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Cytoskeletal reorganisation, 1 α ,25-dihydroxy vitamin D3 and human MG63 osteoblast maturation.

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Abstract

Bone tissue is especially receptive to physical stimulation and agents with the capacity to mimic the signalling incurred via mechanical loading on osteoblasts may find an application in a bone regenerative setting. Recently this laboratory revealed that the major serum lipid, lysophosphatidic acid (LPA), cooperated with $1\alpha,25$ -dihydroxy vitamin D3 (D3) in stimulating human osteoblast maturation. Actin stress fiber accrual in LPA treated osteoblasts would have generated peripheral tension which in turn may have heightened the maturation response of these cells to D3. To test this hypothesis we examined if other agents known to trigger stress fiber accumulation cooperated with D3 in stimulating human osteoblast maturation. Colchicine, nocodazole and LPA all co-operated with D3 to promote MG63 maturation in a MEK dependent manner. In contrast, calpeptin, a direct activator of Rho kinase and stress fiber accumulation did not act with D3 to secure MG63 differentiation. Herein we describe how the signalling elicited via microtubule disruption cooperates with D3 in the development of mature osteoblasts.

1. Introduction

Osteoblast differentiation in response to $1\alpha,25$ -dihydroxy vitamin D3 (D3) secures competent bone collagen mineralization (van Driel et al., 2004; van Driel et al., 2006); D3 deficiency in childhood leads to rickets and an inadequate D3 status in adults results in osteomalacia (Berry et al., 2002). Although it is widely recognised that D3 is essential for the provision of a mechanically sound mineralised skeleton the application of D3 to cultured osteoblasts in the absence of serum or selected growth factors does not drive their differentiation. We recently discovered that the principal serum factor co-operating with D3 in promoting human osteoblast maturation is lysophosphatidic acid (Gidley et al., 2006); in isolation D3 was unable to induce osteoblast differentiation but when used in conjunction with human serum a stark, synergistic maturation response occurred. We subsequently found that osteoblast maturation in response to D3 and serum could be blocked by Ki16425, a lysophosphatidic acid (LPA) receptor antagonist (Ohta et al., 2003). Furthermore the application of commercially available LPA in combination with D3 to MG63 osteoblasts led to striking, synergistic increases in both osteocalcin and alkaline phosphatase (ALP), proteins expressed by the differentiated phenotype (Gidley et al., 2006).

Lysophosphatidic acid was identified as the serum borne agent responsible for triggering actin stress fibre accumulation in fibroblasts via activation of Rho (Ridley and Hall, 1992; Ridley and Hall, 1994; Ridley, 2001). It has subsequently been established that LPA can actually elicit the same intracellular responses as generated by integrin-ECM contacts (Hunger-Glaser et al., 2003; Bershadsky et al., 1996 &

references therein), namely activation of Ras, focal adhesion kinase (FAK) and Rho. Since these important discoveries LPA has found application in many studies aimed at enhancing our understanding of the mechanobiological events accompanying cytoskeletal reorganisation. For example, intracellular microrheological studies have recently reported how LPA enhances the viscosity and stiffness of the cytoplasm in serum starved Swiss 3T3 cells (Kole et al., 2004). Thus LPA could be thought of as a “mechanochemical” by virtue of its ability to both stimulate intracellular physicomachanical changes, via cytoskeletal reorganisation, and to mimic the signalling in response to integrin occupancy. In our previous report we postulated that events accompanying LPA-induced actin stress fibre accumulation somehow linked together with D3 in promoting osteoblast maturation (Gidley et al., 2006). Credence is given to this possibility because of the dependency on (osteoblast) mechanotransduction to the actin microfilaments of focal adhesion complexes (Lipfert et al., 1992; Sinnett-Smith et al., 1993; Chen et al., 1994; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994; Zhu and Assoian, 1995). The actin component in turn acts in concert with the microtubular network in coordinating signal transduction emanating from focal adhesions. It has been proposed that the microtubules control integrin-dependent signalling and that the tension arising from the actin filaments might be under the control of microtubular assembly (Bershadsky et al., 1996).

It is widely recognised that physical stimuli influences bone matrix metabolism and accrual (Liedert et al., 2006; Klein-Nulend et al., 2005; Harada and Rodan, 2003; Pavalko et al., 2003 and references therein). Indeed Pavalko and colleagues (2003) remind us that “bending bone ultimately bends genes”. Osteoblasts are in intimate

contact with the collagenous matrix they secrete via cell surface integrins. Physical stimuli result in changes in the extracellular environment, for example altered matrix orientation and/or initiation of strain and it is these changes that are detected by the integrins. On the cytoplasmic side of the osteoblast the integrin molecules make contact with the actin cytoskeletal network. Thus the integrins are the mechanoreceptors relaying physical stimuli arising from alterations in the extracellular matrix (ECM) directly to the cytoskeleton (Wang et al., 1993). It is becoming clear that the function of mechanotransducing integrins is dependent on their linkage to the cytoskeleton (Alenghat and Ingber, 2002).

