

TGF- β 1 EXPOSURE FROM BONE SURFACES BY CHEMICAL TREATMENT MODALITIES

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Abstract

Growth factors are known to be sequestered to the mineralised matrix of bone. The aim of this study was to investigate the ability of citric acid, EDTA, calcium hydroxide and sodium hydroxide to release active growth factors from bone surfaces, able to promote osteoblast differentiation. All chemical treatments increased surface levels of TGF- β 1 (used as a biomarker of growth factor release), compared to control bone surfaces treated with PBS. Differences were observed in the kinetics of TGF- β 1 exposure at the surface and its subsequent release into the aqueous environment for the different chemical treatments. Surface levels of growth factor following chemical treatment were low, but of sufficient concentration to stimulate cell expansion and osteoblast differentiation of bone marrow stromal cells grown on EDTA and calcium hydroxide treated surfaces compared to PBS treated surfaces. The increased osteogenic potential on these surfaces may relate to an increase in growth factor availability and changes to the surface chemistry and topography.

Key words: TGF- β 1, bone, osteoblasts, acid, alkali.

Introduction

The extracellular matrix of bone is known to contain a reservoir of growth factors bound within it (Hauschka *et al.*, 1986; Frolik *et al.*, 1988; Taipale and Keski-Oja, 1997). The release of these proteins, such as bone morphogenetic proteins, platelet derived proteins, insulin-like growth factors and transforming growth factor- β (TGF- β), following injury or trauma, allows presentation of these factors to target cells, initiating optimal bone reparative processes at these sites (Schönherr and Hausser, 2000; Ramirez and Rifkin 2003). This *in vivo* repair contrasts current clinical therapies to enhance bone repair, which utilise single bioactive growth factors to elicit a therapeutic response (Reddi, 1998; Gautschi *et al.*, 2007). These growth factor therapies face problems due to the massive milligram doses required to elicit a therapeutic response in humans; doses which are in stark contrast to the natural *in vivo* situation (Reddi, 1998). As an alternative approach, future clinical treatments could benefit from exposing and releasing the array of growth factors already residing *in vivo* within the bone matrix, rather than by the addition of exogenous growth factors.

Several chemical treatments, including citric acid, ethylenediaminetetraacetic acid (EDTA), and calcium hydroxide, have been demonstrated to solubilise the growth factor TGF- β 1 from the mineralised matrix of dentine (Zhao *et al.*, 2000; Graham *et al.*, 2006). Acids and EDTA are proposed to solubilise growth factors by mineral demineralisation or chelating calcium ions (DeDeus *et al.*, 2008). Growth factor release, however, is likely to involve more complex mechanisms than its simple elution from a demineralising mineral, but also require its release from other extracellular proteins such as collagen, decorin and biglycan, which are known to sequester the bioactive molecules (Schönherr and Hauser, 2000; Baker *et al.*, 2009). This is highlighted from the observations that TGF- β 1 levels released from dentine after treatment with calcium hydroxide differ from those released with EDTA (Graham *et al.*, 2006). TGF- β provides a pertinent growth factor for study as it plays a pivotal role in both dentine and bone repair, complementing and influencing the activity of other growth factors such as BMPs and IGFs also present in the mineralised matrix (Bostrom and Asnis, 1998; Barnes *et al.*, 1999; Janssens *et al.*, 2005; Dimitriou *et al.*, 2005). With reference to bone healing, TGF- β is expressed from the very early stages and throughout fracture healing, and has roles as a potent chemotactic factor of mesenchymal progenitor cells and macrophages to the wound healing site, induction of their subsequent proliferation and stimulates production of the extracellular collagenous

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matrix by osteoblasts (Dimitriou, *et al.*, 2005). TGF- β 1 has also been proposed to initiate signalling for BMP synthesis by osteoprogenitor cells, effecting osteoblast differentiation (Bostrom, 1998) and promote osteoclast apoptosis inhibiting bone resorption (Mundy, 1996). Clinically, the incorporation of TGF- β 1 into critical size defects stimulated *in vivo* bone healing (Gombotz *et al.*, 1994; Blom *et al.*, 2001), which has been shown to be synergistically enhanced by the addition of IGF-1 (Blumenfeld *et al.*, 2002).

Against this background, the present study investigates the release characteristics of TGF- β 1, from bone matrix, using citric acid, EDTA, calcium hydroxide and sodium hydroxide to represent a range of different chemical modalities. TGF- β 1 is selected as an example of one growth factor, amongst others, which may be exposed and released from the bone surface, providing a biomarker to determine release kinetics. The ability of those growth factors exposed on the surface of the bone to influence cellular activity of bone marrow stromal cells is also investigated.

Materials and Methods

Preparation and chemical treatment of bone slabs

Femurs, dissected from 28 day old male Wistar rats, were halved longitudinally; marrow removed and cut into 2mm² slabs. Bone slabs were cleared of soft adherent tissue with 1 mg/ml collagenase/dispase (Sigma-Aldrich, Gillingham, Dorset, UK) for 30 min, thoroughly washed in 70% ethanol and phosphate-buffered saline (PBS), and freeze-thawed three times. Samples were treated for 5, 10, or 15 min with 17% EDTA (pH 7.2), 0.02 M calcium hydroxide (pH 11.7, saturated solution), 0.2 M sodium hydroxide (pH 11.7), 10% citric acid (pH 3) and PBS (pH 7.4) as a negative control (6 bone slabs per treatment, for each time point), rinsed in distilled water and air-dried for 48 h.

