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Secretion of osteopontin from MG-63 cells under a physiological level of mechanical strain *in vitro*—a [^{35}S] incorporation approach

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SUMMARY To gain insight into the early response of osteoblastic cells to a physiological level of mechanical strain *in vitro*, the secretion of osteopontin by MG-63 osteosarcoma cells was assessed by [^{35}S] incorporation and autoradiography. First, osteopontin secreted from MG-63 cells was immunolocalized at 60–64 kDa (Mr) by polyacrylamide gel electrophoresis. A uniform physiological level of strain was generated by a vacuum added to the convex side of a half-ball shaped silicon rubber membrane on which the cells were cultured on the concave side. After labelling proteins with [^{35}S]-methionine/cysteine (147 μCi/ml), the membranes were exposed to a strain of 0.5 per cent (5000 με), 3 cycles/minute (sine wave) with 10 minutes on and off. At 1, 2 and 4 hours after strain, the supernatants were collected and analysed by 10 per cent sodium dodecyl sulphate–polyacrylamide gel electrophoresis and autoradiography.

The results showed that osteopontin was secreted by the strained cells at significantly higher amounts than the non-strained cells at all three time points ($P < 0.05$), with the first hour being the most prominent. A physiological level of mechanical strain increased the secretion of osteopontin from MG-63 cells in an early phase. This finding implies an accelerated process of bone remodelling, which suggests that the application of light and intermittent forces would result in the cellular reaction identified in relation to orthodontic tooth movement. The results indirectly indicate that the level of force presently used might be too high.

Introduction

The relationship between different types of force system (magnitude and frequency) applied for the generation of tooth movement and periodontal/alveolar tissue responses is poorly understood. Through years of study, the essence of orthodontic tooth movement has been recognized as a mechanically induced remodelling of the periodontal ligament (PDL) and alveolar bone, being regulated by a large number of humoral factors (Davidovitch, 1991). Whether the initial reaction is to be found in the PDL of the surrounding bone is still a matter of debate (Melsen, 1999). Orthodontists seem to focus their interests on the primary reaction of the PDL cells (Roberts and Morey, 1985; Basdra and Komposch, 1997), whereas the biological reaction of bone to variation in strain described by Frost (1987) in the so-called 'mechanostat' has assigned the primary reaction of bone to mechanical perturbation of the osteocytes (Marotti, 1996).

At the cellular and molecular levels, the mechanism of mechanically induced bone remodelling has been investigated in both *in vivo* (Takano-Yamamoto *et al.*, 1994; Terai *et al.*, 1999) and *in vitro* experiments, in an attempt to answer the question 'how do bone cells respond to different regimens of mechanical stimuli?' *In vitro*, mechanical perturbations have been executed on osteoblast-like cells, mainly by three types of culture mechanostimulation model, namely the hydrostatic pressure model, the fluid shear stress model and the substrate-deformation strain model (Brown, 2000). As the questions asked are related to the delivery and the quantity of strain, these variables have to be controllable when delivering a mechanical perturbation. This can best be done in relation to the substrate-deformation model, although the delivery of a uniform strain has only been possible with a few of the described appliances. Uniaxial strain was adapted to test soft tissue cells. Biaxial strain was generated as in-plane substrate distension (Pitsillides *et al.*, 1995) and as out-of-plane circular distension (Banes *et al.*, 1985). Among the latter, the Flexcell® gained high popularity due to its broad range of applications. As the problem was, however, the heterogeneity and the lack of uniformity in various parts of the circular membrane when the 2 mm thick membrane was submitted to a vacuum (Gilbert *et al.*, 1994), a thinner membrane was suggested (Brown, 2000). A better solution to overcome this problem was, however, the design of a half-ball shaped membrane able to generate biaxial uniform strain in the range 0.5–1.99 per cent.

The biological reaction to mechanical stimulation has been studied in various ways. Initial changes in the cytoskeletal structure are responsible for the signal transduction pathways first reflected in the integrin profile and in the mRNA level of osteopontin (OPN).