One of our main goals is to identify novel D3-growth factor combinations for improving human osteoblast responses to bone implant materials. Both LPA and D3 co-operate in stimulating human osteoblast maturation, furthermore LPA exhibits many of the cell responses elicited via ECM-integrin contacts. The principle aim of this study therefore is to determine whether actin stress fiber accumulation per se provides a nexus with D3 in generating mature human osteoblasts and/or whether alterations in the microtubular component can fulfil D3 induced cellular differentiation. The information gleaned from such an investigation will assist in the identification of suitable small molecules for their subsequent application in a bone biomaterials context aimed at improving osseointegration.

2. Materials & Methods

2.1 Tissue culture reagents

Tissue culture medium and fetal calf serum were obtained from Gibco (Paisley, Scotland) and essentially fatty acid free human serum albumin (FAFA) from Sigma (Poole, UK). Stocks of D3 (Sigma, Poole UK) were prepared in ethanol (100 μ M) and stored at 4°C, LPA (Biomol, UK) was prepared in 1:1 ethanol:water (10mM) and stored at -20°C and calpeptin (Calbiochem) reconstituted to 5mM in DMSO prior to storage at -20°C. Colchicine (Sigma, Poole, UK) was dissolved in serum free culture medium to prepare a stock of 1mg/ml, filter sterilised and left refrigerated and both taxol and nocodazole (Sigma, Poole, UK) reconstituted in DMSO (1mg/ml) and stored at -20°C. Thiocolchicoside was kindly provided by Alchem International (Faridabad, India) and prepared in ethanol to a working stock concentration of 100 μ g/ml. Colchicine (EIN) was prepared in house from colchicine exactly as described by Boyland and Mason (1938); briefly colchicine was prepared in 100mM HCl at a concentration of 100 μ g/ml in a screw-cap microcentrifuge tube and placed in a boiling water bath for one hour. The resultant EIN preparation was immediately diluted 1000 fold in serum-free tissue culture medium and applied to MG63 cells. Inhibitors of Raf (GW5074) and MEK (UO126) were reconstituted to 10mM in DMSO whereas the Ras inhibitor (FTI-277) was prepared as a 10mM stock in serum free culture medium. All were obtained from Calbiochem and aliquots stored at -20°C. The focal adhesion kinase (FAK) inhibitor, TAE226, was kindly provided by Novartis Pharma AG (Switzerland) and reconstituted to 5mM using DMSO.

2.2 MG63 cell culture

Human osteoblast-like cells (MG63) were cultured in conventional tissue culture flasks (250 ml, Greiner) in a humidified atmosphere at 37°C and 5% CO₂. Cells were grown to confluence in DMEM/F12 nutrient mix supplemented with sodium pyruvate (1 mM final concentration), L-glutamine (4 mM), streptomycin (100 ng/ml), penicillin (0.1 units/ml) and 10% v/v fetal calf serum. The growth media (500ml final volume) was also supplemented with 5ml of a 100 X stock of non-essential amino acids. Cells were grown to confluence and subsequently dispensed into 24-well plates (Greiner, Frickenhausen, Germany) such that each well contained 1ml of a 2×10^4 cells/ml suspension (as assessed by haemocytometry). Cells were then cultured for 65 hours, the media removed and the cells treated with the same medium but lacking serum for 24 hours. In addition to the 24-well plate experiments cells were also seeded into 12-well plates for reporter assays, 60mm dishes or 4-well chamber slides for Erk phosphorylation and cytoskeletal studies respectively.

2.3 Co-treating MG63 cells with D3 and cytoskeletal reorganising agents – 24 –well plate experiments

The establishment of MG63 cells for stimulations within 24 well plates was identical to that described above. These osteoblast-like cells were treated with 100ng/ml of either colchicine, nocodazole, taxol, thiocolchicoside or EIN in the presence or absence of 100nM D3. MG63 cells were also treated with calpeptin (5μM) with or without D3. In some instances MG63 cells were treated with a combination of cytoskeletal agents and/or signal transduction inhibitors. For a positive control of MG63 maturation, cells were co-stimulated with D3 and LPA (20μM) in the presence of 100μg/ml FAFA. Cultures were left for a maximum of 72 hours prior to an

assessment of cellularity, osteocalcin production and alkaline phosphatase activity as we have detailed previously (Yarram et al., 2004, Gidley et al., 2006)

2.4 *Assessment of cell number*

An assessment of cell number was performed using a combination of the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, Promega, UK) and the electron coupling reagent phenazine methosulphate (PMS). Each compound was prepared separately in pre-warmed (37°C) phenol red free DMEM/F12, allowed to dissolve and then combined so that 1 ml of a 1mg/ml solution of PMS was combined to 19 ml of a 2mg/ml solution of MTS. Immediately prior to treating cells with the MTS/PMS reagent, medium was aspirated from each of the wells and replaced with 0.5ml pre-warmed (37°C) phenol red free DMEM/F12. Each well was subsequently treated with 0.1ml of the MTS/PMS reagent mixture and the plates returned to the incubator for one hour. A blank consisted of media alone (0.5 ml) plus 0.1 ml of the MTS/PMS reagent mixture. Once incubated, samples (0.1 ml) from each well were dispensed onto a 96 well microtitre plate and the absorbances at 492 nm read using a multiplate reader. Plates were staggered to ensure that all samples were recovered for 96-well plating within 5 minutes to minimise any error introduced during the formation of further formazan product by the cell monolayer.