Determination of exposure and release of TGF- β 1 from bone surfaces

Bone slabs were treated for 1 h with 5% goat serum, 10% bovine serum albumin (BSA) in Tris-buffered saline (TBS), followed by a 15 min buffer wash (1% goat serum, 1% BSA, 0.1% Tween 20 in TBS). Treated surfaces were incubated with polyclonal rabbit anti-human TGF- β 1 primary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA; diluted 1:100 in 1% BSA, TBS) for 2 h, buffer washed and incubated with gold-labelled secondary antibody (BBI diagnostics, Cardiff, UK, particle diameter 30nm, protein 3.6 μ g/ml; diluted 1:100 in 1% BSA, TBS) for 1 h. Preincubation of the primary antibody with a 10-fold excess of TGF- β 1 peptide (Santa Cruz Biotech) to block further antibody-antigen interaction, provided a negative control. Bone slabs were washed with distilled water, incubated with silver enhancing agent (BBI diagnostics) for 15 min, washed in distilled water and air dried for 24 h. Surfaces were examined by scanning electron microscope at x 550 magnification.

Supernatants recovered from bone slabs treated for 15 min with the above named chemicals (9 slabs per 1 ml of

treatment), were exhaustively dialysed against double distilled water at 4°C and then lyophilised. Samples were re-dissolved in 80 μ l of 0.062M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2- β -mercapthoethanol, 0.002% bromophenol blue and examined by SDS-PAGE and Western blot analysis using a Phastsystem (GE Healthcare, Chalfont St Giles, Bucks, UK) for immunoreactivity to TGF- β 1 antibody (Santa Cruz Biotech), as previously described by Sloan *et al.* (2002).

Isolation and culture of bone marrow stromal cells

Bone marrow stromal cells (BMSCs) were isolated from rat femurs as previously described (Waddington and Langley, 1998) and grown to confluence in α -MEM (Invitrogen, Paisley, UK), 15% heat inactivated foetal calf serum (Invitrogen), 10mM β -glycerophosphate (Invitrogen), 10nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 50 μ g/ml ascorbic acid (Invitrogen), 100U penicillin, 100 μ g/mL streptomycin, 250 ng/mL amphotericin B (Invitrogen), at 37°C, 5% CO₂ in air. Cells were trypsinised (Invitrogen), seeded at 1x10⁴ cells/cm² onto bone slabs pre-treated with EDTA for 15 min, calcium hydroxide for 5 min, or remained untreated (n=5 per treatment) and cultured as above.

Influence of surface treatments on cell attachment, growth and morphology

Cells were cultured on the treated and control bone surfaces for 1, 2, 3 and 7 days. Cells were fixed in 2% paraformaldehyde, 30 min, washed in TBS, permeabilised in 3% Triton-X 100, 30 min and stained, in the dark, for 1 h at room temperature with bisbenzimidazole nuclear counterstain (Sigma-Aldrich; diluted 1:50). After washing in 0.5% Tween 20 in TBS, cells on the bone slabs were viewed by fluorescence microscopy. Viable cells adhering to the bone surfaces were counted on day 1 using image analysis procedure described below. Cell expansion was examined from cell counts on the bone surface at days 1, 2, 3 and 7. As a positive control, bone slabs, cleared of adherent tissue and cells by treatment with collagenase dispase and freeze thawing, as described, were pre-treated with 50 ml of 20 μ g/ml TGF- β 1 (PeproTech, Rocky Hill, NJ, USA) for 5 min. Excess TGF- β 1 solution was removed and cells were cultured on the surface as for treated and PBS negative control surfaces. Cell expansion was measured over days 1, 2, 3 and 7.

Apoptotic cells on the bone surfaces were detected by immunocytochemistry. Following 1 or 7 days in culture, cells on bone surfaces were fixed with 2% paraformaldehyde and permeabilised with 3% triton, as above. Cell coated surfaces were then blocked for 1 h with 1% bovine serum albumin (Sigma-Aldrich) followed by incubation with rabbit anti-rat cleaved Caspase 3 ASP175 (New England Biolabs, Ipswich, ME, USA; diluted 1:400 in 1% BSA, TBS) overnight at 4°C (Gown and Willingham, 2002). Surfaces were washed 3 times 5 min with 0.5% Tween 20 in TBS, incubated in the dark with goat anti-rabbit FITC (Santa Cruz) for 1 h at 4°C. Surfaces were washed again with 0.5% Tween 20 in TBS and treated for 1 h with bisbenzimidazole nuclear counterstain, prior to

viewing using fluorescent microscopy. As a negative control, the primary antibody was replaced with an IgG isotype control (Sigma-Aldrich; diluted to working concentration of primary antibody). As a positive control, BMSC were cultured on untreated bone surfaces in the above media for 1 day followed by a further 1 day in media supplemented with 0.2% DMSO, in order to induce apoptosis (Trubiani *et al.*, 1996). Apoptotic cells were detected by immunocytochemistry.

