A molecular mechanism of integrin regulation from bone cells stimulated by orthodontic forces

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SUMMARY The purpose of this paper is to discuss a molecular mechanism in the signal transduction pathways of the regulation of integrin genes taking place in bone cells as a result of orthodontic or mechanical stimulation. Human osteosarcoma (HOS) TE-85 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 and grown to confluency in Flexercell type I dishes and orthodontic forces were applied to the cells via an intermittent strain of 3 cycles/minute using the Flexercell Strain Unit System for periods of 15 and 30 minutes, 2 and 24 hours and 3 days. Antibodies against β_1 and α_v integrins were immunolocalized in strained and unstrained cultures. Total RNA was extracted and cDNA probes were used to measure at various mRNA expression of β_1 (1.2 kb) and α_v (l.l kb) integrins. A cDNA probe for cyclophylin (750 b) was used for controls of gene expression. Results showed that mechanical stimulation caused a reorganization of integrin distribution in comparison with non-stimulated controls. mRNA for β_1 expression showed a marked increase at 30 minutes and 3 days, while mRNA levels for α_v did not change with strain. The selective expression of integrins mRNA is indicative of a specific gene regulation by mechanical stimulation in the cells studied.

Introduction

The application of mechanically generated forces is central in the prevention and correction of dentofacial discrepancies and dentoalveolar malocclusions. During clinical treatment, these forces are highly effective in determining tooth position. However, they may also generate a wide range of undesired biological reactions associated with the force-induced response. While mechanical forces may be regarded as beneficial in connective tissues such as bone (Wolff, 1892; Lanyon, 1987), the cellular mechanisms involved in the translation of clinical forces into biological responses are poorly understood. The macroscopic studies of orthodontically treated teeth have shown the remarkable potential for connective tissue remodelling both *in vivo* (Yen and Chiang, 1984; Yen *et al.*, 1989a, b) and *in vitro* (Reitan and Kvam, 1971; Yen *et al.*, 1990). Partly due to the effectiveness of clinical treatment, this potential for remodelling has been largely based on the type, duration

and magnitude of applied forces (Storey, 1973), specifically to those descriptions of tissue tension and compression associated with bone apposition and bone resorption, respectively. These concepts stem mainly from early histological observations, including a variety of morphological and histochemical studies undertaken to clarify orthodontic tissue response (Reitan and Kvam, 1971; Storey, 1973; Rygh, 1976; Lilja *et al.*, 1983; Martinez and Jonhson, 1987; Davidovitch, 1991). Meanwhile, the clinically applied force may appear highly 'uncontrolled' at the cellular level, possibly explaining why seemingly 'light' and 'controlled' orthodontic forces may, in addition to acceptable tissue remodelling, generate undesired cellular responses.

Rygh (1976, 1977) observed considerable tissue degradation in the periodontal ligament following orthodontic treatment. Kvam (1972) found that root resorption was present in all orthodontically-stressed teeth, while Sims *et al.* (Langford and Sims, 1982; Sims and Weekes,

1985) determined that root resorption was directly related to the magnitude of orthodontic force applied. A possible explanation for this phenomenon may be associated with injury to the periodontal ligament, since it has been reported that intentional ligament trauma could initiate patterns of root resorption (Nakane and Kameyama, 1987). As yet, theories of trauma and/or magnitude of forces can not explain the changes induced in cell behaviour as a result of orthodontic and especially physiological stimulation. Better understanding of mechanisms of cellular signal transduction in mechanically-stimulated connective tissues may help to explain unexpected cell reactions observed as sequelae in the majority of orthodontic-treated clinical cases.

It has been suggested that the cellular environment of specialized matrix scaffoldings characterizes the cellular responses (Ingber, 1991). Lanyon (1987) has concluded that the matrix provides a source of 'strain memory' which can continue the process of cell stimulation even some time after removal of the initial force. However, physiological and mechanically-induced processes depend on critical recognition and binding of selected cell ligands thereby ensuring appropriate functioning of certain cell properties such as cell adhesion and cell migration. These interactions are manifested by changes in cell morphology, proliferation and gene expression (Ginsberg *et al.*, 1992a). Cell–matrix and cell–cell interaction are known to be mediated by the family of adhesion molecules called integrins.

