma), and 0.2% IPG-buffer (Pharmacia) to a final volume of 350 μl. The first dimensional isoelectric focusing was carried out on an Immobiline DryStrip (18 cm long, pH linear range 3–10; IPG-Phor, Pharmacia). The voltages applied were 6 h, 30 V; 6 h, 60 V; 1 h, 150 V; 1 h, 200 V; 1 h, 500 V; and finally 8 h, 8,000 V.

After focusing, the gel strip was equilibrated for SDS–PAGE as described (Pharmacia). For the second dimension, SDS gel electrophoresis was carried out vertically using a standard 4 % separating/12% stacking gel in a BIO-RAD PROTEAN II-system. During the run, the chamber was cooled to 10°C.

Protein spots were visualized by silver staining using the protocol of Shevchenko et al. [1996]. The spots of interest were cut out with a sterile scalpel and transferred immediately to a microtube and stored at −80°C. Sequence determination was done by PROTANA, Denmark.

Protein expression was analyzed by Western blotting using the anti-annexin II monoclonal antibody (Transductions Laboratories) in combination with enhanced chemiluminescence (WestPico Chemiluminescent Substrate, Pierce).

Reporter gene assay. A fragment of 1,442 bp of the annexin II gene promoter amplified by

PCR was cloned into the commercial pGL3-vector, containing the luciferase reporter gene (Promega, Mannheim, Germany). The forward primer was: 5′-CGACGCGTGGGGCCAACTT-CACAGCTAC-3′ and the reverse primer: 5′-CCCAAGCTTCTGGGTGGGGCTTTTATACC-3′. On the forward primer a MLU site was added and on the reverse primer a HindIII site was added. Luciferase activity was measured in a Berthold luminometer. The transfection was done with lipofectamine (Metafectene, Biontex, Germany). As a control we used the empty vector as well as a cytomegalovirus promoter driven microphtalmia transcription factor expression construct (XMI, derived from the fish Xiphophorus) [Delfgaauw et al., 2003] and unstressed cells as well. The transfection was done prior to stress.

Proliferation Assay

The proliferation assay used is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. It is based on tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). Cells were plated in 96-well plates. At different time points after treatment, 20 μl proliferation solution (Promega, Cell-Titer 96) were added to each well and incubated in 37°C for 1 h. The absorbance at 490 nm was then measured by using a 96-well plate reader (MR 5000 ELISA-READER, Dynatech). All experiments were performed at least three times.

FACS Analysis

Both cell lines were resuspended in 1.5 ml medium and 2 ml FACS-buffer containing PBS, FCS and sodium-acid, and centrifuged 5 min at 1,200 rpm. Cells were washed in FACS-buffer again and resuspended in the same buffer and incubated in 10 μl anti-annexin II antibody for 30 min on ice. Four milliliters of FACS-buffer was added and centrifuged at 1,200 rpm for 5 min. The supernatants were discarded and the pellet incubated with the second antibody for 30 min in the dark. After recentrifugation and resuspension in FACS-buffer, the cells were analyzed in Beckman Coulter, EPICS XL system.

Immunofluorescence

The cell lines were stressed as described above, afterwards grown on coverslips in 6-well plates. Twenty-four hours later, the cells were harvested and washed in PBS, fixed in 4% methanol-free formaldehyde solution in PBS (pH 7.4) for 20 min in a Coplin Jar at 4°C, and washed in fresh PBS for 5 min at room temperature. The cells were permeabilized by immersing in 0.2% Triton X-100 in PBS for 5 min. They were air-dried and incubated with PBS/1%BSA (Sigma) for 20 min. Then the cells were stained with the different antibodies (anti-annexin IImAb, Transduction Laboratories; and anti-1[alpha],25-dihydroxyvitamin D_3, provided by Leo Pharma) [Blaehr et al., 2001, 2003] for 1 h. After three washes in PBS, cells were stained with Cy3 and Cy2 (Jackson Immuno Research) for 1 h, washed in PBS and embedded in Vectashield. The microscopy was done with a Leica CLSM version TCS NT. The signal intensity quantification was done using Image J (http://rsb.info.nih.gov/ij/). On each picture the mean intensity from all cells was determined.

RESULTS

To investigate the stress response of bone forming cells, we isolated primary osteoblasts intra-operatively from bone fractures in human patients. After two or three in vitro passages the cells were subjected to mechanical stress by strain. The 2-D Gel analysis revealed 11 proteins that were reproducibly altered as compared to the non-stressed control cells. Protein-microsequencing identified one of the most prominent spots as annexin II (Fig. 1a). The sequencing of the other spots is not done yet.

To verify that annexin II is indeed upregulated in mechanically stressed osteoblasts, primary human osteoblasts and the mouse osteoblastic cell line, MC3T3, were analyzed by Western blotting after stretching or after using gravitational forces as mechanical stress. An approximate eightfold increase in the level of annexin II was consistently observed (Fig. 1b).

A reporter gene construct containing 1,442 bp of the annexin II upstream regulatory region was transfected into MC3T3 cells to determine whether the increased protein levels in stressed cells were due to transcriptional activation. The activity of the reporter construct, as measured