OPN is a secreted phosphorylated glycoprotein existing predominantly not only in mineralized hard tissues, i.e. bone (Butler, 1989) and teeth (Takano-Yamamoto *et al.*, 1994), but also in the associated PDL (Lekic *et al.*, 1996) and gingival cervicular fluid (Kido *et al.*, 2001). *In vitro* OPN is synthesized by pre-osteoblasts, osteoblasts, osteocytes (Ajubi *et al.*, 1996; Aarden *et al.*, 1996) and PDL fibroblasts (Takano-Yamamoto *et al.*, 1994; Ivanovski *et al.*, 2001). Characteristically, the Arg–Gly–Asp (RGD) motif in the structure of OPN indicates a potential function in cell attachment and migration, as well as transmembrane signal transduction (Butler, 1989). OPN is often chosen as a parameter in studies of bone remodelling. Kubota *et al.* (1993) first reported that OPN and its mRNA were increased by an intermittent hydrostatic pressure. Later, the substrate-deformation strain of different regimens as well as oscillating fluid shear stress were shown to up-regulate OPN protein production. A clear dependence of both strain magnitude and delivery has been demonstrated. Toma *et al.* (1997), who applied 1.3 per cent (13 000 μstrain) mechanical strain, reported the up-regulation of OPN mRNA by 400 per cent in chicken calvarial osteoblasts, whereas a reduction in OPN mRNA expression in UMR-106 osteosarcoma cells was found after delivery of 14 per cent (1 400 00 μstrain) mechanical strain by Liu *et al.* (1998).

The influence of timing has shown that OPN mRNA was increased by 1.3 per cent mechanical strain after 2 hours (Toma *et al.*, 1997), whereas OPN was reduced 60 per cent by the same strain only after 4 consecutive days (2 hours/day) of action (Meazzini *et al.*, 1998).

There is thus no doubt that both the magnitude of the strain and delivery play an important role in the production of OPN. As OPN can be degraded in extracellular matrix (ECM; Meazzini *et al.*, 1998), it is important to focus on the early secretion of OPN into ECM shortly after mechanical perturbation. Therefore, in the present study, the early responses of MG-63 cells to a physiological level of biaxial uniform strain were studied by assessing the extracellular changes in OPN.

Materials and methods

Cell culture

The MG-63 human osteosarcoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and cultured in minimum essential medium (MEM; Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10 per cent foetal calf serum (FCS; PAA Laboratories, Linz, Austria) and antibiotics (25 000 IU/ml penicillin and 25 mg/ml streptomycin; DuraScan Medical Products AS, Odense, Denmark) at 37°C in a humidified atmosphere of 5 per cent CO_2. The medium was changed every 4 days; the cells were subcultured every other week.

Mechanical perturbation

A special half-ball shaped silicon rubber membrane (0.3 mm thick and 9.0 mm in diameter with a total volume of 1.5 ml) made from Elastosil® RT (Drawin Vertriebs GmbH, Ottobrunn, Riemerling, Germany) was produced as described by Nielsen (1996). The membrane was transparent, biosafe and sterilizable and able to generate a homogenous isotropic strain uniformly distributed over the entire surface except the marginal 5 per cent area (Figure 1). The cells grew uniformly on the surface coated with 10 μg/ml fibronectin (Figure 2). To exert a vacuum-generated strain to the