Integrin receptors are transmembrane hetero-dimers formed by an α and a β subunit associated non-covalently (Ingber, 1991; Hynes, 1992; Yamada *et al.*, 1992) In addition to their potential for transducing signals from the extracellular to the intracellular environment (Damsky and Werb, 1992; Sastry and Horwitz, 1993), integrin gene expression in the nucleus and protein post-translational modifications in the cytoplasm also appear to regulate this 'outside-in' mechanism of cellular messages, establishing an 'inside-out' signalling pathway (Damsky and Werb, 1992; Ginsberg *et al.*, 1992b; Sastry and Horwitz, 1993). This concept is highly relevant to orthodontic-stimulated tissue response. The aim of this paper is to discuss a molecular model for the mechanisms of mechanical signal transduction of integrin gene regulation which

takes place in bone cells as a result of orthodontic forces. Such information may be crucial in the understanding of epigenetic control of tissue remodelling, in which both environment and genetic mechanisms may predetermine the optimal cell and tissue response.

Materials and methods

Human osteosarcoma TE-85 cells from ATCC (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Sigma, St. Louis, MO, USA) with 5 per cent FBS, 25 IU/ml penicillin G, 25 μg/ml streptomycin and 100 μg/ml ascorbic acid (all from Sigma). Mechanical strain was applied by using the Flexercell Strain Unit (Flexcell Corp., McKeesport, PA, USA) with cells grown in Type I dishes (flexible bottoms) at 20 kPa at 3 cycles/min (10 s of strain/10 s of relaxation). Cells were strained for periods of 15 and 30 minutes, 2 and 24 hours and 3 days. Control cultures were cultivated for the same time periods using Flexercell type II rigid-bottomed dishes. The properties of stretch (type I) and non-stretch (type II) dishes have been described earlier (Anderson *et al.*, 1992).

Immunohistochemistry was performed on strained and unstrained HOS TE-85 cells. Samples were rinsed with phosphate-buffered saline (PBS) fixed with 4% paraformaldehyde for 10 minutes, washed in PBS, permeabilized in 0.5% Triton X-100 for 20 minutes and washed again in PBS. Monoclonal mouse anti-human β_1-integrin antibodies or anti-human α_v-integrin (both from Gibco, Burlington, ON, Canada) were added for 1 hour at room temperature. Samples were washed and incubated with goat Cy3 conjugated anti-mouse immunoglobulin (Ig) G antibodies for 40 minutes. Negative controls for all antibodies were performed.

To study the effect of mechanical strain on gene expression, total RNA was extracted from TE-85 cells as described previously (Chomzynski and Sacchi, 1987). Cells were rinsed in ice-cold PBS, incubated in a denaturing solution (4 M guanidinium thiocyanate, 25 mM NaCl, pH 7.0, 0.5% sarcosyl and 0.1% β-mercaptoethanol), incubated with water-saturated phenol, 2 M Na acetate and chloroform:isoamyl alcohol (49:1). Samples were centrifuged (10 000 g for 20 minutes) and the water phase, transferred to isopropanol at

−70°C, centrifuged, resuspended in the denaturing solution, incubated with isopropanol, centrifuged, washed in 70% ethanol and dried at room temperature. Samples were resuspended in distilled RNase-free water. RNA was quantified by spectrophotometry (Bio-Rad Laboratories Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd., Japan). RNA (20 μg per sample) was fractionated on a 1.2% agarose gel and RNA was transferred to a nitrocellulose membrane, pore size 0.45 μm (MSI, Fisher Sci., Winnipeg, MB, Canada) overnight at room temperature.

cDNA probes for β_1 integrin of 1.2 kb or α_v integrin of 1.1 kb (Gibco, Burlington, ON, Canada) were labelled with [32P]-γCTP by a random prime kit (Amersham, Oakville, ON, Canada) and the membranes were hybridized overnight at 42°C. In addition, a probe for cyclophylin of 750 b was used as a control. The

excess probe was removed by subsequent changes of SSPE buffer (3 M NaCl, 23 mM NaH$_2$PO$_4$, 2 mM EDTA, pH 7.4) with varying degrees of stringency, and the blots were exposed to Kodak XRP-IX-ray films (Kodak Canada Inc., Toronto, ON, Canada).

Statistical analysis

Data were analysed statistically by a two-way analysis of variance with Tukey's multiple comparison test. T-tests with the Bonferroni correction were also performed to compare mRNA expression from strained and unstrained cells. All data represent the average of four experiments performed under identical conditions.

