Thyroid hormone stimulation of extracellular signal-regulated kinase and cell proliferation in human osteoblast-like cells is initiated at integrin $\alpha_V\beta_3$

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Abstract

The aim of the present study was to examine whether triiodo-L-thyronine (T_3) or L-thyroxine (T_4) rapidly activated the mitogen-activated protein kinase (MAPK) intracellular signalling cascade in osteoblast-like cells and investigate whether this activation was initiated at the integrin $\alpha_V\beta_3$ cell surface receptor. Using PCR and western blotting, the expression of integrin $\alpha_V\beta_3$ mRNA and protein was demonstrated in the human osteoblast-like cell lines MG-63 and SaOS-2. The treatment of MG-63 cells with T_3 (10 nM) or T_4 (100 nM) for 10 min stimulated extracellular signal-regulated kinase activity (ERK, a component of the MAPK pathway) as determined by fluorescent immunocytochemistry and an immunocomplex activity assay (T_3 by 10·7-fold, $P<0·01$ and T_4 by 10·4-fold, $P<0·01$ compared with control). T_3 (10 nM) and T_4 (100 nM) also significantly stimulated thymidine incorporation into MG-63 cells by $2·3\pm0·7$-fold ($P<0·01$) and $2·1\pm0·1$-fold ($P<0·05$) respectively. To establish whether transient ERK activation via the integrin $\alpha_V\beta_3$ cell surface receptor mediated these effects, MG-63 cells were pretreated for 30 min with the specific MAPK kinase inhibitor, U0126 (1 μM), or an anti-integrin $\alpha_V\beta_3$-blocking antibody. Both pretreatments significantly inhibited T_3- and T_4-stimulated ERK activation and abolished T_3-stimulated thymidine incorporation ($P<0·01$). T_4-stimulated incorporation was significantly inhibited from 2·1- to 1·3-fold above control ($P<0·05$). Thus, our results suggest that T_3 and T_4 rapidly stimulate ERK activation in MG-63 cells via integrin $\alpha_V\beta_3$ and that one functional effect of this ERK activation is increased DNA synthesis.

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Introduction

Non-genomic, receptor-independent actions of members of the steroid hormone family are well recognised. For example, triiodo-L-thyronine (T_3) and L-thyroxine (T_4) are reported to rapidly activate intracellular signalling pathways, such as mitogen-activated protein kinase (MAPK; Lin *et al.* 1999, Bergh *et al.* 2005, Mousa *et al.* 2006), independent of the thyroid hormone receptor (TR). A number of molecular mechanisms underlying the non-genomic activation of signalling cascades by thyroid hormone have been reported (reviewed in Davis *et al.* 2005) with one proposed mechanism involving the existence of plasma membrane-binding sites for thyroid hormone. Evidence that the heterodimeric structural proteins, integrins, might contain a cell surface receptor site for thyroid hormones was initially obtained from a study showing T_4-dependent regulation of integrin–laminin interactions (Farwell *et al.* 1995). This study also reported that a RGD (Arg-Gly-Asp) peptide interfered with these interactions. Later studies showed that purified integrin $\alpha_V\beta_3$ protein bound radiolabelled T_4 and that this binding was again blocked by the RGD peptide and integrin antibodies (Bergh *et al.* 2005), thus suggesting that the receptor site was located at or near the RGD recognition site on integrin $\alpha_V\beta_3$. The functional consequences of thyroid hormone binding to a cell surface site were subsequently illustrated by the same authors with knock-down experiments using small interfering RNAs directed against the genes encoding the α_V or β_3 subunits, which prevented thyroid hormone activation of MAPK in CV-1 cells. An alternative approach used a specific integrin $\alpha_V\beta_3$ antagonist to inhibit the activation of MAPK and proangiogenic actions of T_4 in human microvascular endothelial cells (Mousa *et al.* 2006). Thus, increasing evidence would suggest that the extracellular domain of integrin $\alpha_V\beta_3$ can act as a thyroid hormone cell surface receptor and initiate rapid activation of MAPK signal transduction cascades in human cell lines.