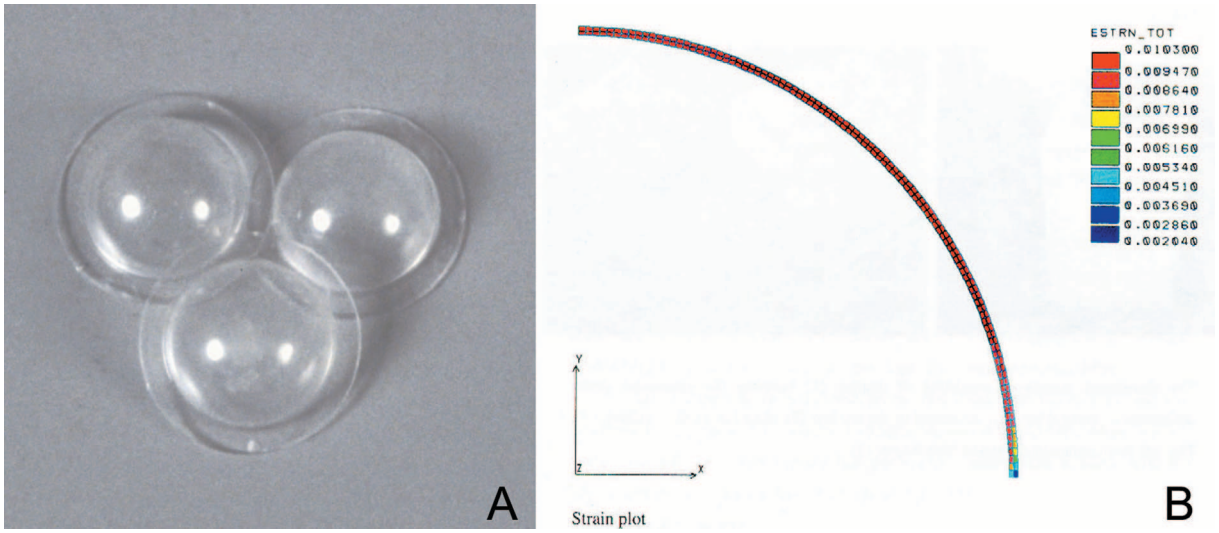

Figure 1 (a) Half-ball shaped silicon rubber membranes (0.3 mm thick, 9.0 mm in diameter, with a total volume of 1.5 ml) which are transparent, sterilizable and biosafe. (b) Finite element analysis showed a homogeneous isotropic strain generated almost on the entire surface of the membrane except for the marginal 5 per cent area.

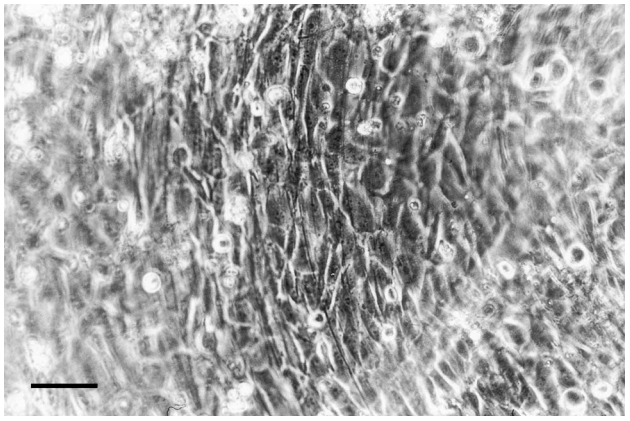

Figure 2 Under normal culture, MG-63 cells grew evenly on the surface of the silicon membrane coated with 10 μg/ml bovine plasma fibronectin. After 3–4 days, a monolayer of confluence was reached. Due to the curvature of the membrane, only a limited area could be focused under the inverted phase-contrast microscope (magnification: ×64, bar = 10 μm).

cultured cells, a custom-made strain-loading system consisting of three parts: a mechanical system (a), a controlling system (Aarhus Engineering Academy, Denmark) (b) and an incubator (c) was constructed (Figure 3). The magnitude and frequency of the strain could be selected from a dialogue menu on the computer screen. Technically, this system is capable of delivering a uniform substrate-deformation based mechanical strain ranging from 0.5 to 1.99 per cent (5000 to 19 000 με), with a free adjustable frequency. In order to simulate an *in vivo* environment of tension, a basal strain of 0.5 per cent (5000 με) was pre-set (Figure 3).

Experimental protocol

MG-63 cells grown in culture flasks were digested with 0.125 per cent trypsin (Difco Laboratories, Sparks, Michigan, USA) and 5 mM EDTA (Sigma, Poole, Dorset, UK) and 2×10^5 cells/membrane were seeded on to the membranes coated with 10 μg/ml bovine plasma fibronectin (type I-S; Sigma). After 3–4 days of culture, confluence was reached (Figure 2).

Of 12 membranes, six were strained each time and six served as the controls. The experiment was repeated three times.

Pulse radiolabelling the proteins synthesized before strain

To radiolabel the cells before strain, [^{35}S] Promix (Amersham Pharmacia Biotech, Bucks, UK) was added to a concentration of 147 μCi/ml to MEM supplemented with 5 per cent FCS. After washing with serum-free MEM, the cells in all 12 membranes were cultured in the radiolabelling medium for 2 hours. The membranes were then gently washed with serum-free MEM and returned to 5 per cent FCS-supplemented MEM free of [^{35}S].