2.5 *Measurement of alkaline phosphatase (ALP) activity*

An assessment of ALP activity is reliably measured by the generation of p-nitrophenol (p-NP) from p-nitrophenylphosphate (p-NPP) under alkaline conditions. The treatment of cells to quantify ALP activity was similar to that described

previously (Yarram et al., 2004; Gidley et al., 2006). Briefly the remaining MTS/PMS reagent was removed and the monolayers rinsed with 1ml of phenol red free DMEM/F12 which was subsequently removed and the monolayers lysed with 0.1 ml of 25 mM sodium carbonate (pH 10.3), 0.1% (v/v) Triton X-100. After 2 min each well was treated with 0.2 ml of 15 mM p-NPP (di-tris salt, Sigma, UK) in 250 mM sodium carbonate (pH 10.3), 1.5 mM MgCl_2 . Lysates were then left under conventional cell culturing conditions for 1 hour. In order to confirm that the enzyme activity being measured was the bone/liver/kidney isoform, lysis buffers supplemented with either 1 mM levamisole or 1 mM phenylalanine were prepared. After the incubation period, 0.1 ml aliquots were transferred to a 96 well microtitre plate and the absorbance read at 405 nm. An ascending series of p-NP (25-400 μM) prepared in the incubation buffer enabled quantification of product formation.

2.6 Osteocalcin (OC) enzyme linked immunosorbent assay (ELISA) – MG63 cells

were seeded into small flasks such that each flask contained 10ml of 20×10^3 cells/ml and the osteoblasts left for three days prior to medium removal and overnight starvation using serum free culture medium (SFCM). Once starved MG63s were either given fresh SFCM (supplemented with 250 $\mu\text{g/ml}$ FAFA), D3 (100nM), LPA (20 μM), colchicine (100ng/ml), nocodazole (100ng/ml) or a combination of D3 with the aforementioned agents and the cells left to incubate for three days. After the treatment period conditioned media (20 μl) from each of the flasks were assayed for OC production using a commercial ELISA exactly as instructed by the manufacturer (immunodiagnostic systems (ids), Boldon, Tyne & Wear, UK). Two independent experiments were performed using 4 replicates per treatment group and the data expressed as the mean osteocalcin concentration (ng/ml) $\pm$ S.D.

2.6 Smad-3 and activator protein-1 (AP-1) reporter assays

MG63 osteoblasts were seeded into 12-well plates such that 2ml of a 2×10^4 cells/ml was dispensed per well. Once the cells had reached approximately 60-70% confluence (typically within 24hr) the media was removed and replaced with serum free culture medium and the cells transiently transfected with 0.5 μ g/well each of a Renilla control vector, pRL-TK-luc (Promega), and either 7AP-1-luc or CAGA₁₂-luc (Smad-3 reporter) using Fugene6 transfection reagent (Roche). After 6 hours the cells were then treated with test reagents in serum free culture media and left for 18 hours prior to processing for luminometry using the Dual luciferase reagents as instructed (Promega). Luminescence was measured and the luciferase values normalised to control for transfection efficiency using the data obtained for the co-transfected control as previously described (Davies et al., 2005).

2.7 Immunoblotting for Smad-2 phosphorylation

Briefly 5ml of a 5000 cell/ml suspension of MG63 cells were dispensed into 60mm dishes and left for 72hrs, the medium removed and replaced with serum free medium and the cells starved for 24hrs. The next day dishes were treated with 100ng/ml TGF β (positive control), 10 μ g/ml colchicine or vehicle for 1, 6 and 18 hours. After the stimulation period the media was removed, and the cells lysed using 0.1ml/dish of hot (~70°C) reducing buffer (3% SDS; 7% sucrose; 45mM Tris, pH 6.8; 35mM DTT; 0.01% bromophenol blue) and the samples subjected to SDS-PAGE and subsequent transfer onto PVDF. The membrane was then probed for phosphorylated Smad-2 using a rabbit polyclonal antibody (Cell Signalling Technologies, UK) and the secondary antibody was an anti-rabbit peroxidase conjugate (Sigma). The membrane

was subsequently reprobed using a mouse monoclonal anti- α -tubulin (Sigma) to confirm equal protein loading.

2.8 *F-actin labelling using FITC-phalloidin.*

Chamber slides, 4-well (Lab-Tek Chamber Slide systems, Nalge Nunc International, Roskilde, Denmark), were seeded with 1ml/well of a 5000 cells/ml suspension and the cells left as described above. After the initial culture period the medium was removed and the cells treated with either colchicine (100ng/ml), nocodazole (100ng/ml) or 5 μ M calpeptin and the cells left for 24hrs. Following the incubation period cells were processed for F-actin detection using the fungal toxin phalloidin conjugated to FITC (Sigma, reconstituted to 0.5mg/ml in DMSO) exactly as described previously (Wulf et al., 1979). Briefly the culture media was removed and the cells rinsed once with PBS and then fixed for 5 minutes with 3.7% formaldehyde in PBS. The fixative was then aspirated and the cells treated with 0.2ml/well FITC-phalloidin (5 μ g/ml) in PBS for 40 minutes at room temperature. After the incubation period the cells were treated with DAPI slide mountant (Vectashield, Vector Laboratories, Peterborough, UK) and viewed under a DMLB fluorescence microscope (Leika, Bucks, UK) and images taken using Provia 400F 35mm film (Fuji).