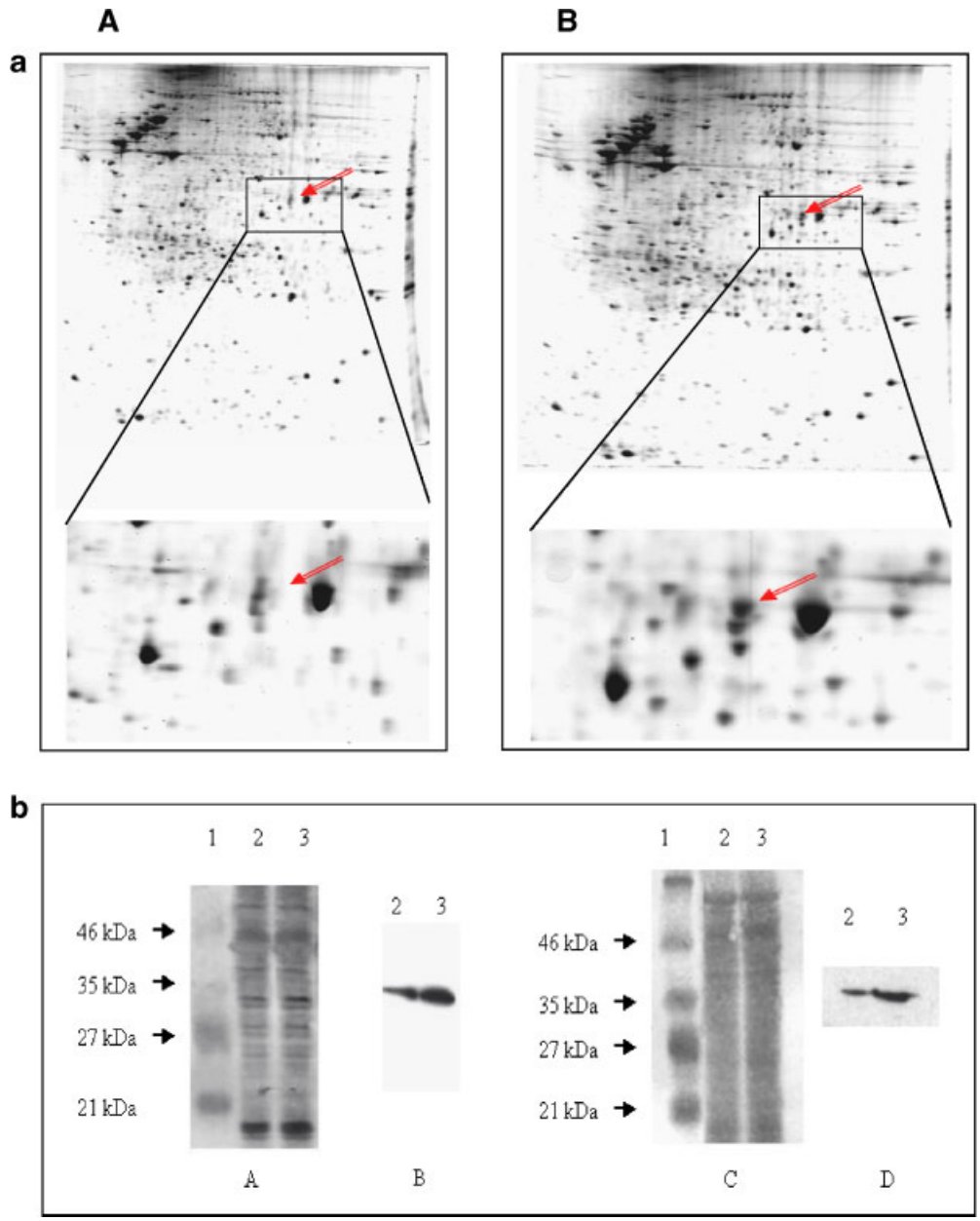


Fig. 1. **a**: 2D-gel electrophoresis of whole cell extracts isolated from untreated (**A**) and mechanically stimulated (**B**) primary osteoblasts. The arrow demarcates the spot corresponding to annexin II. **b**: Western blot analysis of primary osteoblasts (**A** and **B**) and MC3T3 cells (**C** and **D**) stimulated by centrifugation. To confirm equal loading of protein amounts, the nitrocellulose membrane was stained using Ponceau S after blotting. **A**: **Lane 1**: Marker; **2**: untreated osteoblast cells; **3**: stimulated osteoblast cells. **B**: **2**: untreated osteoblast cells; **3**: treated osteoblast cells. **C**: **Lane 1**: Marker; **2**: untreated MC3T3; **3**: stimulated MC3T3. **D**: **2**: untreated MC3T3; **3**: treated MC3T3.

by luciferase assay (Fig. 2), was increased in stressed cells with the maximum response seen 24 h after the gravitational stress. When an irrelevant expression construct (a fish transcription factor driven by the CMV promoter) was used, reporter gene expression was unaffected by mechanical load, indicating the specificity of the upregulation. Also non-stressed, transfected cells revealed no induction of annexin II after various time points.

We next determined whether the apparent transcriptional upregulation of annexin II in response to mechanical stress resulted in physiological consequences. We therefore analyzed the proliferation of mouse and rat osteoblasts after mechanical stress. A significant