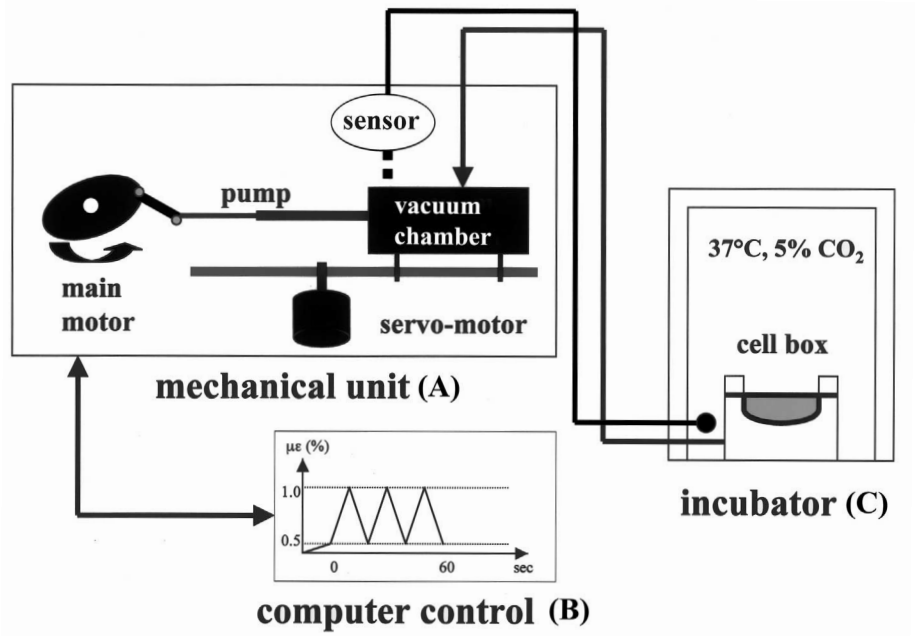

Figure 3 A schematic drawing of the vacuum-driven computer-monitored strain-loading system which consisted of three parts: (a) the mechanical unit, (b) the computer control and (c) an incubator. The mechanical unit included a main motor generating a sine wave vacuum pressure and a servo-motor providing vacuum compensation in case of air leakage occurring in the system. The system was capable of generating a strain range of 0.5–2.0 per cent (5000–19 000 με), 3 cycles/minute (sine wave) with 10 minutes on and off, with a free adjustable frequency.

Application of the mechanical strain to the cells

After labelling, the six membranes in the strain group were connected to the vacuum chamber (Figure 3). A strain of 0.5 per cent (5000 με) was exerted at a frequency of 3 cycles/minute (sine wave) with 10 minutes on and off. The six control membranes were seated in another cell box and cultured under exactly the same conditions. The culture conditions during the strain-loading period were identical to normal culture. At 1, 2 and 4 hours after strain, two membranes from the strain and control groups were detached. The cells on the membranes were photographed under an inverted phase-contrast microscope (Leitz, Wetzlar, Germany). The total amount of supernatant was collected from each membrane and centrifuged at 10 000 rpm for 5 minutes at room temperature, with the secondary supernatant being re-collected and stored at –20°C for further testing.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

To detect OPN in the supernatant, separation of the proteins was achieved by 10 per cent SDS–PAGE (Andersen *et al.*, 1987). To identify OPN, 300 μl of supernatant was precipitated by acetone (Merck, Darmstadt, Germany) at –20°C for 30 minutes (Andrianarivo *et al.*, 1992). After 10 minutes of centrifugation at 20 000 rpm, the pellets were dried in a Speed Vac Concentrator (Savant™, Teknunc A/S, Denmark) at 37°C for 20 minutes, then completely dissolved in 20 μl of 1 × SDS sample buffer containing β-mercaptoethanol (pH 7.4) and boiled at 100°C for 2 minutes. The boiled samples were loaded on to a 10 per cent SDS gel. As a molecular weight marker, 15 μl of SeeBlue™ pre-stained standards (Novex™, San Diego, California, USA) was used. The gels were run at 3 W/gel until the bromophenol blue tracking dye reached the bottom of the gels. To quantify the secreted OPN of the control and strain groups, 15 μl of supernatant was directly mixed with 15 μl of sample buffer without precipitation.