Results

Confluent TE-85 cell cultures formed bone-like material as evidenced by bone nodules shown
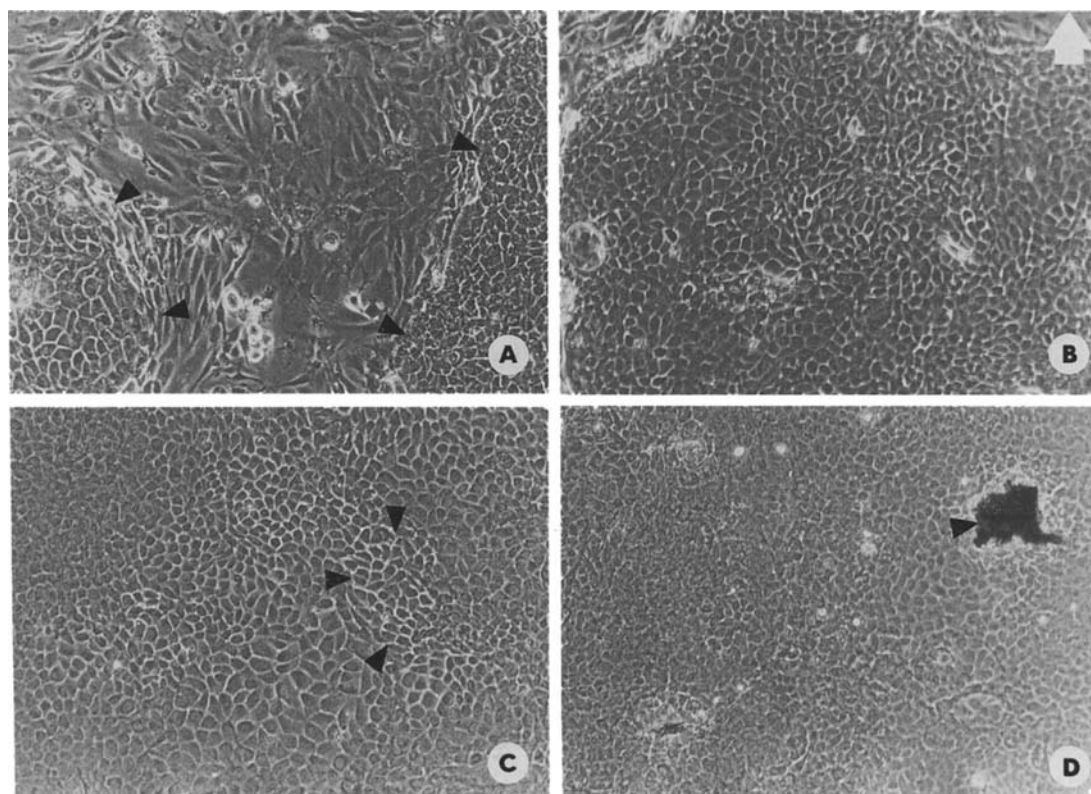

Figure 1 Phase contrast micrographs of human osteosarcoma (HOS)-TE 85 cells in culture. (**A**) 24 hours after seeding of the cells, there is rapid growth and the cells start forming clusters (arrows). (**B**) The cell clusters from (**A**), grow and coalesce with other clusters until complete confluency is reached. (**C**) At 48–72 hours of the initiation of the cultures, increased cell proliferation takes place at localized sites (arrows) in which mineral is deposited and bone nodules are formed (arrow) in (**D**).

in Figure 1. Mineralized matrix was characterized by alkaline phosphatase activity and Von Kossa stain (not shown). Immunohistochemical fluorescence microscopy showed that the application of mechanical strain for 24 hours appeared to induce a reorganization of integrin distribution. β_1 integrin from untreated cultures (Fig. 2a) appeared to cluster in the centre of cells after mechanical strain (Fig. 2b). The intensity of staining also seemed to increase. α_v integrin subunit staining was redistributed with strain when untreated cultures (Fig. 2c) were mechanically strained (Fig. 2d). Labelling of α_v integrin showed that clusters were more intense at the periphery of the cells in unstrained cultures (Fig. 2d). Following the application of strain, some cells did not show labelling for α_v at their periphery (not shown), but in the majority of cells α_v redistributed as indicated by white arrows (Figure 2d). Northern blots revealed that the application of mechanical

strain caused a significant increase in hybridization ($P < 0.005$) of β_1 integrin mRNA probe at 30 minutes and 3 days of culture in HOS TE-85 cells when compared with other time periods studied as shown in Figures 3a and 3b. On the other hand, the application of mechanical stimulation did not change the expression of α_v integrin mRNA in experimental cultures compared with untreated controls (Figs 3c and 3d). The same amount of total RNA was used in this study for both strained and unstrained cultures of α_v and β_1 integrin subunits. The expression of β_1 integrin and α_v integrin mRNA based on the optical density data are also shown in Figure 4.