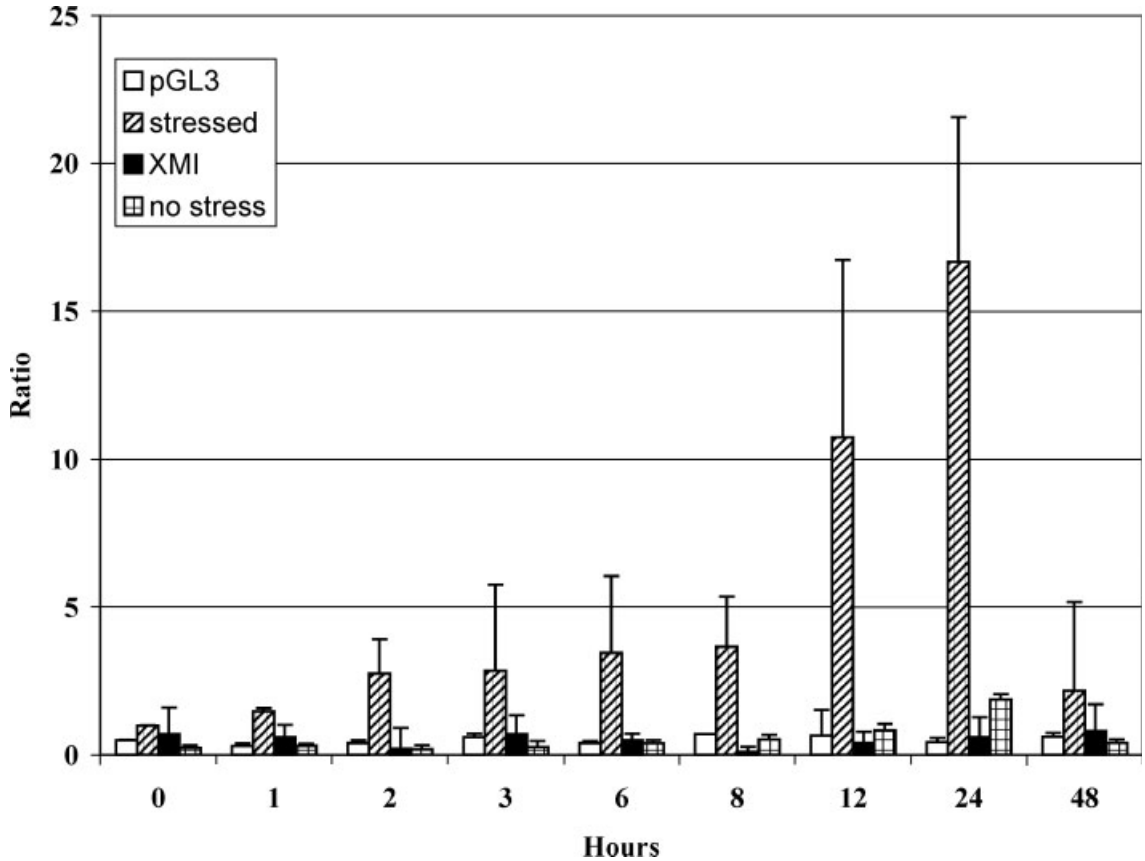


Fig. 2. Luciferase-reporter-gene assay of annexin II 1,442 bp promoter fragment in pGL3-vector system in MC3T3-cells. The cells were stressed by centrifugation as described and harvested at different time points. Transfection efficiency was normalized by cotransfection of the β-galactosidase vector system. As a control the empty pGL3-vector and a CMV driven Xiphophorus microphtalmia expression construct were used. No stress: transfected, no mechanical stress. Time point 0 refers to the unstressed cells. The mechanical stress was applied 12 h after the transfection.

approximate twofold stimulation of proliferation in stressed cells compared to non-stressed cells was observed with the maximum at 24 h after treatment (Fig. 3a). For a control HaCaT cells were used which revealed no proliferation at any of the same time points. 1[alpha],25-dihydroxyvitamin D_3 is a well-known regulator of bone formation and metabolism. Addition of 100 pM of 1[alpha],25-dihydroxyvitamin D_3 20 h after stress application led to a further twofold increase of the proliferation response of the mechanically stressed bone cells (Fig. 3b). Interestingly, there is only a very narrow time window in which the 1[alpha],25-dihydroxyvitamin D_3 effect can be observed. This effect is statistically highly significant ($P < 0.0006$). Application of an anti-annexin II antibody to the cells totally abolished the stimulatory action of 1[alpha], 25-dihydroxyvitamin D_3 (Fig. 3c). This effect is independent from the observed mechanical stress induced annexin II expression, as the antibody did not interfere with the proliferation response. However, transfection of a dominant negative mutant form of annexin II, XM-mutant [Harder and Gerke, 1993], resulted in a significant decrease of the stress induced proliferation (Fig. 3d).

Because cell stretching is a mechanical stress that would be difficult to perform for potential therapeutic application, we also exposed the cells to ultrasonic treatment. Interestingly, the proliferative response was more pronounced (1.5×) than stressing them with gravitational forces (Fig. 3e). In addition, application of 1[alpha],25-dihydroxyvitamin D_3 exactly as it was done in the cell stretching experiments also enhanced the proliferative response more than 2.5-fold.