2.9 *Statistical analysis*

Unless stated otherwise, all experiments described above were performed at least three times, each on separate days and using different passage numbers of cells. The minimum replicate number for each of the treatment groups and controls was four. All data were subject to a one-way analysis of variance (ANOVA) to test for

statistical significant. When a p value of <0.05 was found, a Tukey multiple comparisons post-test was performed between all groups.

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3. Results

(i) *Both colchicine and nocodazole cooperate with D3 in stimulating MG63 maturation whereas calpeptin is without effect* - The initial phase of this study was to ascertain if molecules reported to generate actin stress fibers could link with D3 in promoting human osteoblast maturation. To this end MG63 cells were treated with two structurally dissimilar microtubule disrupting agents or calpeptin, a direct activator of ROCK induced actin stress fiber accrual. Synergistic increases in ALP activity, and therefore osteoblast maturation, were observed for cells co-stimulated with D3 and either colchicine or nocodazole but not for MG63 cells treated with a combination of calpeptin and D3 (Fig. 1A). The differential ALP response was not attributed to altered cell numbers between the treatment groups (Fig. 1B). Confirmation of calpeptin bioactivity was supported by FITC-phalloidin stained actin stress fiber accrual of treated cells (Fig. 2B).

(ii) *Osteocalcin (OC) expression in response to D3 is blunted by either LPA or microtubule disruption* – Osteocalcin is expressed by committed osteoblasts but whether levels of this molecule are influenced by events surrounding cytoskeletal reorganisation have not been reported. As expected the treatment of MG63 cells with 100nM D3 led to a statistically significant increase ($p < 0.001$) in OC production compared with vehicle controls (Fig. 3). However when MG63 cells were co-treated with D3 and colchicine/nocodazole/LPA the level of OC expression, although still greater than control cultures, was significantly attenuated ($p < 0.005$). In each instance the co-stimulation of MG63 cells with D3 and LPA/colchicine/nocodazole lead to the expected synergistic increase in ALP activity compared to all other treatment groups (results not shown).

(iii) *Events surrounding microtubule disruption are key to D3 induced osteoblast maturation* – To determine if the signalling arising from microtubule disruption links up with D3 in generating mature osteoblasts these cells were treated with colchicine derivatives (EIN and thiocolchicoside) reported to have a trivial influence on tubulin dynamics. Furthermore cells treated with either colchicine or nocodazole were pre-treated with the microtubule stabilising drug taxol. Both EIN and thiocolchicoside were unable to cooperate with D3 in generating mature MG63 cells (Fig 4A). Also, the pre-treatment of osteoblasts with taxol prevented the synergistic increase in ALP activity for those cells co-stimulated with D3 and the microtubule disrupting agents (Fig. 4C). In each instance the very low ALP activities could not be explained by reduced cell numbers (Figs. 4B & 4D).

(iv) *Evidence for the participation of Ras, Raf and MEK upon osteoblast maturation in response to microtubule disruption and D3* – We previously reported that osteoblast maturation in response to D3 and LPA could be blocked by UO126, an inhibitor of MEK. Similarly we also found that the same compound could neutralise the synergistic increase in ALP for MG63 cells co-treated with D3 and epidermal growth factor. The inclusion of UO126 to cells treated with both D3 and colchicine led to a marked inhibition of ALP activity ($p < 0.001$, Fig. 5A) The Ras inhibitor, FTI-277, led to a significant attenuation in ALP activity ($p < 0.01$, Fig 5B) and the pre-treatment of MG63 cells with GW5074, an inhibitor of Raf, led to a stark inhibition of ALP activity for cells co-stimulated with D3 and colchicine ($p < 0.001$, Fig. 5C). In each instance the very low or attenuated ALP activities could not be explained by reduced cell numbers (Fig. 5D).

(v) *Microtubule disruption in MG63 cells does not influence Smad 2 and 3 activity but does promote activator protein-1 activity* – There are studies reporting on increased Smad transcriptional activity in response to microtubule disruption. To ascertain whether colchicine might influence osteoblast Smad 2 and 3 activity cells were stimulated with TGF β and colchicine and appropriately prepared to detect phosphorylated Smad-2 by immunoblotting and Smad-3 activation via dual luciferase reporter assay respectively. As expected the treatment of MG63 cells with TGF β resulted in a clear increase in phosphorylated Smad-2 at all treatment points whereas colchicine and vehicle treated groups were without effect (Figs 6A&B). Activator protein-1 (AP-1) plays a key role in bone development and homeostasis and it has been reported that colchicine can activate AP-1, albeit in non-bone cells. The application of 7AP-1-luc transfected MG63 cells reveals that the events associated with microtubule disruption do result in increased activity of AP-1 ($p < 0.01$) in osteoblasts (Fig 7).