Cell morphologies on the treated and PBS control surfaces were assessed after 7 days of culture. Cells were fixed and permeabilised (as above) and the actin cytoskeleton was stained with phalloidin-FITC (Sigma-Aldrich; diluted 1:16), for 2 h, 4°C, in the dark, followed by 1 h incubation in the dark at room temperature with bisbenzimidazole nuclear counterstain (Sigma Aldrich; diluted 1:50). Cells were washed with 0.5% Tween 20 in TBS and viewed by fluorescence microscopy.

Osteogenic potential of bone treated surfaces

After 7 days in culture, cells on treated and PBS control bone slabs cells were fixed with paraformaldehyde, permeabilised with 3% Triton-X 100 and blocked with 1% BSA, as described in the previous section. Cells were incubated overnight with primary antibodies diluted in 1% BSA in TBS: monoclonal mouse anti-human osteopontin (clone LF-124; 1:50) (Fisher *et al.*, 1995); polyclonal rabbit anti-human alkaline phosphatase (Santa Cruz Biotech; 1:50). Negative controls comprised replacement of the primary antibody with IgG isotype control (Sigma-Aldrich; diluted to working concentration of primary antibody). Cells were washed in 0.5% Tween 20 in TBS and incubated for 1 h with the appropriate secondary antibody, diluted in 1% BSA in TBS: goat anti-mouse IgG FITC-conjugated for osteopontin (Sigma-Aldrich; 1:50); goat anti-rabbit IgG FITC-conjugated for alkaline phosphatase (Sigma-Aldrich; 1:50). Cells were treated with bisbenzimidazole nuclear counterstain (Sigma-Aldrich; 1:50). Bone surfaces were washed in 0.5% Tween 20/TBS and viewed by fluorescence microscopy.

Image analysis

Image analysis was performed on images obtained above using Image ProPlus software (Media Cybernetics, Bethesda, MD, USA). Following immunogold labelling, gold particles were counted within five random 20 μ m² areas per bone slab. Cells stained with bisbenzimidazole were counted on bone slabs within five random fields measuring 200 μ m². For immunocytochemistry, total number of cell nuclei (blue) and the number of cell nuclei immunopositive for either alkaline phosphatase or osteopontin (green pericellular staining) were counted within five random fields of 200 μ m² per bone slab, and expressed as a percentage of immuno-positive cells. Standard errors and means were calculated and analysed using one-way analysis of variance (ANOVA) and Tukey's *post hoc* test to analyse differences in TGF- β 1 release, differences in cell numbers, and cellular differences in response to chemical treatments.

Results

Release of TGF- β 1 onto bone surfaces

SEM images of immunogold-labelled bone surfaces revealed that all chemical treatments exposed TGF- β 1, when compared with PBS treated controls (selected images shown in Fig. 1). When considered as a function of time, different exposure kinetics were observed (Fig. 2b). EDTA demonstrated highest surface levels of TGF- β 1 after 15 min. Citric acid demonstrated a similar trend, with surface levels of TGF- β 1 significantly higher at 10 min compared with EDTA ($p < 0.001$). In contrast, calcium hydroxide (pH 11.7) demonstrated highest counts for TGF- β 1 immunolabelling after 5 min, with subsequent treatment times revealing a sharp reduction ($p < 0.001$). This pattern of exposure was not seen for sodium hydroxide (pH 11.7) with TGF- β 1 surface levels increasing between 5 and 10 min treatments (peak 10 min; $p = 0.0476$ comparing 5 and 10 min), and a slight, but insignificant, decrease after 15 min ($p = 0.3072$). Maximal levels of TGF- β 1 observed with sodium hydroxide treatment (10 min) were significantly lower than that exposed with calcium hydroxide 5 min treatment ($p < 0.0412$). Fig. 2b shows Western blot analysis for the immunodetection of TGF- β 1 in supernatants recovered after 15 min of treatment with the various chemicals. All demonstrated the presence of a band at 45kDa which is equivalent to the molecular weight of TGF- β 1. The intensity of the band was considerably weaker for supernatants recovered from bone slabs treated with PBS, indicating an increased ability of EDTA, calcium hydroxide, sodium hydroxide and citric acid, to release growth factors from the surface.