Discussion

Therapeutic corrections in clinical orthodontics and dentofacial orthopaedics attempt to stimulate cell and tissue remodelling by manipulation
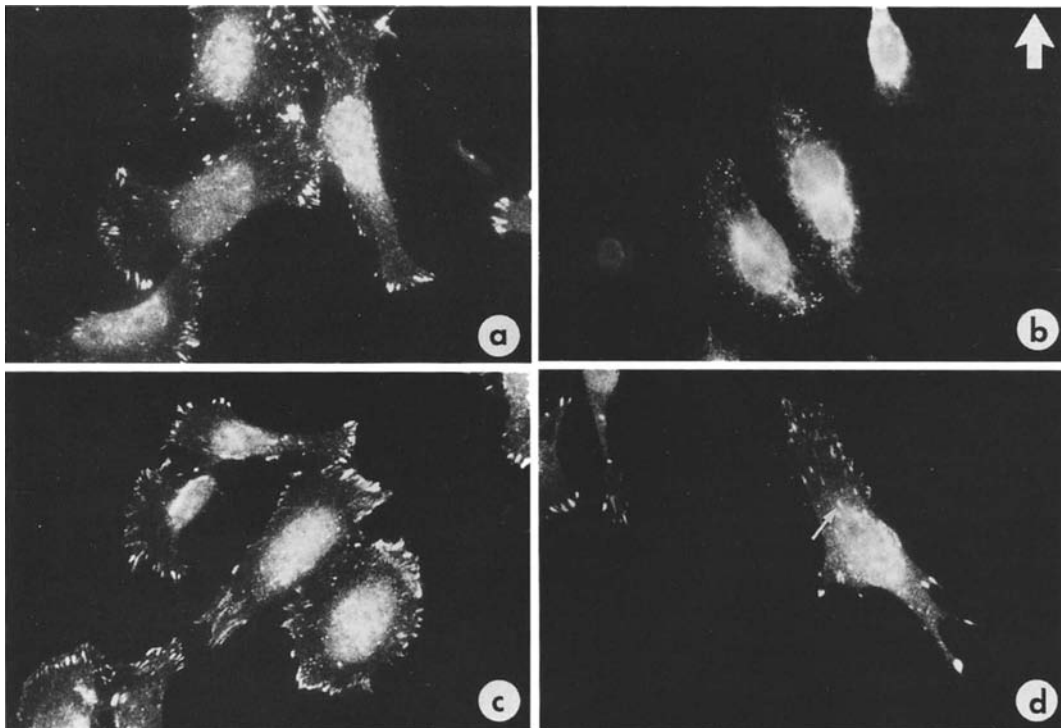