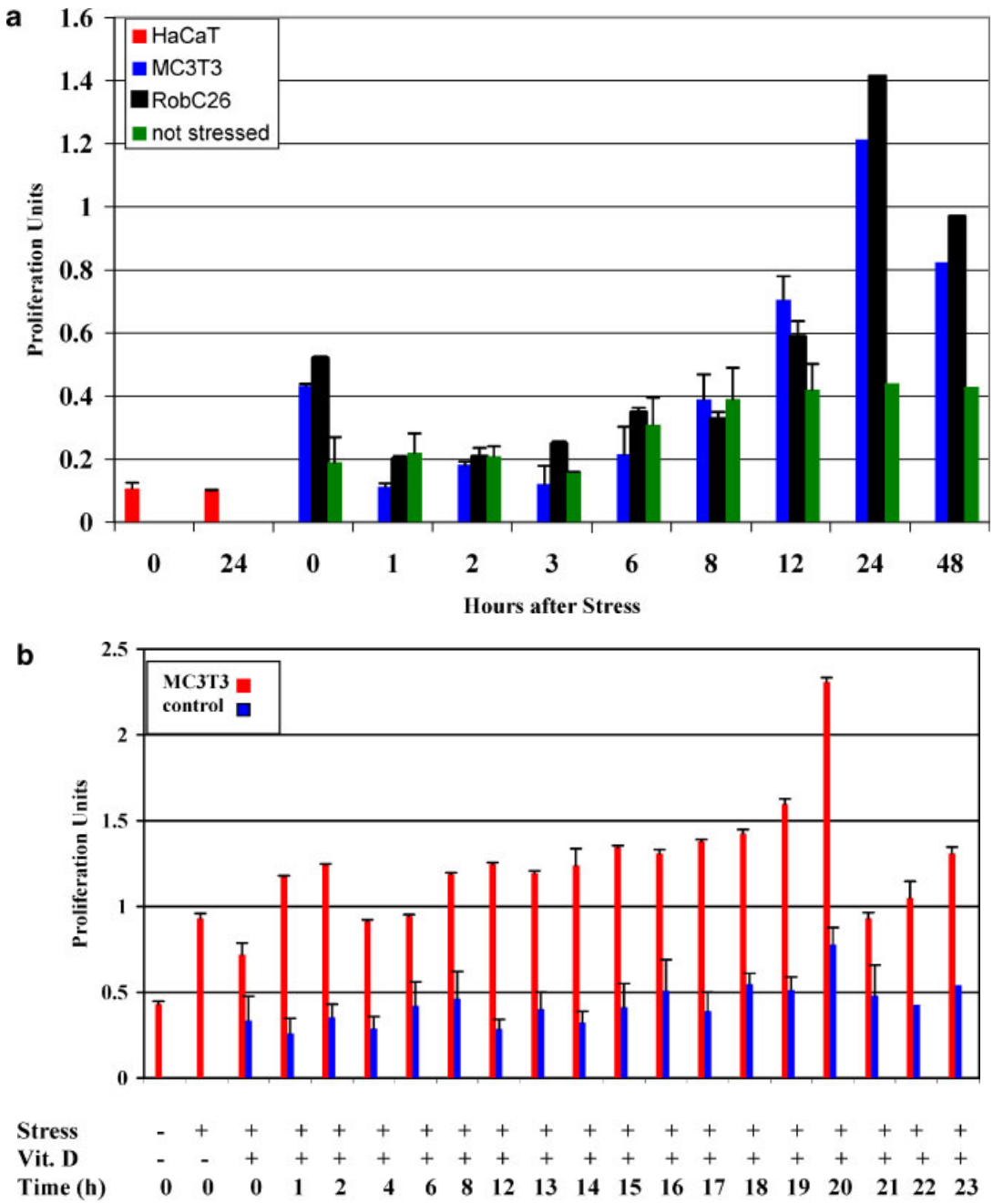
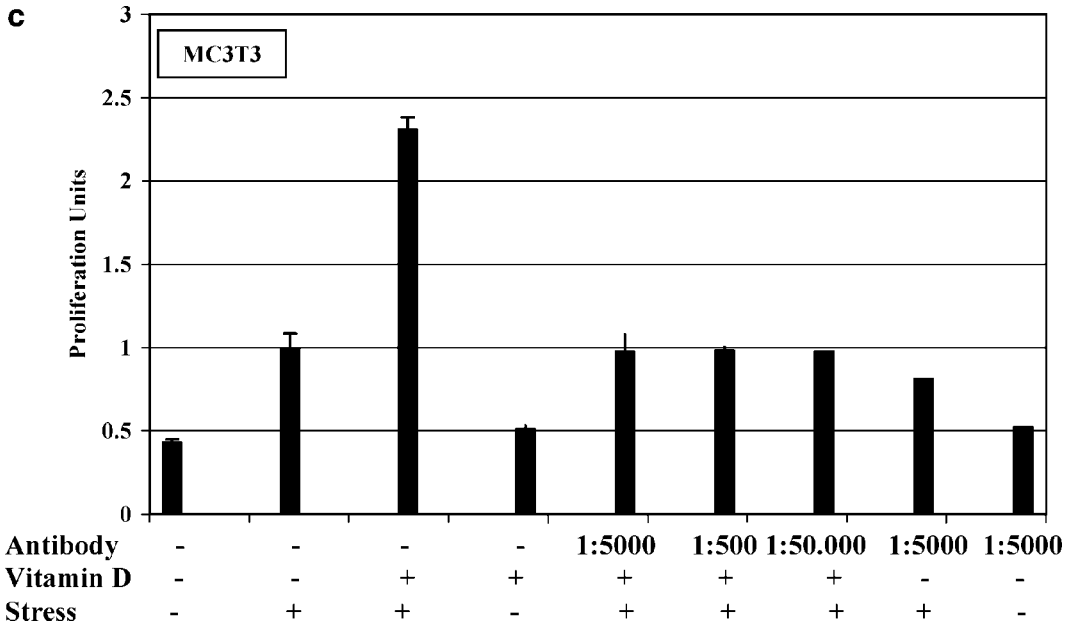


Fig. 3. **a**: Proliferation assay in MC3T3 and ROBC26 cells at different time points. Cells were stressed by centrifugation. HaCaT: control not stressed at time 0 and stressed at 24 h. Not stressed: MC3T3 without application of mechanical stress was also used as a control. The highest proliferation rate was seen 24 h after application of stress. **b**: Proliferation assay in MC3T3 in the presence of 100 pM 1[alpha],25-dihydroxyvitamin D_3. For the stressed MC3T3 cells each measurement was done 24 h after stress. The time line indicates the point of time when 1[alpha], 25-dihydroxyvitamin D_3 was added. For the control, MC3T3 cells were treated with MC3T3 cells treated with 1[alpha],25 dihydroxyvitamin D_3 at the same time points without mechanical stress the measurement was also done after 24 h. The difference between 18, 19, 21, and 22 to 20 h hormone application is highly significant. 18:20, $P < 0.0006$; 19:20, $P < 0.002$; 20:21, $P < 0.0001$; and 20:22, $P < 0.0002$ (*t*-test). **c**: Proliferation assay in MC3T3 in the presence of anti-annexin II antibody with or without 1[alpha],25-dihydroxyvitamin D_3; proliferation measured 24 h after mechanical stress. **d**: Proliferation assay in MC3T3 in the presence of a dominant negative form of annexin II. As a control, the Xiphophorus vector (XMI) was transfected by the lipofectamine procedure. **e**: Effect of ultrasonic treatment on proliferation in MC3T3 cells in the presence and absence of 1[alpha],25-dihydroxyvitamin D_3 and anti-annexin II antibody. The measurement was done 24 h after the application of ultrasound.