Influence of surface treatments on cell attachment, growth and morphology

Cells were cultured on surfaces treated with either 0.02M calcium hydroxide for 5 min or 17% EDTA for 15 min (treatment time providing optimum TGF- β 1 surface levels). As a positive control, surfaces were treated with TGF- β 1 for 5 min prior to seeding of cells onto the surface, whilst untreated bone surfaces provided a negative control. Counts of cells following bisbenzimidazole nuclear staining are shown in Fig. 3 (average counts from 5 x 200 μ m² fields of view on 5 repeat bone surfaces). Cells were allowed to adhere to surfaces for 1 day of culture. There was no significant difference in the number of cells adherent to the various treated and control surfaces ($p > 0.05$). At this time immunocytochemical analysis indicated that no cells on these surfaces stained for the apoptotic marker, cleaved caspase 3 (results not shown). Induction of apoptosis with DMSO resulted in positive immunostaining for a high proportion of cells (positive control). On all surfaces cell counts increased with time in culture (Fig. 3). However, at day 3 and day 7 there were significantly more cells present on surfaces treated with either calcium hydroxide, EDTA or TGF β 1, compared with untreated surfaces ($p < 0.001$) indicating that surface treatment enhanced cell growth. No statistical differences were noted on comparing the individually treated surfaces with each other ($p > 0.05$). No apoptotic cells were detected on the bone surfaces at day 7.

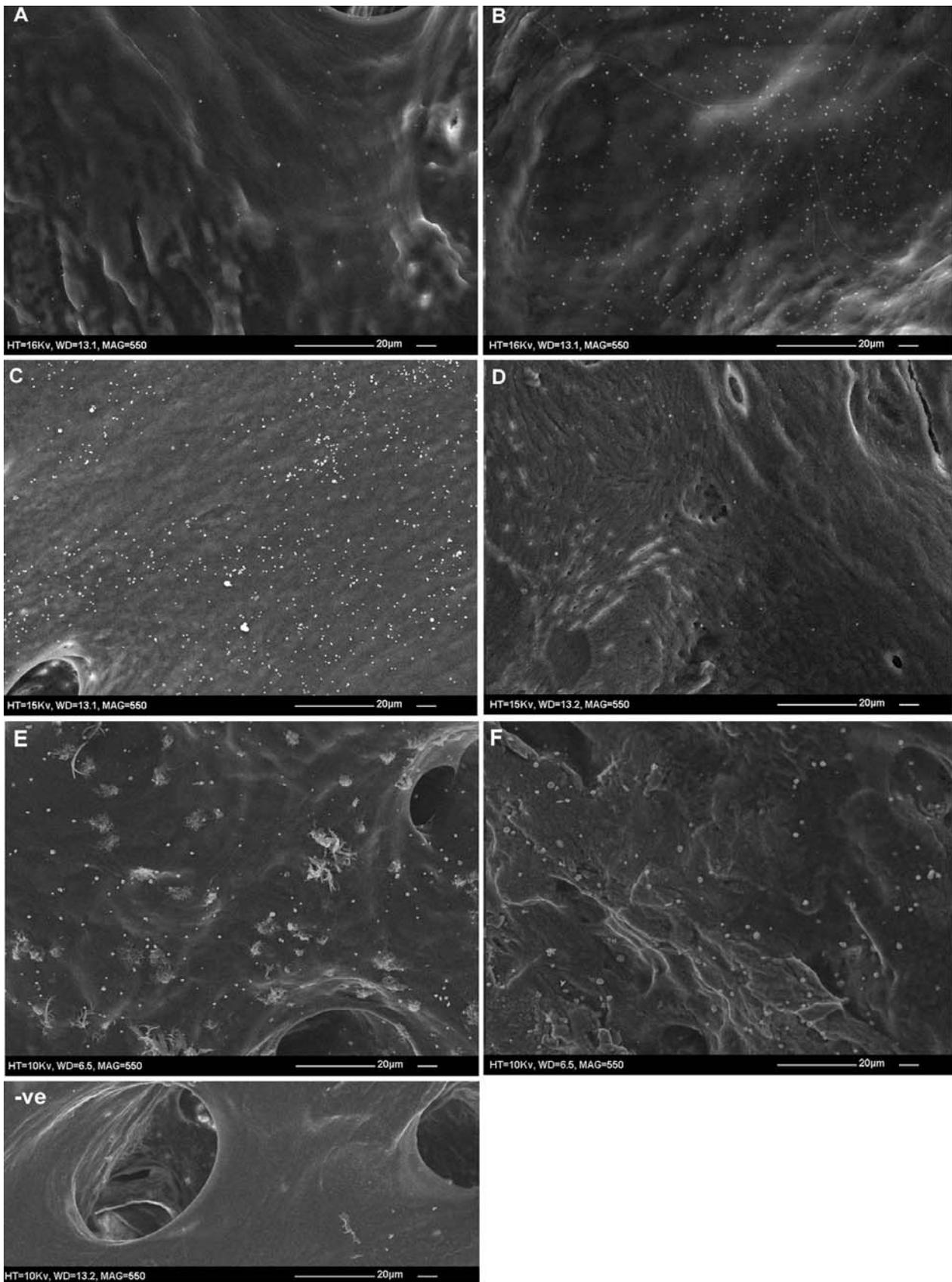


Fig. 1. Immunogold labelling of TGF- β 1 on bone surfaces treated with (A) 17% EDTA for 5 min, (B) 17% EDTA for 15 min, (C) 0.02M calcium hydroxide for 5 min, (D) 0.02M calcium hydroxide for 15 min (E) 10% citric acid for 5 min, (F) 0.2M NaOH for 5 min. -ve: negative control where primary antibody was incubated with a 10 fold excess of specific peptide to block immuno-reactivity of antibody with surface TGF- β 1.