(vi) *Inhibition of FAK with TAE226 does not prevent osteoblast maturation* – Physical stimuli are detected by FAK, but whether the events encompassing cytoskeletal reorganisation can influence osteoblast maturation via FAK activation has not been reported. As expected the co-stimulation of MG63 cells with either D3 and colchicine or LPA and D3 led to a stark increase in ALP activity. However when cells were exposed to 500nM TAE226, a FAK inhibitor, the differentiation response was not prevented. The application of higher concentrations of TAE226 ($> 1\mu\text{M}$) resulted in cell losses (data not shown).

Discussion

Research conducted in our laboratory identified lysophosphatidic acid (LPA) as a potent mediator of D3 induced human osteoblast maturation (Gidley et al., 2006). At the time we postulated that LPA might influence osteoblast differentiation via its reported actions on the actin cytoskeleton (Ridley and Hall 1992; Ridley and Hall 1994, Ridley, 2001) and/or its ability to mirror the signalling initiated through ECM-integrin contacts (Hunger-Glaser et al., 2003; Bershadsky et al., 1996). Credence is given to either of these possibilities because of the important role of mechanical loading and the cytoskeleton in the regulation of bone mass (Pavalko et al., 2003). To this end our primary focus was to ascertain whether other molecules reported to trigger actin stress fiber assembly and resultant cell tension could in some way link up with D3 in stimulating immature osteoblast differentiation. In parallel with LPA we screened the ability of three agents known to alter cytoskeletal reorganisation on their ability to cooperate with D3 in supporting human osteoblast maturation.

Calpeptin, colchicine and nocodazole are well known to stimulate actin stress fiber assembly. Originally developed as a cell permeable calpain inhibitor (Tsujinaka et al., 1988), calpeptin was serendipitously reported to stimulate actin stress fiber accumulation in fibroblasts via the inhibition of Shp-2 upstream of the small GTPase Rho (Schoenwaelder and Burridge, 1999; Schoenwaelder et al., 2000). Both the naturally occurring alkaloid colchicine and the synthetic agent, nocodazole, both inhibit microtubule assembly and promote actin stress fiber accrual in a Rho-dependent manner (Bershadsky et al., 1996; Enomoto et al., 1996; Zhang et al., 1997; Liu et al., 1998). As anticipated the co-treatment of MG63 osteoblast-like cells with LPA and D3 led to a robust maturation response as supported by a clear, synergistic

increase in ALP activity. Similar results were obtained for cells co-treated with both colchicine and D₃ and nocodazole and D₃ whereas calpeptin, either alone or in combination with D₃, was without effect on ALP activity. Treatment of MG63 cells with calpeptin and the subsequent detection of F-actin accumulation via staining with FITC-phalloidin gave a clear indication of calpeptin bioactivity (Fig 2B).

Interestingly the heightened expression of OC on receipt of D₃ was significantly attenuated by LPA, colchicine or nocodazole. Although OC is expressed by committed osteoblasts it is well known that mice nullizygous for OC have no skeletal phenotype at birth but raised bone mass by six months of age (Ducy et al. 1996). Thus it is possible that a dampening of OC production by mature cells is associated with bone matrix accrual in adult life. Recently Wolf (2008) has reviewed the importance of OC in energy metabolism rather than having a role to play in skeletal mineralisation. Suffice it to say ALP deficiency does result in hypophosphatasia, a skeletal abnormality characterised by an inadequately mineralised bone matrix (Whyte 2001) whereas a paucity of OC is without influence on skeletal development. Thus agents with a propensity to raise ALP expression have far greater potential for promoting bone tissue mineralisation to those molecules which up-regulate OC production. With the exception of calpeptin the other factors reported to provoke actin stress fiber accumulation acted with D₃ in stimulating MG63 differentiation. Thus any signalling event(s) accompanying stress fiber accumulation per se appeared unsupportive of osteoblast maturation. What seemed more likely was that the signalling incurred by microtubule disruption in some way connected with D₃ in generating the mature osteoblast phenotype. Credence is given to such a possibility because signalling molecules interact with microtubules, furthermore drugs that

influence microtubule stability are known to trigger and/or influence intracellular signalling cascades (Gundersen and Cook, 1999).

To ascertain whether the processes accompanying microtubule disruption cooperated with D3 in promoting increased ALP in osteoblasts we established two independent but complimentary approaches. In the first instance MG63 cells were treated with naturally occurring colchicine derivatives that have little or no impact on microtubule assembly; both colchicine (EIN) and thiocolchicoside occur in the tissues and seeds of the Autumn Crocus, *Colchicum autumnale*. Although these agents exhibit, at most, a trivial action on the cytoskeleton they are biologically active and have proven efficacy in treating, for example, inflammatory, fibrogenic and hepatic diseases (Chandrasekaran and Porkodi, 1994; Dvorak Z, et al., 2002). In parallel with these compounds, MG63 cells were pre-treated with taxol, a microtubule stabilising drug, prior to their co-treatment with D3 and colchicine or D3 and nocodazole.

Neither thiocolchicoside nor EIN co-operated with D3 in stimulating an increase in ALP expression in osteoblasts. Similarly the pre-treatment of cells with taxol prevented the ability of both nocodazole and colchicine to act in concert with D3 in generating a maturation response. Collectively these data suggest that events accompanying the actual process of microtubule disruption in some way provide the nexus from which D3 is able to bolster a differentiation response in human osteoblasts.