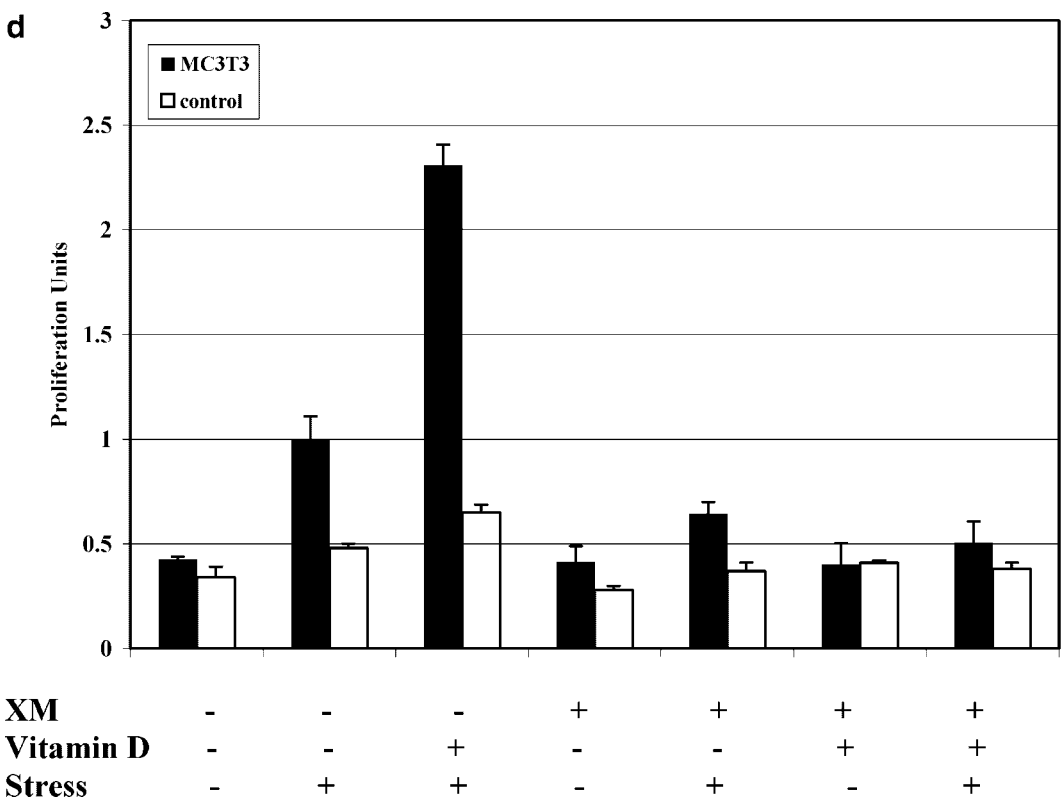


Fig. 3. (*Continued*)

A possible link between annexin II and 1[alpha],25-dihydroxyvitamin D_3 emerged from studies on the subcellular localization of annexin II. In unstressed cells the protein is evenly distributed over the whole cytoplasm and only low amounts can be detected on the cell surface. We performed immunolocalization studies using anti-annexin II polyclonal antibodies to determine the position of annexin II after mechanical stress and found increased amounts of annexin II at the outer cell membrane. This phenomenon was further enhanced if the cells

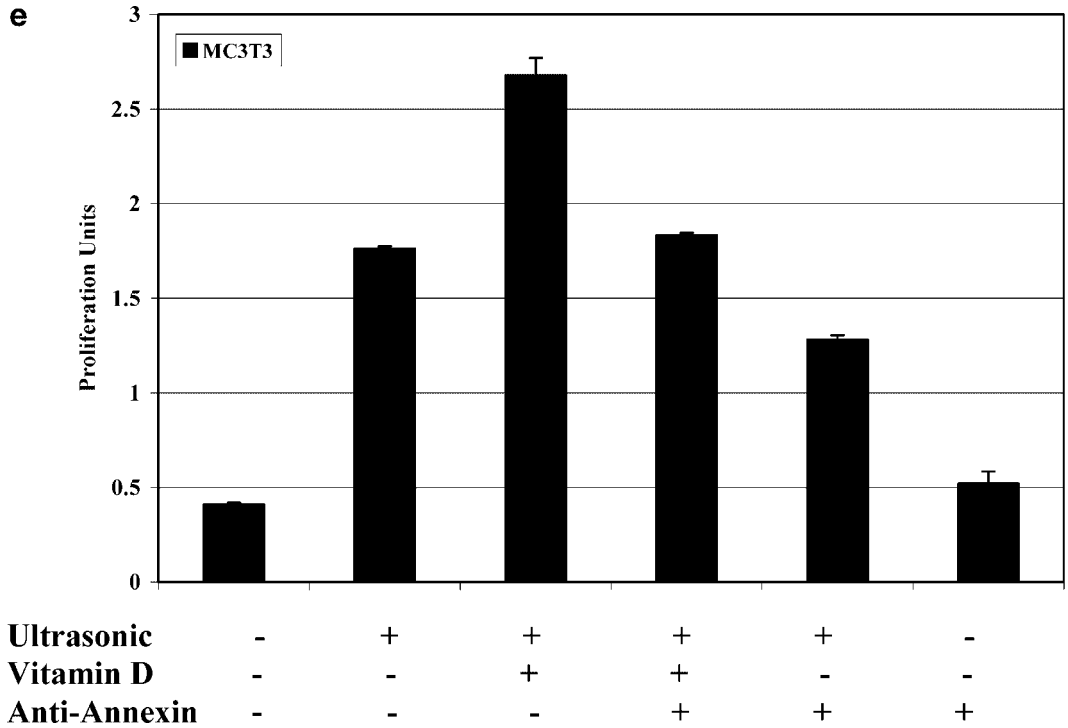


Fig. 3. (*Continued*)

were given the proliferation stimulatory 1[alpha],25-dihydroxyvitamin D_3 treatment (Fig. 4a–c). The increase was determined by image analysis as averaged signal cell intensity between the mechanical stressed cells (Fig. 4b) and the cells additionally treated with 1[alpha],25-dihydroxyvitamin D_3 (Fig. 4c) and revealed a 1.98-fold upregulation.