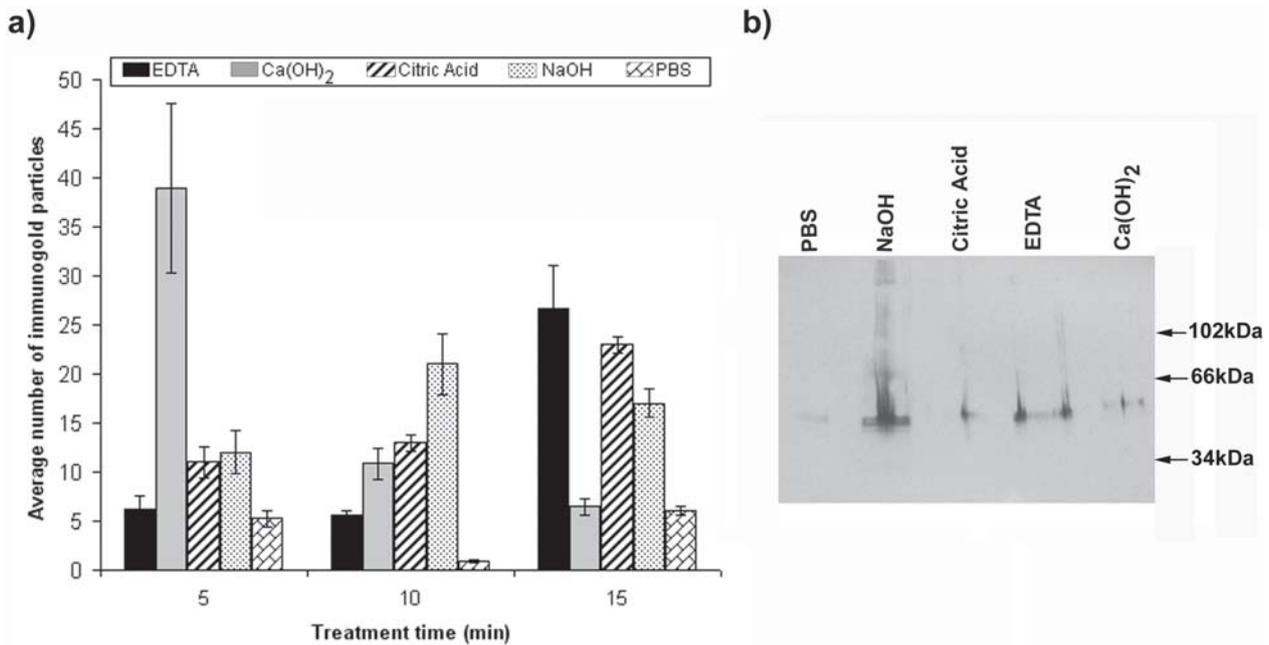


Fig. 2. (a) Average counts for TGF- β 1 labelled immunogold particles per 20 μm^2 area of bone treated surfaces, exemplified in Fig. 1. (b) Immunodetection of TGF- β 1, at 45kDa, released from the bone surfaces following treatment for 15 min.

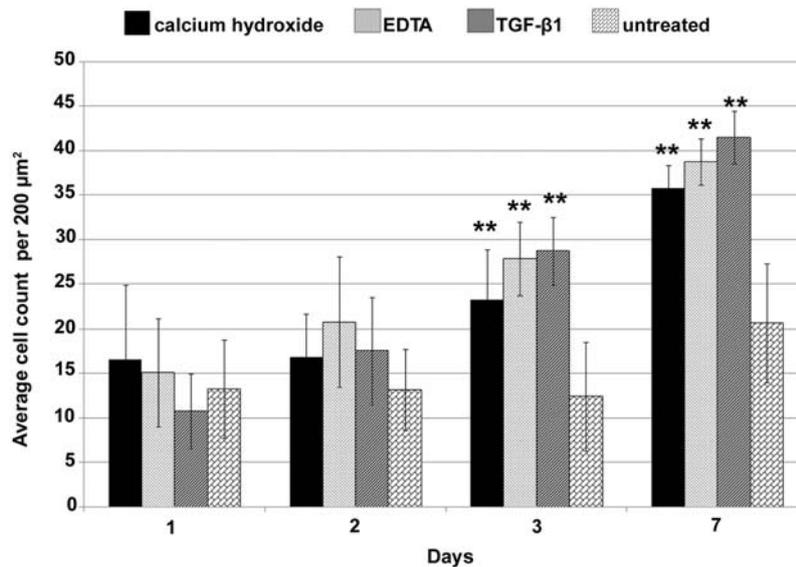


Fig. 3. Average number of phalloidin stained cells per 200 μm^2 area cultured on bone surface for 1-7 days following treatment calcium hydroxide for 5 min, EDTA for 15 min, TGF- β 1 for 5 min or untreated surface. * $p < 0.001$ when statistically compared with untreated surface.