Figure 2   Immunolocalization studies of human osteosarcoma (HOS) TE-85 cells. Photomicrographs of unstrained (**a** and **c**) and strained (**b** and **d**) TE-85 HOS cells. (**a**) unstrained cultures incubated with β_1 integrin antibodies showed a change in the labelling distribution when these cells were mechanically strained, as integrin subunits appeared to redistribute towards the cell centre (**b**). Similarly, control unstrained cultures incubated with α_v integrin antibodies (**c**) also showed a change in labelling after mechanical stimulation (**d**), however, α_v labelling was still evident at the cell periphery in both unstrained and strained cells. Magnification (**a–b**) × 1250.
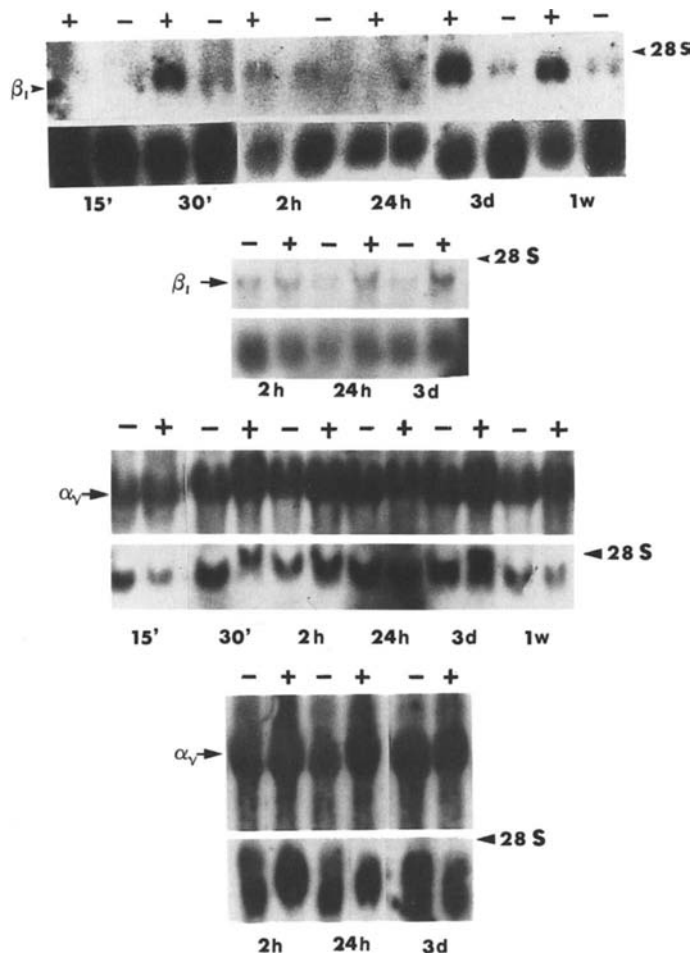
Figure 3 Photomicrographs of Northern blots showing mRNA of β_1 and α_v integrin subunits. (a) effects of orthodontic strain in β_1-integrin subunit mRNA expression. Note that strain (+) causes an increase of β_1 mRNA at 30 minutes and again at 3 days and 1 week, when compared with unstrained controls (–). (b) same as that shown in (a). Note the increase in β_1 mRNA at 3 days of strain application. (c) effects of orthodontic strain in α_v integrin subunit mRNA expression. Note that strain (+) does not change the mRNA expression at any given time period when compared with unstrained controls (–). (d) same as that shown in (c). Note that no changes are seen with the application of strain. Control mRNA (probe for cyclophylin) indicates comparable amounts of RNA per lane and 28 S indicates the position of the 28 srRNA. 15' = 15 minutes; 30' = 30 minutes; 3 d = 3 days and 1 w = 1 week.

of mechanical forces. Since early studies showed that these forces produced a series of undesirable tissue reactions (Kvam, 1972; Rygh, 1977; Langford and Sims, 1982; Sims and Weekes, 1985; Nakane and Kameyama, 1987), the lack of knowledge between physiological and clinical force systems has been evident. Of special interest in orthodontics is the response of cells from supporting structures of teeth, such as periodontal ligament and bone, to mechanical stimulation. Our understanding in this field has

increased rapidly from a few years ago, however, as this area expands more questions arise.