Identification of OPN by Western blot

All the chemical reagents were obtained from Sigma unless otherwise specified. To localize OPN, a Western blot was performed (Andersen *et al.*, 1987). After electrophoresis, the proteins in the SDS–PAGE slab gel were transferred to a nitrocellulose membrane (Protran™, Schleicher & Schuell GmbH, Bassel, Germany, pore size 0.2 μm) by means of a Tank Transphor Unit (Hoefer Pharmacia Bioteck Inc., San Francisco, California, USA). The transfer buffer was 0.025 M Tris, 0.192 M glycine (pH 8.3). A voltage of 15 V/cm was applied for 4 hours (current 0.34–0.75 A) at 6°C. The nitrocellulose membrane was blocked (20 mM Tris, 150/500 mM NaCl, 3 per cent gelatin, pH 7.5) at 37°C for 20 minutes and washed twice (10 minutes each) with washing buffer (20 mM Tris, 150 mM NaCl, 0.05 per cent Tween-20, pH 7.5) at 37°C and soaked in antibody buffer (20 mM Tris, 150 mM NaCl, 0.05 per cent Tween-20, 0.2 per cent gelatin, pH 7.5) at 37°C for 1 hour. The primary antibody, OPN polyclonol antibody LF-123 (NIDCR, Maryland, USA) diluted 1:500 in antibody buffer was applied for 1 hour at 37°C. The membrane was washed twice and incubated with an alkaline phosphatase-conjugated secondary antibody (1:2000 diluted) at 37°C for 1 hour. After two washes, the membrane was coloured with a mixture of nitroblue tetrazolium (30 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (15 mg/ml) in buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5).

Autoradiography

To quantify the radiolabelled OPN in the supernatant, electrophoresis and autoradiography were performed. The gels were soaked in a fixation buffer (65 per cent H_2O, 25 per cent 2-propanol, 10 per cent acetic acid) under gentle agitation for 30 minutes at room temperature and soaked in Amplify™ (Amersham Pharmacia Biotech) for 20 minutes. The gels were then placed between blotting paper (Advantec 1514A, Friservette, Denmark) and plastic sheets and dried on a Bio-Rad gel dryer (model 583, Bio-Rad, Bristol, UK) at 75°C for 4 hours. The dried gels were exposed to Kodak BioMax MR films (Eastman Kodak Company, New York, USA) at –70°C for 1 week and developed in a Kodak X-OMAT 270 RA processor. The optical densities of the autoradiographic signals were measured using a densitometer (ImageQuant™ analysis software, version 2.0, Molecular Dynamics, San Dimas, California, USA). The nitrocellulose membrane for immunoblotting was air-dried before exposure to BioMax MR film (Figure 4).

Statistical analysis

The optical densities of OPN gel bands were presented as mean ± standard deviation ($n = 6$). A comparison between the strain and non-strain groups was performed using Student's *t*-test for paired samples (SPSS, version 10.0; Chicago, Illinois, USA). The difference was considered to be significant at $P < 0.05$.

Results

A biaxial uniform strain was generated when the half-ball shaped silicon rubber membranes were submitted to a vacuum, which guaranteed the delivery of a homogeneous isotropic strain of 5000–19 000 με (Figure 1).

The MG-63 cells were evenly distributed on the 10 μg/ml fibronectin-coated membranes and appeared to be growing in a circular pattern, probably due to the circularly machined surface of the aluminium modules

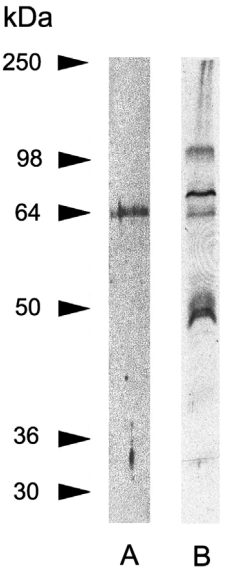

Figure 4 Identification of the radiolabelled osteopontin (OPN) secreted from MG-63 cells in the supernatant on an autoradiogram. Immunoblotted with whole rabbit anti-human polyclonol antibody LF-123, OPN was localized at 60–64 kDa according to the molecular weight markers (A). The same nitrocellulose membrane exposed to an X-ray film correspondingly localized OPN among all the radiolabelled secreted proteins in the supernatant (B).