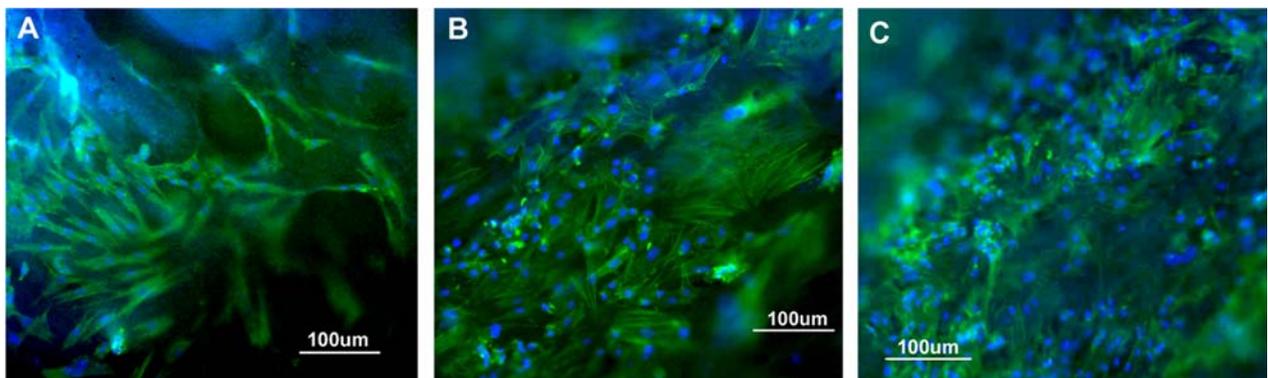


Fig. 4. A typical image of cells stained with phalloidin-FITC and bisbenzimidazole nuclear counterstain following culture on (A) untreated, (B) calcium hydroxide treated, and (C) EDTA treated bone surfaces.