Even though the pathway or pathways which are responsible for translation of extracellularly applied mechanical forces into intracellular signals are still not clear, it is beyond the scope of this paper to discuss this question in detail. These concepts have been reviewed elsewere (Carvalho et al., 1995). This study is based on the proposition that cellular changes in morphology, proliferation and synthetic activity are
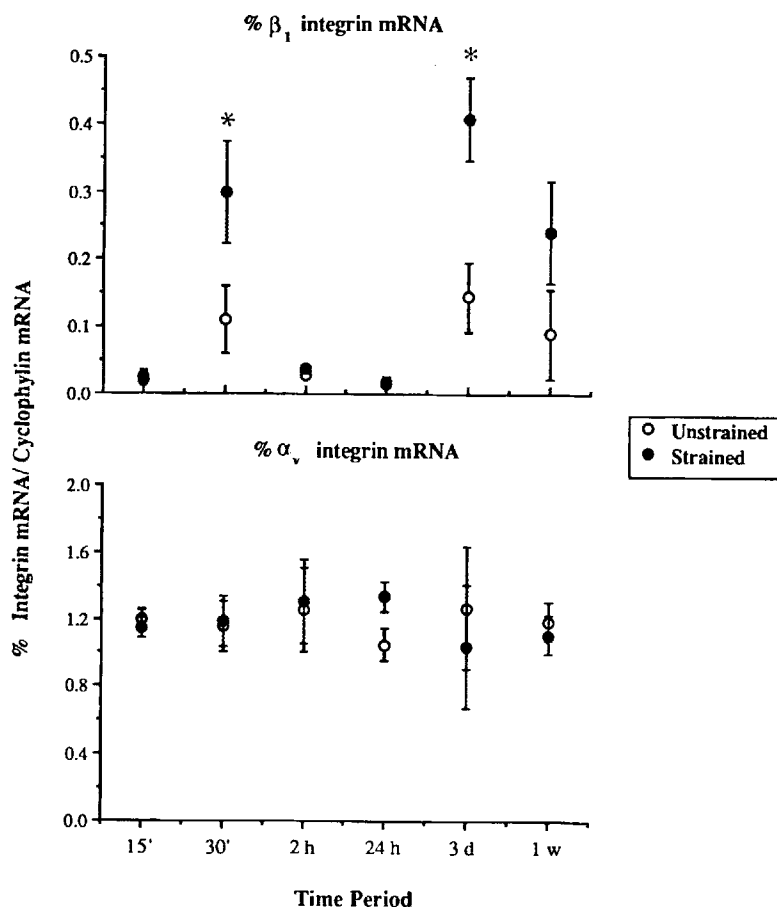
Figure 4 Effect of mechanical strain in the expression of β_1 and α_v integrin subunits mRNA. Values indicate at least four cultures for each time period studied. β_1 and α_v integrin mRNA expression is represented by its ratio (%) of to the internal control (cyclophylin expression) from densitometric scans of X-ray films. *Significantly greater ($P < 0.005$) in strained cultures in comparison with unstrained controls.

correlated directly or indirectly to gene expression. By understanding mechanisms of mechanically-stimulated gene regulation, we believe that ultimately, the clinical application of forces will be delivered in such a manner as to communicate with the cells in their 'language'. This scenario will allow the most appropriate results with a minimum of biological tissue damage.

The cytoskeleton serves not only to provide positional information for intracellular organelles including the nucleus, but also it has been shown to bind directly and regulate the expression of various mRNAs (Biegel and Patcher, 1992; Simpson *et al.*, 1994). Nuclear positioning within the cell has also been shown to be essential in regulation of protein synthesis and gene expression (Russel and Dix, 1992). This positioning is partly regulated by binding of the cytoskeleton to the nuclear membrane (Bissel *et al.*, 1982; Berezny, 1991) which allows transduction of signals to the nucleus (Ingber, 1991; Simpson *et al.*, 1994). Therefore, the association of integrin receptor proteins with the underlying cellular cytoskeleton (Ingber, 1991; Lotz *et al.*, 1989; Hynes, 1992; Yamada *et al.*, 1992) partly explains their regulation of gene expression (Ng-Sikorski *et al.*, 1991).

In addition to stimulating gene expression, integrin receptors have functions as signal transduction channels (Kornberg *et al.*, 1991; Hynes, 1992) in a series of critical recognition events of cell–substratum and cell–cell adhesion. These events are the key in a variety of biological processes such as embryonic development, pro-

liferation, differentiation, wound healing, tumour cell metastasis, cell polarity, cell migration, organ function, tissue organization and immunological recognition (Ingber, 1991; Kornberg et al., 1991; Milam et al., 1991; Clover et al., 1992; Ginsberg et al., 1992a; Hynes, 1992; Yamada et al., 1992; Majda et al., 1994).

The mechanically-induced distribution and expression of both α_v and β_1 integrins shown here suggests that there is a specificity of response amongst strain-induced integrin regulation. The presence of α integrin subunits in a particular cell type indicates that such cell type may bind to a variety of attachment proteins (Felding-Habermann and Cheresh, 1993). However, this diversity is regulated by the β subunit. In addition to ligand specificity, α integrins may participate in the process of focal contact assembly (La Flamme et al., 1992). Similarly, β_1 actively participates not only in the regulation of focal contacts but also in the maintenance of cytoskeletal interactions. In Figure 2, we indicate that mechanical strain alters β_1 integrin 'focal contact' distribution (Fig. 2a) to a more perinuclear labelling (Fig. 2b). In contrast, the stimulation of α_v subunits by strain does not follow the same distribution, and patches of labelling similar to a 'focal contact' protein distribution can still be seen following strain application (Fig. 2d). In cell types responsive to strain, such differences possibly demonstrate a strain-dependent regulatory mechanism, involving gene regulation and protein maturation mechanisms.