Recently, Baran et al. demonstrated that annexin II can act as a membrane receptor for 1[alpha],25-dihydroxyvitamin D_3 in osteoblast-like cells, thus mediating the action of 1[alpha],25-dihydroxyvitamin D_3. This results in a fast Ca^{2+} influx at the cell membrane [Baran et al., 2000]. We performed FACS-analysis of stressed osteoblasts to determine the cellular location of annexin II before and after treatment with 1[alpha],25-dihydroxyvitamin D_3. The results indicated that annexin II in mechanically stressed cells appeared at the cell surface only in the presence of D_3, further supporting the idea that annexin II may act as a VDR (Fig. 5). Interestingly, when the cells were transfected with the dominant negative version of annexin II (XM-mutant), no cell surface localization of annexin II was detectable after stress and in the presence of 1[alpha],25-dihydroxyvitamin D_3 (Fig. 5g). This supports the crucial role of annexin II. In addition, we carried out immunolocalization studies on stressed cells and found that 1[alpha],25-dihydroxyvitamin D_3 colocalizes with annexin II at the cell membrane (Fig. 6a).

Taken together our data indicate that the induction of proliferation is independent of the nature of the mechanical stress and gives therapeutic options for treating bone fractures or patients suffering from osteoporosis. The results provide evidence of a novel interaction between 1[alpha],25-dihydroxyvitamin D_3 and annexin II in the proliferation response of osteoblasts as well as a novel function of annexin II in the stress response.

DISCUSSION

In this study we demonstrated that annexin II expression increased in osteoblast cell lines and cultivated primary osteoblasts 24 h after mechanical stimulation. Furthermore, the proliferation of osteoblasts is coincident with the increase in annexin II expression, implicating a direct connection between annexin II expression and proliferation of osteoblasts after mechanical stress. This finding is corroborated by experiments, which show that cells expressing a dominant negative mutant form of

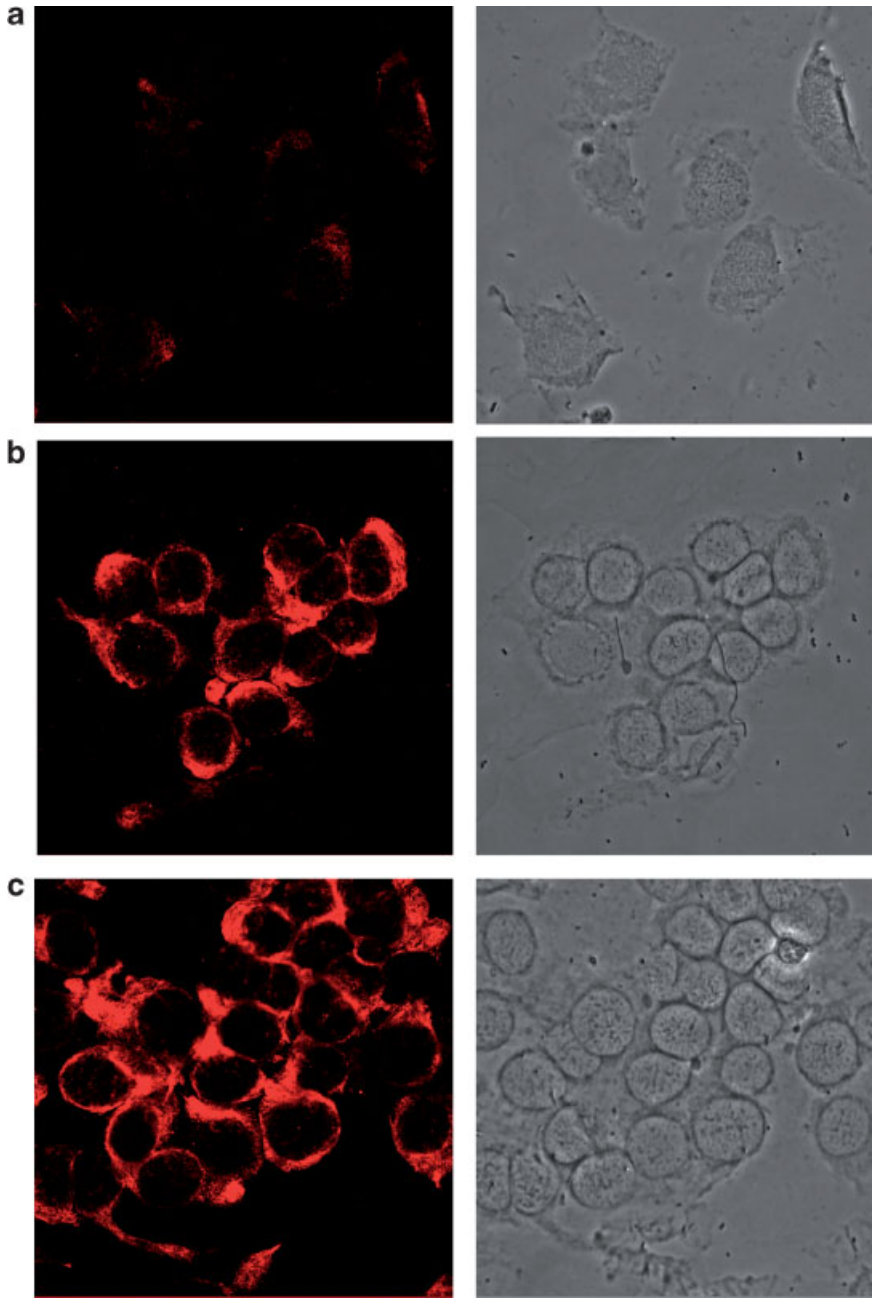


Fig. 4. Laser scanning experiments in MC3T3 cells. Immunofluorescence of annexin II, MC3T3 cells, stressed with mechanical loading, 24 h after stress and fixed in 4% PFA. **a**: control, −centrifugation, −1[alpha],25-dihydroxyvitamin D3, immunofluorescence, and phase contrast; (**b**) +centrifugation, −1[alpha],25-dihydroxyvitamin D_3; (**c**) +centrifugation, +100 pM 1[alpha],25-dihydroxyvitamin D_3.

annexin II have little or no proliferative response to mechanical stress.