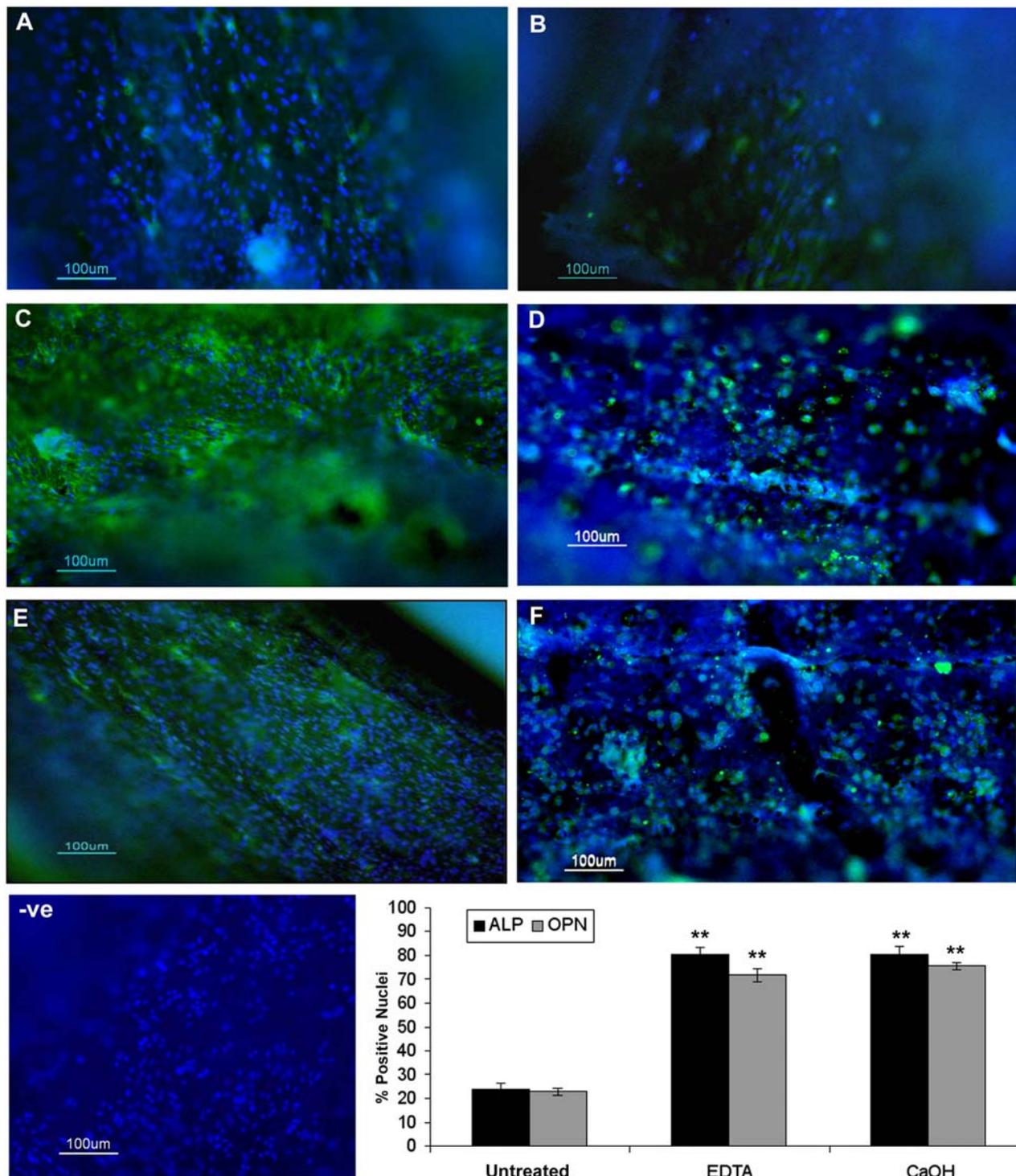


Fig. 5. Immunocytochemical characterisation of cells cultured on treated bone surfaces. Immunolocalisation for (A) alkaline phosphatase on 17% EDTA treated surfaces, (B) osteopontin on 17% EDTA treated surfaces, (C) alkaline phosphatase on calcium hydroxide treated surfaces, (D) osteopontin on calcium hydroxide treated surfaces, (E) alkaline phosphatase on untreated surfaces, (F) osteopontin on untreated surfaces. Graphical insert displays the average percentage positive cell nuclei for alkaline phosphatase and osteopontin on the three surfaces.

Cells cultured on untreated bone surfaces had elongated morphologies, appearing as small discrete cell clusters on the surface (Fig. 4). In contrast, cells cultured on bone surfaces treated with either 0.02M calcium hydroxide for 5 min or 17% EDTA for 15 min (treatment time providing optimum TGF- β 1 surface levels) demonstrated more cuboidal morphologies, covering the entire surface.

Immunocytochemical characterisation of cells cultured on bone surfaces

Cells cultured on all chemically treated and untreated surfaces expressed bone markers alkaline phosphatase and osteopontin (Fig. 5). However, following culture on EDTA treated surfaces, the percentage of alkaline phosphatase and osteopontin positively expressing cells was

significantly increased compared with cells cultured on untreated surfaces ($p < 0.001$ for both proteins). A similar significant increase was observed for cells cultured on calcium hydroxide treated surfaces compared with the control ($p < 0.001$ for both proteins). The percentage of osteopontin or alkaline phosphatase positive cells following culture on EDTA or calcium hydroxide treated surfaces were not significantly different ($p > 0.05$).

Discussion

This study provides evidence that short-term treatment of bone surfaces with either alkaline, acid or acidic calcium chelating chemicals has the ability to augment the release of TGF- β 1 from the mineralised matrix when compared to PBS, representing a control bone surface equivalent to physiological conditions. It should be noted that this study investigated the release of TGF- β 1 as a biomarker of growth factor release. It is probable that other growth factors trapped within the mineralised matrix, such as bone morphogenetic proteins, platelet derived proteins and insulin-like growth factors also contribute to the observed increase in osteogenic potential. Significantly, the results for the first time demonstrate that the short-term treatment of bone surfaces with either EDTA or calcium hydroxide leads to the increased surface exposure and subsequent release of active growth factors, which are able to increase the osteogenic potential of bone marrow stromal cells.

Differences were observed in the release kinetics between treatments, which may reflect their mechanism of exposure to the surface, as demonstrated by immunogold labelling, and subsequent release, as demonstrated by Western blot analysis of the supernatant. EDTA and citric acid displayed similar patterns of TGF- β 1 solubilisation, with surface levels increasing with time, probably through the gradual dissolution of apatite crystals present in mineralised bone. In acid environments both apatite crystals and proteins are likely to carry a positive surface charge (Doss, 1976; Harding *et al.*, 2005), providing a repulsive effect facilitating the release of growth factors from the bone surface. Conversely, alkali treatments demonstrated different patterns of exposure and release from the bone surface. For treatment with calcium hydroxide, highest levels of TGF- β 1 were observed at 5 min, which subsequently declined, probably through loss into the aqueous environment. Following treatment with sodium hydroxide, highest surface counts for TGF- β 1 were observed at 10 min. Sodium and calcium hydroxide are capable of extracting proteins from tissue matrices (Partridge and Elsdon, 1961) and our results probably represent growth factor solubilisation from the protein matrix near the surface. Of note, collagen has been demonstrated to swell more in sodium hydroxide compared with calcium hydroxide (Bowes, 1950), which may account for the delayed exposure of TGF- β 1 on the sodium hydroxide treated bone surfaces. In sodium hydroxide, apatite surface charge would be expected to be negative (Doss, 1976; Harding *et al.*, 2005) repelling proteins which also adopt a negative charge in alkali pH. However, studies

suggest that hydroxyapatite behaves differently in calcium hydroxide, acquiring a more positive charge due to the specific adsorbance of Ca^{2+} (Doss, 1976), providing an explanation for the observed higher levels of TGF- β 1 on calcium hydroxide treated surface. The low levels of TGF- β 1 detectable on the PBS control surface may be as a consequence of freeze thawing and collagenase/disypase treatment in preparing the bone surfaces, or simple solubilisation of growth factors into a physiologically equivalent fluid. However, all chemical treatments indicated a significant increase in the level of TGF- β 1 compared with PBS treated surfaces, indicating increased ability of these chemical modalities to expose and subsequently release TGF- β 1 from bone surfaces. The clinical application of these chemical modalities to a fractured bone surface has the potential to increase growth factor availability within the wound healing environment.