Several *in vitro* studies have demonstrated direct effects of thyroid hormones on cells of the osteoblast lineage. In response to T_3, osteoblasts are able to increase DNA synthesis and alkaline phosphatase activity (Sato *et al.* 1987), osteocalcin production (Ohishi *et al.* 1994) and cytokine synthesis (Siddiqi *et al.* 1998). While the expression of TR isoforms has been documented in both rodent (Williams *et al.* 1994) and human (Siddiqi *et al.* 2002) osteoblast cell lines and primary cultures of

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rat (Bland *et al.* 1997) and human (Siddiqi *et al.* 2002) osteoblasts, there is only one report from more than a decade ago, which has provided evidence for non-genomic effects of T_3 on bone (Lakatos & Stern 1991). More recently, Hoffman *et al.* (2002) reported that the RGD peptide blocked bone demineralisation in rats induced by thyroid hormone raising the possibility that the integrin $\alpha_V\beta_3$ protein might be the initiation site for non-genomic actions of thyroid hormone on osteoblasts. The aim of our study therefore was to examine whether T_3 or T_4 were able to rapidly activate the MAPK intracellular signalling cascade and investigate whether this activation was initiated at the integrin $\alpha_V\beta_3$ cell surface receptor.

Materials and Methods

Materials

Epidermal growth factor (EGF) was purchased from CN Biosciences (Nottingham, UK), T_3 and T_4 were purchased from Sigma–Aldrich Company Ltd. The specific MAPK kinase (MEK) inhibitor, U0126, was obtained from Promega and used at 1 μM, a concentration previously reported to produce almost 100% suppression of MAPK kinase 1 (also known as MKK1 or MEK1 (Davies *et al.* 2000)). All the above components were prepared as stock solutions in sterile water and applied to the cells in culture media. Specific goat polyclonal antibodies to the α_V (SC-6618) and β_3 subunits (SC-6627) with their respective blocking peptides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and a monoclonal blocking antibody to $\alpha_V\beta_3$ (ab20143) was obtained from Abcam (Cambridge, Cambs, UK). Horseradish peroxidase (HRP)-conjugated donkey anti-goat Ig and HRP-conjugated rabbit anti-mouse Ig were purchased from Dako (UK) Ltd (Ely, Cambs, UK).

Cell culture

The human osteoblasts (hOb)-like cell lines, MG-63 and SaOS-2 (both kindly donated by F J Hughes, School of Dentistry, Queen Mary, University of London, UK), and human dermal fibroblasts (kindly donated by L E Russell, Centre of Dermatology, Queen Mary, University of London, UK) were maintained in αMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 1000 U/l penicillin and 1 mg/l streptomycin (hereafter referred to as medium). All culture reagents were purchased from Invitrogen Life Technologies Inc. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. The medium was changed every 3 days, and the cell lines were passaged at 70% confluence.

Reverse transcription and real-time PCR

Total RNA was extracted from cells using the RNeasy kit from Qiagen according to the manufacturer's instructions. Three hundred nanograms of total RNA were transcribed using the First Strand DNA synthesis kit (Amersham Biosciences) according to the manufacturer's instructions. Reverse transcription was also performed on diethylpyrocarbonate (DEPC)-treated water for use as a negative control (NC) in subsequent PCRs. The presence of integrin α_V and β_3 subunits and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in the samples was confirmed by PCR. The primer sequences used in the PCR were based on published human-specific sequences: α_V (NM_002210): forward 5′-CACCAGCAGT-CAGAGATGGA, reverse 5′-GGCAACCGTGTCATT-CTTTT (441 bp product); β_3 (NM_000212): forward 5′-AAGGATAACTGTGCCCCAGA, reverse 5′-CACAG-GCTTGTCCACAAATG (348 bp product); GAPDH (AF261085): forward 5′-TGCACCACCAACTGCTTAG, reverse 5′-CACCACAATGTTGTCGTAG (523 bp product). After a 3-min incubation at 95 °C, the PCR was run for 35 cycles under the following conditions: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, followed by a final extension step at 72 °C for 10 min. The PCR products were visualised on a 1·4% (wt/vol) agarose gel stained with ethidium bromide. The identity of each PCR product generated was confirmed by direct DNA sequencing.

Total protein extraction and western blotting

Total proteins were harvested from dermal fibroblasts, MG-63 and SaOS-2 cells, subjected to SDS-PAGE (on a 10% (w/v) resolving gel) and transferred to nitrocellulose membranes as described previously (Fowkes *et al.* 2003). After blocking for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline containing 0·1% (v/v) Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with the indicated primary antibodies (in the presence or absence of blocking peptides), washed and incubated for 1 h at room temperature (RT) with the appropriate secondary antibodies. Immunoreactive protein was detected using enhanced chemiluminescence (ECL Western Blotting Reagents, Amersham Bioscience).