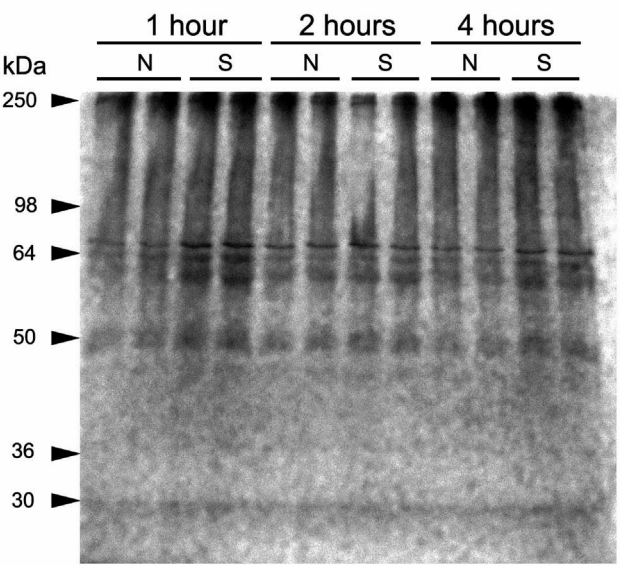

Figure 5 A representation of the pulse-labelled proteins secreted in the supernatant by MG-63 cells exposed to a mechanical strain (0.5 per cent, 3 cycles/minute, 10 minutes on and off) for 1, 2 and 4 hours. A 30 µl sample (15 µl of supernatant + 15 µl of sample buffer) was loaded on each lane of a 10 per cent sodium dodecyl sulphate–polyacrylamide gel electrophoresis slab gel, electrophoresed and autoradiographed. Osteopontin (60–64 kDa) bands were obviously increased in the strain groups (S) at all three time points (N, non-strain group).

on which the membranes were prepared (Figure 2). Confluence was reached after 4 days of normal culture. No difference between the non-strained and strained cells with respect to size and shape was found during the 4 hours of the experiment.

It was critical to determine the localization of OPN on the gel as a first step in this study, as this was later used to confirm the radiolabelled secreted OPN. OPN at 60–64 kDa was identified using the antibody LF-123 (Figure 4).

To quantify the radiolabelled secreted OPN, the proteins synthesized in the cells were pulse-labelled before strain. Hence, a secretion rather than a protein synthesis was monitored. As gel electrophoresis was performed in this study under the same conditions as the Western blot analysis, the labelled secreted OPN could be read at 60–64 kDa. There were no differences between the amounts of OPN corresponding to the three time points in the control groups which were relatively low and stable (Figure 5 and Table 1). Under mechanical strain, however, the amounts (optical densities) of OPN gel bands at 1 (0.247 ± 0.053), 2 (0.204 ± 0.040) and 4 hours (0.211 ± 0.045) were all significantly higher than those in the non-strain control groups (0.080 on average) ($P < 0.05$). The increase at 1 hour was the most prominent, with no significant differences found between groups. The larger standard deviation (0.040–0.053) in the strain groups than in the controls (0.011–0.021) indicated some variation in the cells responding to the strain over time (Table 1).

Table 1 Optical densities of the osteopontin gel bands by 10 per cent sodium dodecyl sulphate–polyacrylamide gel electrophoresis and autoradiography (non-strain versus strain groups) (mean ± standard deviation, $n = 6$)*.

	1 hour	2 hours	4 hours
Non-strain	0.082 ± 0.016	0.080 ± 0.021	0.078 ± 0.011
Strain	0.247 ± 0.053	0.204 ± 0.040	0.211 ± 0.045

*Significance was found between the non-strain and strain groups at all three time points ($P < 0.05$), but not within each group.