During the course of this study, it became clear that colchicine was capable of eliciting many diverse effects upon mammalian cell behaviour. Although colchicine is

widely recognised to prevent microtubule assembly there are many studies reporting how this microtubule-disrupting agent influences a variety of signalling events in mammalian cells (Table 1). With regard to the signalling capabilities of colchicine, there are certainly two areas that deserve special attention in the context of D₃ induced MG63 maturation. First is the study by Dong and colleagues (2000) who reported increased Smad transcriptional activity in response to colchicine. Transforming growth factor beta (TGF- β) is one of the important factors for bone formation and remodelling (Deng et al., 2008; Zhang et al., 2003). TGF- β signalling is initiated by TGF- β binding to the type II receptor, T β RII, this interaction leads to the transphosphorylation of the TGF- β type I receptor (T β RI). Phosphorylated T β RI in turn phosphorylates the Smad proteins that are in fact the transducers of the TGF- β signalling cascade. It has been known for many years that a combination of D₃ and TGF- β synergistically increases MG63 alkaline phosphatase activity (Bonewald et al., 1992). Consequently we examined whether colchicine could act as a TGF- β mimetic by activating Smads.

The second noteworthy property of colchicine is its influence on the mitogen activated protein kinase (MAPK) pathway; in particular the activation (phosphorylation) of extracellular signal regulated kinase (Erk). This laboratory has already demonstrated the importance of Erk activation in D₃ induced osteoblast differentiation; co-stimulation of MG63 cells with epidermal growth factor and D₃ (Yarram et al., 2004) and the treatment of these cells with a combination of D₃ and LPA promotes osteoblast differentiation (Gidley et al., 2006). In both instances the maturation response could be completely blocked using U0126, an inhibitor of mitogen activated protein kinase kinase (MEK) and therefore Erk phosphorylation.

Since colchicine is reported to stimulate Erk activation it was tempting to speculate that colchicine-mediated microtubule disassembly might induce the activation of the entire Ras-dependent cascade which in turn links up with D₃ in promoting MG63 maturation. Nocodazole, like colchicine impedes microtubule polymerisation (Jordan et al., 1992). It is also known that nocodazole activates the small G protein Rho and stimulates tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin and Ras signalling pathways (Vasquez et al., 1997; Wang et al., 1998).

In the first instance MG63 cells were pre-treated with an inhibitor of T β RI which selectively neutralises the kinase activity of this receptor protein. As expected the inhibitor was able to prevent the increase in MG63 ALP activity for cells co-treated with D₃ and TGF β (data not shown). When the inhibitor was applied to MG63 cells co-stimulated with either colchicine or nocodazole and D₃ the synergistic rise in ALP persisted. These findings indicated that microtubule disruption does not trigger a possible TGF β mimetic response via T β RI (data not shown). Osteoblasts were also probed for Smad2 phosphorylation in response to colchicine, nocodazole or TGF β treatment. Whereas TGF β led to Smad2 phosphorylation within 15 minutes there was no evidence for Smad2 phosphorylation in response to either colchicine or nocodazole. The final experiment in this particular series involved the application of a CAGA-12 Smad3 reporter assay. As anticipated the treatment of MG63 cells with TGF β led to a striking increase in luciferase activity. However the application of either nocodazole or colchicine failed to generate a response. Thus far the findings would suggest that microtubule disruption does not, albeit for MG63 cells, generate a TGF β mimetic event. Although there is evidence for raised Smad transcriptional activity in Mink lung epithelial cells experiencing microtubule disruption (Dong et

al., 2000) the conflicting findings for MG63 cells might be attributed to differences in adaptor and/or substrate molecules that participate in TGF β signalling.

In parallel with the studies aimed at investigating a potential TGF β signalling response, a series of experiments were set up to explore whether microtubule disruption might activate MAPK pathways. To this end MG63 cells were initially pre-treated with UO126, an inhibitor of MEK and resultant Erk phosphorylation. The inclusion of UO126 negated the influence of microtubule disruption and D₃ on MG63 differentiation. Thus colchicine and nocodazole were stimulating MEK activation. Both colchicine and nocodazole appeared to activate MEK at the level of Raf in MG63 cells; the application of GW5074 blocked the ability of either colchicine or nocodazole and D₃ on MG63 maturation whereas FTI-277, a Ras inhibitor, resulted in a modest, yet statistically significant, attenuation of the differentiation response. These findings are in support of those from Hayne and colleagues (2000) in which mitotic arrest to nocodazole requires Raf/MAPK activation. Similarly the data presented agree with other studies reporting Erk activation in response to colchicine (Samarakoon and Higgins, 2002; Schmid-Alliana et al., 1998). This laboratory has previously reported that MEK activation is key to the maturation of MG63 cells co-treated with LPA and D₃ (Gidley et al., 2006) and EGF and D₃ (Yarram et al., 2004). It now appears that both colchicine and nocodazole also activate MEK in MG63 cells.