Immunocytochemistry

Specific rabbit polyclonal anti-phospho-extracellular signal-regulated kinase (pERK) antibody (V803A) was obtained from Promega. MG-63 cells were plated at a density of 2×10^5 cells onto sterile coverslips in 35-mm culture dishes and allowed to settle overnight in medium, followed by incubation for 24 h in serum-free αMEM. Subsequently, the cells were pretreated for 30 min in the presence or absence of 1 μM U0126. The cells were then treated with either no stimulant (NC), T_3 (10 nm) or EGF (10 nM), as a positive control, for 10 min before being fixed in 4% (v/v) paraformaldehyde for 30 min. Ice-cold methanol was then applied to the fixed cells for 10 min to permeabilise them. The coverslips were then incubated in blocking buffer (1% (w/v) BSA and 5% (v/v) donkey serum in PBS) for 2 h, washed in PBS and incubated overnight in anti-pERK antibody (Promega) diluted 1:500. Control cells were

processed in the absence of primary antibody. After three 15-min washes in PBS, the cells were incubated for 1 h in donkey anti-rabbit Cy3 secondary antibody (Vector Laboratories Ltd, Peterborough, Lincs, UK) before mounting the coverslips onto microscope slides with Fluoromount-G (Cambridge Bioscience Ltd, Cambridge, UK) and viewing with a fluorescent microscope.

Total protein extractions and measurement of ERK activity

MG-63 cells were plated in 35-mm dishes (2 million cells per dish) and allowed to grow overnight in culture medium containing 10% (v/v) FCS, which was then changed to serum-free medium for 24 h. Subsequently, the cells were pretreated for 30 min in the presence or absence of 1 μM U0126 or a monoclonal blocking antibody to $\alpha_V\beta_3$ (diluted 1:500). Following this pretreatment period, the cells were treated for 10 min with serum-free medium alone, 10 nM T_3 or 100 nM T_4, in the continued presence or absence of 1 μM U0126 or the monoclonal blocking antibody to $\alpha_V\beta_3$. Total proteins were then extracted in the presence of phosphatase inhibitors as described previously and ERK activity was measured using the p44/42 MAP Kinase Assay kit (New England Biolabs, Hitchin, UK) as described previously (Fowkes *et al.* 2001). Briefly, active MAPK was immunoprecipitated from cell lysates with an immobilised phospho-p44/42 MAP kinase (ERK; Thr202/Tyr204) monoclonal antibody. The immunoprecipitated pellets were incubated with 200 μM ATP and 2 μg Elk-1 fusion protein, before terminating the reaction with SDS loading buffer. The levels of phosphorylated Elk-1 were analysed by immunoblotting with anti-phospho-Elk-1 antibody. Immunoreactive protein was visualised by enhanced chemiluminescence, using the supplied LumiGLO and peroxidase reagents. Autoradiographs were analysed by scanning densitometry using Gel Base/Gel Blot Pro software (Synoptics Ltd, Cambridge, UK). Peak height intensities were used to calculate ERK phosphorylation of Elk-1, and compared with the untreated controls.

[^{3}H]thymidine incorporation

MG-63 cells were plated in 24-well cell culture dishes (10 000 cells/dish) and allowed to grow overnight in media containing 10% FCS, which was then changed to serum-free medium for 24 h. Subsequently, cells were treated with serum-free medium alone, T_3 (1, 10, 100 nM), T_4 (1, 10, 100 nM) or medium containing 10% (v/v) FCS as a positive control for 18 h before the addition of 1 μCi/well of [^{3}H]thymidine (Amersham Bioscience) for a further 6 h. Cells were trypsinised and harvested, before counting in the presence of scintillation fluid using a MicroBeta 1450 β-counter (Wallac, Beaconsfield, Bucks, UK). In later experiments, the cells were pretreated for 30 min in the presence or absence of 1 μM U0126 or a monoclonal blocking antibody to $\alpha_V\beta_3$ (diluted 1:500) prior to treatment with serum-free medium alone, T_3 (10 nM), T_4 (100 nM) or medium containing 10% FCS as a positive control in the continued presence or absence of U0126 or the blocking antibody to $\alpha_V\beta_3$ for 18 h before the addition of 1 μCi/well of [^{3}H]thymidine (Amersham Bioscience) for a further 6 h.