Discussion

Although the response of a single integrin molecule to mechanical drag has been studied (Lehenkari and Horton, 1999), the exact deformation of an individual cell is immeasurable. The substrate on which cells are cultured can be deformed mechanically and a number of culture mechanostimulation devices have been developed (Brown, 2000) in an attempt to elucidate the mechanical signal transduction. Substrate-deformation strain devices are most commonly used, but one drawback of this model is its inability to deliver a force level comparable with those generated *in vivo* (less than 0.3 per cent, 3000 µε) (Turner *et al.*, 1995) and lack of homogeneity. In this study, when the half-ball shaped silicon membrane was loaded, a homogeneous and biaxial isotropic strain was generated. The regimen (5000 µε,

3 cycles/minute with 10 minutes on and off) delivered to the MG-63 cells simulated the phase of active orthodontic tooth movement and within the mild overuse window according to Frost's mechanostat theory (Frost, 1987). To mimic the *in vivo* scenario of cells living in tensegrity generated by ECM (Ingber, 1997), the model studied provided the cells with a basal tension on which an active strain was exerted. Compared with the deformation of a flat membrane, the increased radius of the half-ball shaped membrane (0.9 cm in depth, 1.5 ml in volume) resulted in less confounding fluid shear stress (Brown, 2000).

Topographical and chemical features of the substrate surface affect adhesion and migration, as well as the response of cells to mechanical strain (Curtis and Wilkinson, 1997). The adhesion of MG-63 cells to the inert silicon membrane was facilitated by a coating of 10 μg/ml fibronectin which was found to maximally increase the attachment of the cells to the membrane (Nielsen, 1996). Whether fibronectin was able to affect OPN production is unknown, but a fibronectin coating (10 μg/ml) did not change OPN mRNA expression in UMR-106 cells (Traianedes *et al.*, 1995).

On the coated membranes, the MG-63 cells were evenly distributed only a few hours after seeding and grew as well as in a culture flask, indicating the potential action of fibronectin on cell attachment and growth. The cells grew in a circular pattern, possibly due to the orientation of the microgrooves on the concave side of the membrane, which might influence transmission of the strain.

Among human-derived osteosarcoma cell lines, one of the most extensively characterized, in terms of osteoblastic phenotypes, is MG-63 (Andrianarivo *et al.*, 1992). However, the secretion of OPN from MG-63 cells has only been reported by Gotoh *et al.* (1990). In that study, OPN secreted from MG-63 cells was identified as a 60–64 kDa protein by Western blot, in agreement with some (Ramakrishnan *et al.*, 1995; Sodek *et al.*, 2000) but different from other reports (Ecarot-Charrier *et al.*, 1989; Kubota *et al.*, 1993; Wozniak *et al.*, 2000; Walker *et al.*, 2000), possibly due to the post-translational modification and anomalous behaviour on SDS–PAGE (Ramakrishnan *et al.*, 1995). The production of OPN is affected by hormones, cytokines and growth factors on the level of gene transcription, mRNA processing, stability, translation and post-translational modification (Ramakrishnan *et al.*, 1995), but its response to mechanical stimulation has only recently attracted interest. *In vivo*, the OPN-containing lamina limitans (presenting at bone surfaces subjacent to bone-lining cells and surrounding osteocytes and their processes) might serve to accurately position and tightly adhere these cells to the adjacent ECM via an integrin/RGD-mediated attachment mechanism, thus acting as a 'mechanosensor' for bone strain (Aarden *et al.*, 1994; McKee and Nanci, 1996; Terai *et al.*, 1999). *In vitro*, many studies have shown that mechanical strains of different regimen up-regulate OPN mRNA expression and protein production. Toma *et al.* (1997) found that a single 2 hours of 1.3 per cent mechanical strain increased OPN mRNA expression by 400 per cent, but Meazzini *et al.* (1998) reported a reduction in OPN by 60 per cent when the same strain was prolonged for 4 days. A possible explanation, according to Aeschlimann and Paulsson (1994), could be the degradation of OPN by tissue transglutaminase existing intra- and extracellularly in many tissue and cell types. This could also account for the fact that the OPN increases at 2 and 4 hours were seemingly lower than at 1 hour in the present study (Table 1).

Conclusions

Because the secreted form of OPN plays an ultimate role in bone remodelling (Butler, 1989), the present investigation focused on the secretion of labelled OPN. In fact, of the two consecutive processes, synthesis and secretion, it cannot be determined which single process contributes more to the ultimate increase in OPN in the supernatant when responding to strain. The findings therefore only demonstrate that the secretion of OPN is significantly increased by mechanical perturbation. On the basis of the results it can be concluded that a physiological level of mechanical strain can favourably evoke an early response of osteoblast-like cells, which suggests the application of light and intermittent forces.

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