Part of our studies tackled whether FAK might also be implicated in the differentiation events generated in response to either colchicine or LPA. It is becoming clear that FAK is a component of the cellular mechanosensory apparatus in that it is able to detect and respond to local forces (Tilghman and Parsons, 2008). In

order to ascertain whether FAK might be implicated in the maturation response of MG63 cells to D₃ and LPA/colchicine we used TAE226. This recently developed low molecular weight inhibitor is well characterised as preventing FAK activation via blockade of tyrosine phosphorylation (Halder et al., 2007; Shi et al., 2007). However the application of 500nM TAE226 did not inhibit MG63 maturation in response to LPA/ colchicine and D₃ (data not shown). Although Bershadsky and colleagues (1996) reported phosphorylation of FAK in response to LPA and nocodazole in serum starved Swiss 3T3 cells it appears that FAK does not participate in the differentiation response of MG63 cells to D₃ and cytoskeletal reorganising agents.

What remains unresolved is how does active MEK/Erk cooperate with D₃ in driving a maturation response in osteoblasts? One possible explanation for the synergy observed for ALP in response to D₃ and cytoskeletal reorganisation could be MEK dependent stimulation of activator protein-1 (AP-1). It is well known that the AP-1 family of transcription factors plays an important role in the development and maturation of osteoblasts (Wagner, 2002; Marie, 2008). With specific reference to the cytoskeleton and mechanotransduction it has emerged that the AP-1 component, c-Jun, is phosphorylated by colchicine-induced cytoskeletal reorganisation (Lee et al., 1993). Furthermore increased AP-1 binding activity in the promoter region of the human ALP gene has been reported for stretched periodontal ligament cells (Peverali et al., 2001). The application of 7AP-1-luc transfected osteoblasts revealed that cytoskeletal reorganisation precipitated by either colchicine or nocodazole does lead to activation of the AP-1 transcription factor complex. We hypothesise that the mechanism accounting for the synergistic increase in ALP activity in the models described is a consequence of two transcription factors acting at two different loci of

the ALP promoter. It is likely that agents with the capacity to cooperate in this way are likely to find an application in bone tissue repair and regeneration by fuelling the provision of a mechanically competent bone matrix at the implant site. In so doing the agent pairings will facilitate superior osseointegration with resultant improvements in orthopaedic implant longevity.

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Figure & table legends

Table 1. The impact of colchicine on mammalian cell physiology and signalling.

In addition to disrupting the assembly of microtubules and stimulating actin stress fiber accumulation it is becoming clear that colchicine is capable of activating and/or modulating diverse signalling systems in a variety of mammalian cells. Of particular relevance to osteoblast development and differentiation are the reports identifying colchicine as a stimulator of Smad transcriptional activity (Dong et al., 2000) and Erk activation (Schmid-Alliana et al. 1998; Samarakoon & Higgins 2003).

Figure 1. Not all cytoskeletal reorganising agents cooperate with D3 in

stimulating MG63 osteoblast maturation. To test the hypothesis that intracellular events accompanying stress fiber formation interact with D3 in supporting human osteoblast maturation, MG63 cells were treated with calpeptin, nocodazole and colchicine with D3. As expected the co-stimulation of osteoblasts with LPA and D3 led to a stark, synergistic increase in alkaline phosphatase (ALP) activity (* $p < 0.001$, Fig. 1A) compared to controls and for cells treated with either D3 or LPA alone. Similar results were obtained for cells co-treated with D3 and either colchicine (** $p < 0.001$) or nocodazole (§ $p < 0.001$). However cells treated with a combination of calpeptin and D3 did not undergo a maturation response. The large increases in ALP activity for cells co-treated with LPA and D3, colchicine and D3 and nocodazole and D3 are independent of cell number (Fig. 1B). All data are expressed as the mean $\pm$ the standard deviation.

Figure 2. Calpeptin, colchicine and nocodazole trigger actin stress fiber

accumulation in human osteoblasts. MG63 osteoblast-like cells were seeded into wells of chamber slides and left to attach and spread over a 24-hour period. After this period the media was removed and replaced with fresh serum-free culture medium either alone (A) or supplemented with 100ng/ml calpeptin (B), colchicine (C) or nocodazole (D). The cells were left for one hour, the media removed and the cells fixed and processed for F-actin detection using FITC-conjugated phalloidin. Scale bar: 20 μ M.

Figure 3. Co-treating human osteoblasts with D3 and microtubule disrupting

agents attenuates osteocalcin (OC) expression. The application of D3 led to the expected rise ($^*p<0.001$) in OC production by approximately 8 fold over vehicle control cultures. However, when MG63 cells were co-stimulated with D3 and LPA the level of OC expression was significantly ($^{**}p=0.001$) attenuated. Similarly the treatment of osteoblasts with D3 and either colchicine or nocodazole ($^{\$}p<0.005$) resulted in a decline in OC production. This significant reduction in OC could not be explained by diminished cellularity. In addition the co-treatment of MG63 cells with D3 and LPA/colchicine/nocodazole yielded the expected synergistic increase in ALP activity compared to all other treatment groups (results not shown).