Data presentation and statistical analysis

All graphical data were prepared using GraphPad Prism 4.03 (GraphPad, San Diego, CA, USA) and analysed using pre-programmed analysis equations within Prism. Data are presented as results which are representative of several experiments or as normalised data pooled from multiple experiments. Where appropriate, an ANOVA was performed on data followed by Tukey's multiple comparisons test, accepting $P<0{\cdot}05$ as significant.

Results

Expression of integrin α_V and β_3 subunits in MG-63 and SaOS-2 cells

To investigate the presence of α_V and β_3 subunit mRNAs in these human osteoblast-like cell lines, RT-PCR was performed on MG-63 and SaOS-2 cDNA using specific intron-spanning primers designed to amplify the different subunits. After 30 cycles, DNA fragments of the expected size were detected for integrin α_V (441 bp) and integrin β_3 (348 bp) in both cell lines (Fig. 1A). As reported previously (Asano *et al.* 2005), both subunits were also detected in the positive control, dermal fibroblast cells (Fig. 1A). When NCs were performed by omitting reverse transcriptase from the RT reaction no PCR products were detected (data not shown). RT-PCR of GAPDH (expected product size, 523 bp) was also performed to confirm loading of cDNA from each cell type (Fig. 1B). Sequencing of the DNA fragments confirmed product identity in all cases. Western blotting with integrin receptor subunit-specific antibodies (Fig. 2) confirmed the presence of proteins of the expected size for integrin α_V (125–135 kDa) and integrin β_3 (125 kDa) in both cell lines and in the positive control, dermal fibroblasts. Western blotting of dermal fibroblast cell lysates in the presence of specific integrin α_V or integrin β_3 blocking peptides confirmed the identity of the protein.

Thyroid hormones stimulate pERK in MG-63 cells

In preliminary studies, we used fluorescent immunocytochemistry with a specific antibody to pERK to investigate the effects of T_3 on MG-63 cells. MG-63 cells were treated for 10 min with either serum-free media alone, T_3 (10 nM) or EGF (10 nM) as a positive control. To demonstrate the specificity of these effects, MG-63 cells were also pretreated for 30 min with U0126 (1 μM), a MEK inhibitor, and then for 10 min with either serum-free media alone, T_3 (10 nM) or EGF (10 nM) in the continued presence of U0126. Following

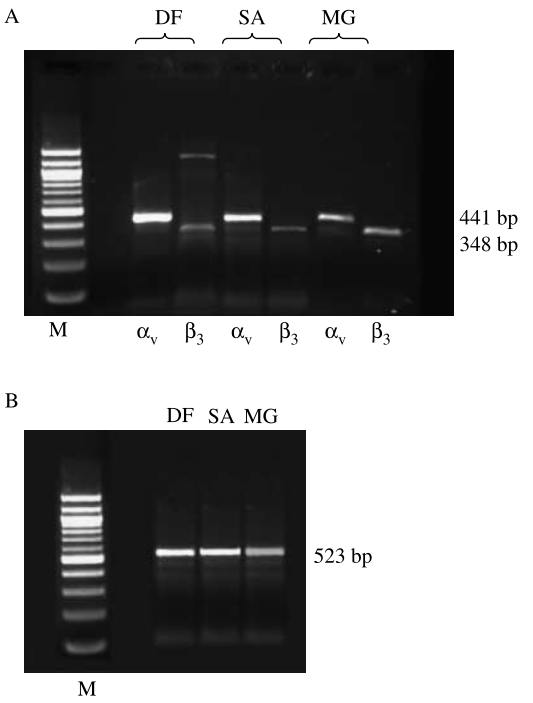

Figure 1 The presence of integrin α_V and β_3 mRNA in human osteoblast-like cell lines. (A) Amplification of cDNA for integrin α_V and integrin β_3 subunits by PCR in dermal fibroblasts (DF), SaOS-2 cells (SA) and MG-63 cells (MG). (B) Amplification of cDNA for the reference housekeeping gene GAPDH in dermal fibroblasts (DF), SaOS-2 cells (SA) and MG-63 cells (MG). M is the DNA size marker.

treatment, the cells were permeabilised, fixed and incubated overnight with rabbit anti-pERK antibody. The cells were then incubated with rhodamine-labelled donkey anti-rabbit Cy3-conjugated antibody. They were subsequently examined using a fluorescent microscope at 100× magnification. As shown in Fig. 3, both T_3- and EGF-stimulated the phosphorylation of ERK in MG-63 cells following 10 min incubation (Fig. 3B and C respectively). The specific MEK inhibitor, U0126, was able to inhibit these stimulatory effects (Fig. 3E and F). There was no staining visible on control slides processed in the absence of primary antibody (Fig. 3, NC).