Annexins are a family of structurally related proteins characterized by a Ca^{2+}-dependent binding to phospholipids. A variety of cellular functions have been proposed for annexin proteins, such as the regulation of membrane traffic and calcium channel activity [Blackwood and Ernst, 1990], mitogenic signal transduction, and the regulation of cytoskeletal-membrane interaction [Mohiti et al., 1995]. The actin cytoskeleton and the integrin family of cell adhesion molecules have been shown to play important roles in mechano-transduction [Pavalko et al., 1998]. Annexin II is a Ca^{2+}-binding protein and could therefore be involved

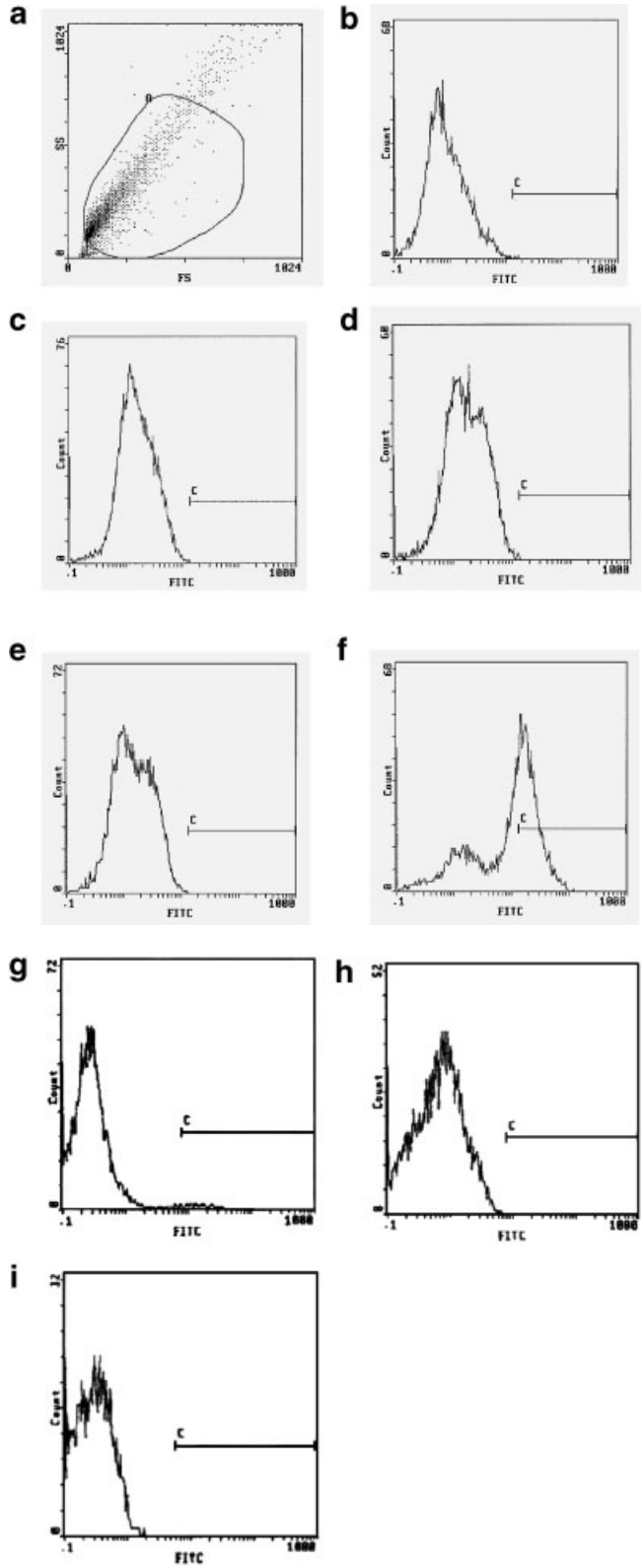


Fig. 5. FACS analysis of MC3T3 cells. Cells were stressed with centrifugational forces with and without 1[alpha],25-dihydroxyvitamin D_3, 24 h after stress detection with anti-annexin-II antibody; (**a**) gate; (**b**) autofluorescence of MC3T3; (**c**) −stress, −1[alpha],25-dihydroxyvitamin D_3, anti-annexin; (**d**) −stress 100 pM 1[alpha],25-dihydroxyvitamin D_3, anti-annexin; (**e**) +stress, −1[alpha],25-dihydroxyvitamin D_3, anti-annexin; (**f**) +stress, 100 pM 1[alpha],25-dihydroxyvitamin D_3 and stained with anti annexin II antibody; (**g**) +stress, 100 pM 1[alpha],25-dihydroxyvitamin D_3, XM-transfected, anti-annexin; (**h**) anti-annexin antibody; (**i**) secondary antibody.