Figure 4. Events accompanying microtubule disruption interact with D3 in

securing MG63 maturation. As expected the co-treatment of MG63 cells with colchicine and D3 led to a clear, synergistic increase in ALP activity ($^*p<0.001$, Fig. 4A) compared to controls and for cells treated with either D3 or colchicine alone. Both colchicine (EIN) and thiocolchicoside (Thio) are colchicine derivatives with

very little influence on microtubule assembly. When either of these molecules were added in conjunction with D3 they were unable to promote MG63 maturation. Neither EIN or Thio had an adverse effect on cell number (Fig. 4B). To further substantiate that the process of microtubule disruption is somehow linked with D3 in generating mature osteoblasts, MG63's were pre-treated with taxol (100ng/ml) for one hour prior to (and during) their stimulation with colchicine and D3. The data obtained clearly show how taxol pre-treatment blocks the ability of D3 and colchicine (* $p < 0.001$, Fig. 4C) or D3 and nocodazole (** $p < 0.001$, Fig. 4C) to elicit osteoblast maturation. Collectively the data support the events accompanying microtubule disruption as interacting with D3 in generating mature osteoblasts. All data are expressed as the mean $\pm$ the standard deviation.

Figure 5. Osteoblast maturation in response to D3 and colchicine is dependent upon the Ras-Raf-Mek pathway. There are reports that colchicine can activate Mek, a kinase known to participate in osteoblast maturation. To ascertain if the increase observed for ALP activity following colchicine and D3 might be attributed, albeit in part, to Mek and upstream kinases Ras and Raf, MG63 osteoblasts were treated with colchicine and D3 in the presence of UO126, FTI-277 and GW5074 respectively. The inclusion of UO126 inhibited the maturation response (* $p < 0.001$, Fig. 5A) of cells stimulated with colchicine and D3. Exposure of MG63's to the Ras inhibitor, FTI-277, resulted in a modest, yet significant (* $p < 0.005$, Fig. 5B) reduction in ALP activity and therefore cellular maturation. Inhibition of Raf with GW5074 resulted in a marked suppression of MG63 maturation to D3 and colchicine (* $p < 0.001$, Fig. 5C). The clear reduction in ALP activity for cells treated with the kinase inhibitors was not

attributed to any adverse effects on cell number (Fig. 5D). All data are expressed as the mean $\pm$ the standard deviation.

Figure 6. Cytoskeletal reorganising agents do not activate Smad 2 and Smad 3 in MG63 osteoblasts. Osteoblasts were seeded into 60mm dishes for the subsequent detection of phosphorylated Smad2 by western blot (Fig. 6A). As expected MG63 cells treated with 1ng/ml TGF β for the duration of the experiment generated active, phosphorylated Smad 2. However cells treated with either colchicine or nocodazole (100ng/ml) did not produce phosphorylated Smad 2. In a parallel set of experiments MG63 cells were transfected with a CAGA₁₂-luc reporter construct to assess whether cytoskeletal reorganising agents might activate Smad 3 (Fig. 6B). As anticipated the treatment of cells with 1ng/ml TGF β generated a clear increase in Smad 3 activation over control cultures. However cells treated with either nocodazole or colchicine failed to generate a response. For both sets of data the figures presented are a representative from three independent sets of experiments.

Figure 7. Nocodazole and colchicine activate activator protein-1 (AP-1) in human osteoblasts. To ascertain whether cytoskeletal reorganising agents might influence AP-1 activity in MG63 osteoblasts these cells were transfected with an AP-1-luc reporter construct prior to a 16-hour stimulation with either colchicine or nocodazole (100ng/ml). For a positive control MG63 cells were stimulated with phorbol myristate acetate (PMA 50ng/ml). As expected PMA treated cells displayed a clear increase in AP-1 activity relative to control cultures. The data also indicate that MG63 cells respond to both colchicine and nocodazole by increasing AP-1 activation. The data presented is a representative from three independent sets of experiments.

Table 1.

Cell type	Colchicine dose used	End point(s)	Reference
Human monocyte derived macrophages	50-500 ng/ml	Increased expression of PDGF-B mRNA & protein	Wangoo et al., 1992
Human monocytes (PBMC)	1 μ M-10 μ M	IL-1 expression increased, PKA stimulation	Manie et al., 1993
Human fibroblasts	500nM-10 μ M	Increased expression of the urokinase plasminogen activator receptor	Bayraktutan and Jones 1995
Mink lung epithelial cells	10-100 μ M	Increased Smad transcriptional activity	Dong et al., 2000
Rat cardiac myocytes	1 μ M	Reversible enhancement of calcium signalling	Kerfant et al., 2001
A549 (human lung cancer cell line)	1 μ M	HIF-1 α protein stabilisation via NF κ B dependent pathway	Jung et al., 2003
R22 smooth muscle cells	10 μ M	Increased expression of PAI-1 – MEK dependent	Samarakoon and Higgins 2002
Murine kidney fibroblasts	1 μ M	Increased expression of CTGF	Graness et al., 2006
Rat pup cortical neurons	10-1000nM	Apoptosis via p38 MAPK and JNK MAPK activation	Yang et al., 2007
Human monocytes & THP1 cells	100nM - 1 μ M	IL-1 induced via activation of Erk and Src kinases	Schmid-Alliana et al., 1998

Fig. 1A

Fig. 1B

Fig. 2

Fig. 3

Fig. 4A

Fig. 4B

Fig. 4C

Fig. 4D

Fig. 5A

Fig. 5B

Fig. 5C

Fig. 5D

Fig. 6A **Fig. 6B**

Fig 7.