Subsequent quantitative studies were performed with an immunoprecipitation kinase activity assay. MG-63 cells were pretreated for 30 min with serum-free media alone in the presence or absence of U0126 (1 μM) or anti-integrin $\alpha_V\beta_3$ antibody (1:500 dilution). Subsequently, the cells were treated for 10 min with serum-free media alone, T_3 (10 nM) or T_4 (100 nM) in the continued presence or absence of U0126 (1 μM) or anti-integrin $\alpha_V\beta_3$ antibody (1:500 dilution). The presence of activated ERK was quantitated by its ability to phosphorylate Elk-1, which was detected by western blotting with phospho-Elk-1 antibody and scanning densitometry. The activation of ERK at 10 min was enhanced by T_3 (10·7-fold, $P<0·01$) and T_4 (10·4-fold, $P<0·01$) compared with the control (Fig. 4). T_3-stimulated ERK activation was completely blocked by pretreatment with U0126 (1·1-fold above control, $P<0·01$ compared with T_3 stimulation alone) and partially inhibited by pretreatment with anti-integrin $\alpha_V\beta_3$ antibody (5·2-fold above control, $P<0·05$ compared with T_3 stimulation alone; Fig. 4). T_4-stimulated ERK activation was also inhibited following pretreatment with U0126 (3·1-fold above control, $P<0·01$ compared with T_4 stimulation alone) and showed a partial reduction to 7·3-fold above control with anti-integrin $\alpha_V\beta_3$ antibody, although this decrease did not achieve statistical significance.

Thyroid hormones stimulate thymidine incorporation in MG-63 cells

Previous studies have reported that T_3 can stimulate DNA synthesis in MG-63 cells (5) but we could find no published reports for effects of T_4 on DNA incorporation in osteoblast-like cells. Thus, our initial studies investigated dose–response effects of T_3 and T_4 on MG-63 cells over a 24-h time period. Both 10 and 100 nM T_3 significantly stimulated thymidine incorporation (by 1·8- and 1·9-fold respectively, $P<0·001$; Fig. 5A). The positive control (10% serum) increased thymidine incorporation by 2·6-fold ($P<0·001$). T_4 significantly increased thymidine incorporation at all three concentrations tested (by 1·3-fold at 1 nM, $P<0·01$; by 1·6-fold at 10 nM, $P<0·001$ and by 1·5-fold at 100 nM, $P<0·01$; Fig. 5B). Subsequent experiments using T_3 at 10 nM and T_4 at 100 nM were performed in the presence or absence of U0126 (1 μM) or anti-integrin $\alpha_V\beta_3$ antibody (1:500 dilution; Fig. 6). Again, T_3 and T_4 significantly stimulated DNA synthesis by 2·3-fold ($P<0·01$, compared with control) and 2·1-fold ($P<0·05$, compared with control) respectively. Neither U0126 nor anti-integrin $\alpha_V\beta_3$ antibody affected basal thymidine incorporation. Treatment of MG-63 cells with U0126 blocked the effects of T_3 entirely (to 0·95-fold compared with control, $P<0·01$ compared with T_3 stimulation alone) and significantly attenuated the effects of T_4 (to 1·3-fold above control, $P<0·05$ compared with T_4 stimulation alone), suggesting that ERK activation is involved in mediating the effects of both T_3 and T_4 on DNA synthesis in MG-63 cells. The effects of the positive control were also significantly reduced by U0126, suggesting that serum-stimulated thymidine incorporation in MG-63 cells is partially mediated by ERK activation. Treatment of MG-63 cells with anti-integrin $\alpha_V\beta_3$ antibody also blocked the effects of T_3 (to 0·96-fold compared with control, $P<0·01$ compared with T_3 stimulation alone) and significantly reduced the effects of T_4 (to 1·5-fold above control, $P<0·05$ compared with T_4 stimulation alone). As expected, the serum-positive control was unaffected by anti-integrin $\alpha_V\beta_3$ antibody. These results suggest that blocking the integrin $\alpha_V\beta_3$ cell surface receptor inhibits the effects of T_3 and T_4 on thymidine incorporation in MG-63 cells, thus supporting a role for integrin $\alpha_V\beta_3$ as the initiation site for T_3- and T_4-induced ERK activation and cell proliferation in human osteoblast-like cells.