In mechanically-induced signal transduction, signals begin in the extracellular matrix (ECM). The predominantly physical stimulus may be translated by integrins to some form of chemical signals which are propagated through the cytoskeleton and a variety of second messenger molecules. These signals are progressively enhanced or repressed to reach finally the nucleus in which they regulate gene expression. It has been recently hypothesized that integrins translate mechanical stimulation from outside to inside the cells ('outside-in') (Sastry and Horwitz, 1993; Carvalho et al., 1995). On the other hand, the regulation of gene expression by integrins (Damsky and Werb, 1992) has revealed the potential for the 'inside-out' regulation of mechanically-induced signal transduction (Ginsberg et al., 1992b; Sastry and Horwitz, 1993). The latter involves effectors in the cyto-

plasm which modulate affinity and/or specificity for its ECM ligand (Damsky and Werb, 1992). Thus, changes in integrin mRNAs could regulate the cellular protein synthetic machinery through mRNA-cytoskeleton binding upon mRNA translation (Bissel et al., 1982). By contrast, inside-out signalling of integrins may not be controlled by control of gene expression in certain cell types, such as in platelets (Ginsberg et al., 1992b).

In addition to nuclear positioning, arrangement of the ECM also appears to provide positional information for transmission of mechanical forces. Moreover, the three-dimensional arrangement of DNA, a major part of the nuclear matrix, is believed to play a significant role in gene regulation (Simpson et al., 1994). Thus, regulation of 'inside-out' signalling by integrins may take place by a mechanism of interaction of nuclear factors in the nuclear matrix and cytoplasmic proteins. Resnick et al. (1993) have identified a region of DNA in the PDGF-B gene promoter, which appears to be required in order to confer responsiveness to this gene as a result of mechanical stimulation. This region appears to behave as a regulatory 'responsive element'. The results of PDGF-B are similar to our studies on β_1 integrin seen here. We have found that expression of β_1 integrin starts as early as 30 minutes (Figs 3 and 4). However, the mechanically-stimulated response for the distribution of the integrin subunits does not appear to follow the same pattern (Fig. 2). The latter may be expected since such distribution is taking place following gene regulation. A partial explanation may be the interaction of mechanical stimulation with other systems including changes in membrane fluidity causing integrin redistribution as a result of mechanical stimulation (Bissel et al., 1982).

Changes in mRNA levels of β_1 integrin as seen in Figures 3a and 3b may be due to differences in kinetics of the maturation process of integrins. β proteins appear to be synthesized in large numbers in response to growth and differentiation factors (Koivisto et al., 1994), but remain as immature precursors in the endoplasmic reticulum, prior to dimer formation with α subunits (Santala et al., 1994). Application of mechanical stimulation appears to stimulate integrin regulation to a given requirement of the precursor pool at selected

time periods. We hypothesize that strain stimulates genetic regulation by a 'responsive element' coupled to a transcription factor in responsive genes, such as the integrin gene, in mechanically responsive cells. Meanwhile, as the search for these answers progresses, we have been able to show to date that mechanical stimulation is responsible for selective expression of integrins (Fig. 4) corroborating earlier findings of stimulatory and/or inhibitory expression differences of certain genes by strain (Komuro *et al.*, 1991).

Although we have discussed the effects of mechanically-induced gene regulation of selected integrin subunits, it is important to remember that mechanical stimulation is not an isolated phenomenon. Stimulation by mechanical signals at cell level is also regulated by a variety of other morphogens, such as growth factors, hormones and morphoregulatory molecules (Simpson *et al.*, 1994). In addition, a single system probably does not solely explain the complexity of the mechanically-induced cellular response.

New studies on the effects of mechanical stimulation upon these basic cellular mechanisms are starting to shed some light on the principles of cellular behaviour that are frequently taken for granted at the clinical level. Future experimentation both at the basic and clinical levels will greatly enhance our understanding of both physiological and therapeutic application of mechanical forces. Nevertheless the main goals remain to deliver clinical management through controlled loading patterns which could prevent and/or reverse tissue breakdown yet provide the desired functional and aesthetic results

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