Levels of TGF- β 1 exposed at the surface may be considered very low as immunogold localisation was required to detect surface growth factor. Chemical treatments are also likely to have exposed and released low levels of other growth factors proposed to be entrapped within the mineralised matrix (Hauschka *et al.*, 1986; Frolik *et al.*, 1988; Taipale and Keski-Oja, 1997). Compared with cells grown on untreated surfaces, both EDTA and calcium hydroxide treated bone surfaces stimulated cell proliferation. Cells expanded to cover the surface uniformly, adopted a more cuboidal morphology and synthesised increased levels of alkaline phosphatase and osteopontin, suggesting that the low levels of growth factor release were sufficient to increase the osteogenic potential of the BMSCs. Working synergistically, the release of growth factors, such as the FGFs (Mansukhani *et al.*, 2000), IGFs (Hughes *et al.*, 2006), and TGF- β 1 (Barnes *et al.*, 1999), may increase cellular proliferation, whilst BMPs (Wozney, 1992; Cheng *et al.*, 2003) and the IGFs (Celil and Campbell, 2005), may stimulate osteoblast differentiation of the BMSCs. As a positive control, surfaces were pre-coated for 5 min with TGF- β 1. Cells grown on these surfaces demonstrated an increase in cell expansion, with cell numbers significantly higher at days 3 and 7 compared with untreated surfaces. The exposure and release of TGF- β 1 on the bone surface following calcium hydroxide and EDTA treatment may therefore be significant for stimulating mesenchymal cell expansion. However, considering the roles of TGF- β 1, the overall increase in osteogenic potential on treated surfaces is probably attributable to the exposure of a range of growth factors working synergistically. It is possible that this is not the only mechanism facilitating an increased osteogenic potential. Other potential factors affecting cell behaviour of BMSCs may be due to roughening of the surface brought about through demineralisation by EDTA, or increased surface hydrophilicity on EDTA and calcium hydroxide surfaces, facilitating cell proliferation and differentiation down the osteoblast lineage (Anselme, 2000; Deligianni *et al.*, 2001; Kim *et al.*, 2007; Tsukimura *et al.*, 2008). Of note, our results indicated that changes to the surface, through alteration in topography, chemistry or the tertiary structure of bone matrix proteins exposed to the surface,

did not alter cell attachment on the four different surfaces, measured after 24 h. In addition, treatment of the surfaces did not induce apoptosis.

Whilst chemical treatments studied herein are not used in bone repair therapies, calcium hydroxide is widely used in dental endodontics procedures. Application of calcium hydroxide has been demonstrated to stimulate the recruitment and differentiation of mesenchymal progenitor cells within the dental pulp to differentiate and synthesise a reparative mineralised tissue at the dentine-pulp interface, which shares many histological and matrix composition similarities with osseous tissues (reviewed by Goldberg and Smith, 2004). The mechanism by which calcium hydroxide stimulates this mineralised tissue formation remains unclear, but may be multi-factorial. The results described herein confirm the ability of calcium hydroxide to release active growth factors from the mineralised matrix which have also been proposed to play an important role in the formation of reparative dentine (Kardos *et al.*, 1998). Additionally, calcium hydroxide in the presence of exogenous TGF- β 1 appears to have an autocrine effect on osteoblasts, stimulating further synthesis of TGF- β 1 (Jaunberzins *et al.*, 2000) and thus favouring the reparative process. EDTA is also used in dental procedures as a cavity cleanser to remove smear layers. However, the application of EDTA to exposed pulp has been suggested to induce a severe pulpal reaction, and dentine bridge formation takes three times longer than that seen following application of calcium hydroxide to the pulpal tissue (Kiba *et al.*, 2000).

The results presented in this study demonstrate that growth factors exposed at the bone surface can actively influence the behaviour of BMSCs cultured on the bone surface. Such data supports developments for future clinical therapies for impaired bone repair, utilising chemical treatments to release bioactive growth factors residing *in vivo* within the bone matrix. In this clinical scenario, a 'cocktail' of bioactive molecules may be released into the site of injury, enabling these factors to work synergistically, ultimately stimulating optimal repair processes within the injured bone.

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Discussion with Reviewer

Reviewer I: In the experiments including cultures of bone marrow stromal cells onto bone matrix, only EDTA and calcium hydroxide treated surfaces were selected. Is there any particular reason why these two were selected over the other treatments (i.e., NaOH and citric acid) for this series of experiments?

Authors: EDTA and calcium hydroxide were only taken through to examine cell activity as these treatment modalities are used in clinical practice in dental treatments. Moreover, if we are to take any of these treatment modalities through to treatment of bone fractures, then it is more likely to be EDTA and calcium hydroxide due to the research available on these etchants in dental pulp cells. Sodium hydroxide is not currently clinically used, but we used it in our study to demonstrate that extraction is not solely attributable to alkaline pH conditions. Citric acid was used to help establish that the mechanism of release was due to demineralisation, as release kinetics for the two chemical treatments were very similar.