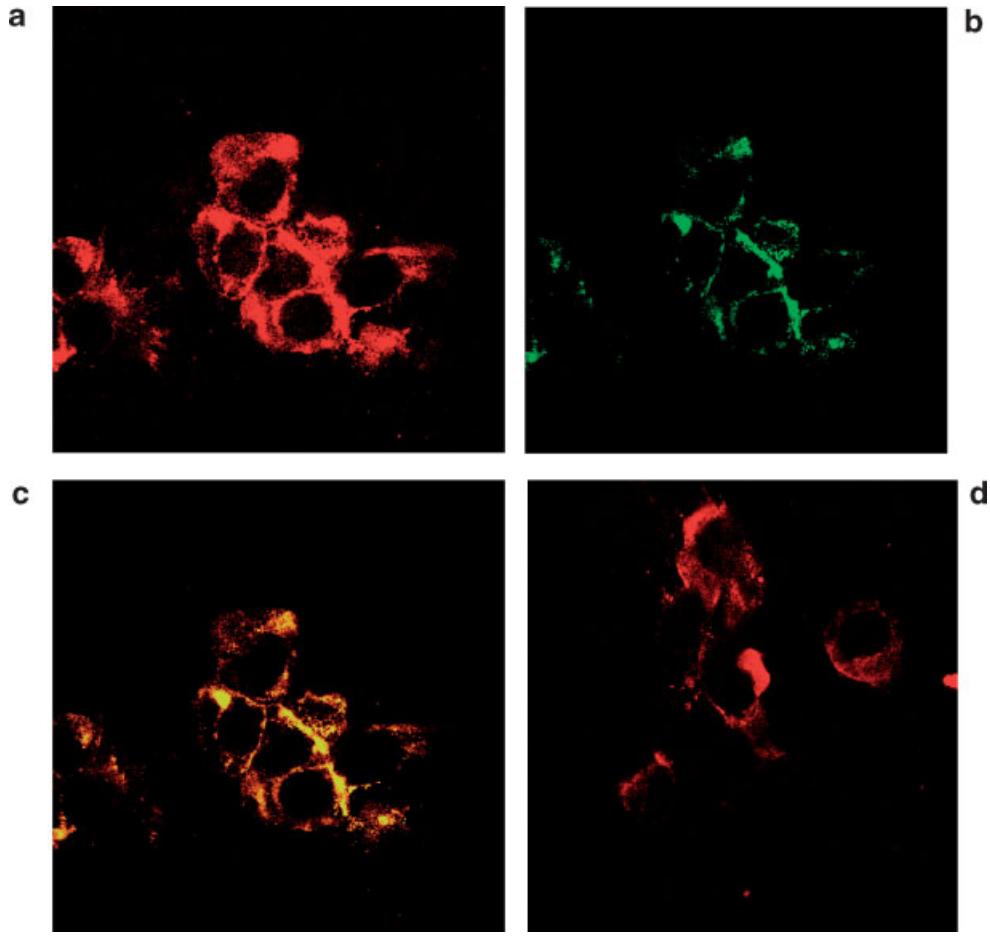


Fig. 6. Laser scanning experiments in MC3T3 cells. Immunofluorescence of annexin II and 1[alpha],25-dihydroxyvitamin D_3. MC3T3 cells, stressed with mechanical loading, 24 h after stress and fixed in 4% PFA. **a–c**: The cells were treated with 100 pM 1[alpha],25-dihydroxyvitamin D_3 20 h after mechanical stress and stained 24 h after mechanical stress with anti-annexin antibody; (b) or anti-1,25-dihydroxyvitamin D_3 antibody b, as indicated; (c) overlay of a and b; (**d**) negative control, no 1[alpha],25-dihydroxyvitamin D_3 added, stained with anti-annexin antibody.

in mediating cellular reactions to Ca^{2+}-concentration changes. Previous work has highlighted the possible role for Ca^{2+} in mediating the response of osteoblasts to mechanical and hormonal stimuli [Hung et al., 1995]. Annexin II is expressed in osteoblasts [Suarez et al., 1993; Mohiti et al., 1995], but also in other cell types, where it displays a broad subcellular localization range. Several studies reported the localization of annexin II to the nucleus [Vishwanatha et al., 1993; Baran et al., 2000], at the plasma membrane; inner and outer surfaces [Thiel et al., 1992] as well as in the extracellular matrix [Siever and Erickson, 1997]. It was shown that annexin II can act as an extracellular receptor for polypeptide ligands [Siever and Erickson, 1997] and for plasminogen on the surface of endothelial cells [Fitzpatrick et al., 2000]. In addition, annexin II was shown to function as an activator of osteoclast formation [Menaa et al., 1999; Baran et al., 2000]. A complex balance between formation and resorption regulates bone strength. Involvement in both processes suggests that annexin II plays a central role in bone metabolism.

Moreover, the annexin II gene promoter has an AP-1 binding site. Fos protein is important in bone cells because recognition elements for the AP-1 complex are found in the promoter region of several genes involved in the growth and mineralization of bone. The *c-fos* gene is also induced by mechanical loading [Ruther et al., 1987; Grigoriadis et al., 1994; Fitzgerald and Hughes-Fulford, 1999], and experiments with transgenic mice indicated that regulation of c-fos expression is important

for normal bone development [Ruther et al., 1987].

The role of 1[alpha],25-dihydroxyvitamin D_3 in bone metabolism is well accepted. 1[alpha],25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is known to initiate a biological response via regulation of gene transcription [Lowe et al., 1992] as well as "rapid" pathways [Norman et al., 1992]. Interestingly, the annexin II promoter region contains a vitamin-D binding site, indicating that annexin II is a target gene for 1[alpha],25-dihydroxyvitamin D_3. In addition, Baran et al. [2000] have shown that annexin II might serve as a membrane receptor for rapid action of $1,25(OH)_2D_3$. According to Baran et al. the 100 pM concentration of $1,25(OH)_2D_3$ that we used throughout the majority of our experiments is well below the proposed equilibrium concentration for $1,25(OH)_2D_3$. However, we obtained similar results using 10 nM (data not shown). Although our data suggest a receptor function of annexin II for 1[alpha],25-dihydroxyvitamin D_3 the highly effective 100 pM concentration might point to an unknown interaction between annexin II and $1,25(OH)_2D_3$. Antibodies against annexin II inhibited $1,25(OH)_2D_3$-induced increases in intracellular calcium in ROS 24/1 cells.

We propose a model whereby increased levels of annexin II after mechanical stimulation result in an enhanced localization of the protein at the cellular membrane of osteoblasts with the addition of 1[alpha],25-dihydroxyvitamin D_3 to the cells, causing annexin II to migrate to the outer membrane and act as membrane receptor for 1[alpha],25-dihydroxyvitamin D_3. Our data suggest that the most beneficial therapeutic course for osteoblastic regeneration would include the concurrent administration of mechanical stress and 1[alpha],25-dihydroxyvitamin D_3.

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