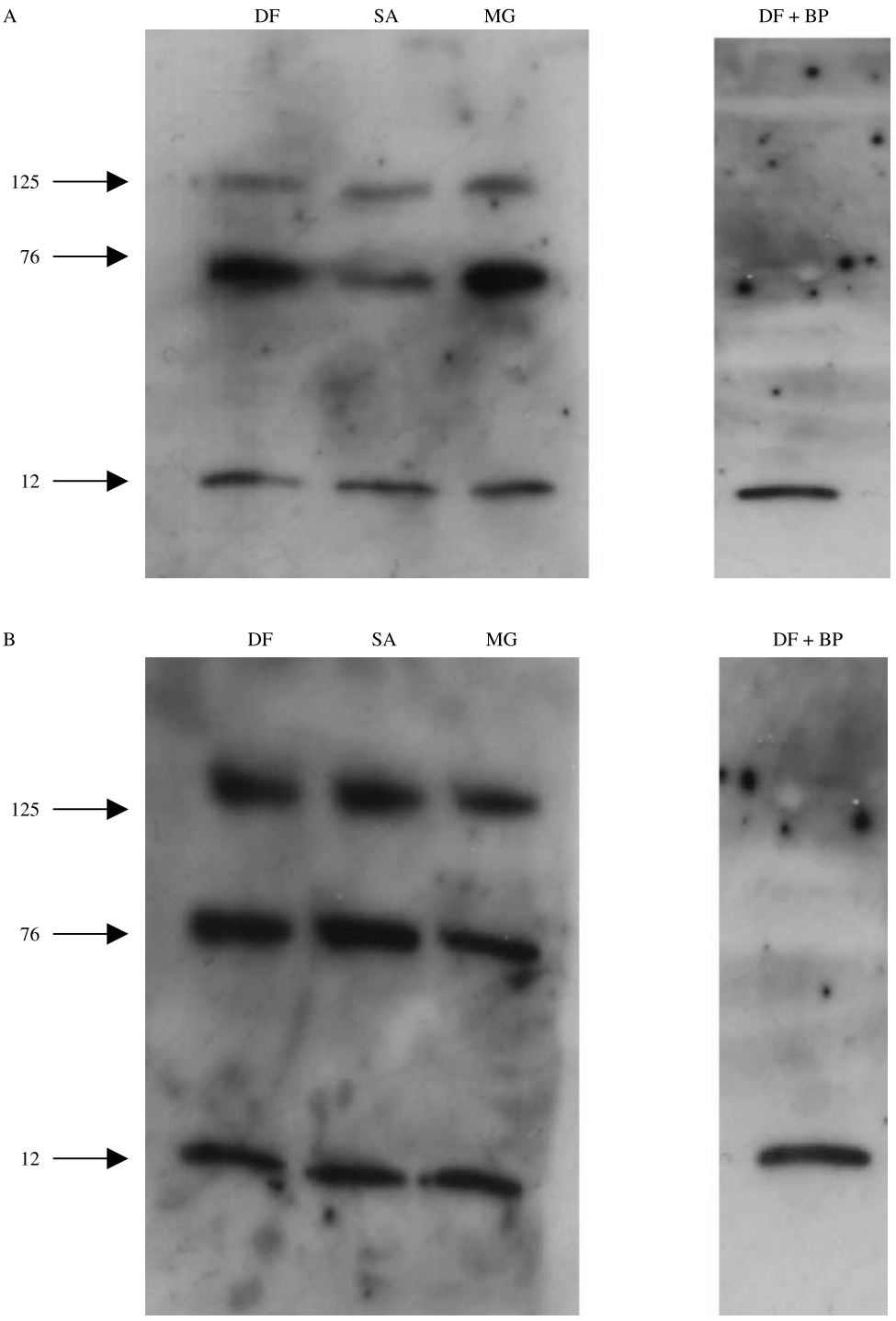

Figure 2 Expression of integrin α_V and β_3 proteins in human osteoblast-like cell lines. Whole-cell lysates from dermal fibroblasts (DF), MG-63 cells (MG) and SaOs-2 cells (SA) were electrophoresed through a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-α_V (A) or anti-β_3 (B) antibodies. The blots shown are representative of three independent experiments. Western blots of dermal fibroblast cell lysates were also performed in the presence of blocking peptides to integrin α_V or β_3 (DF + BP).

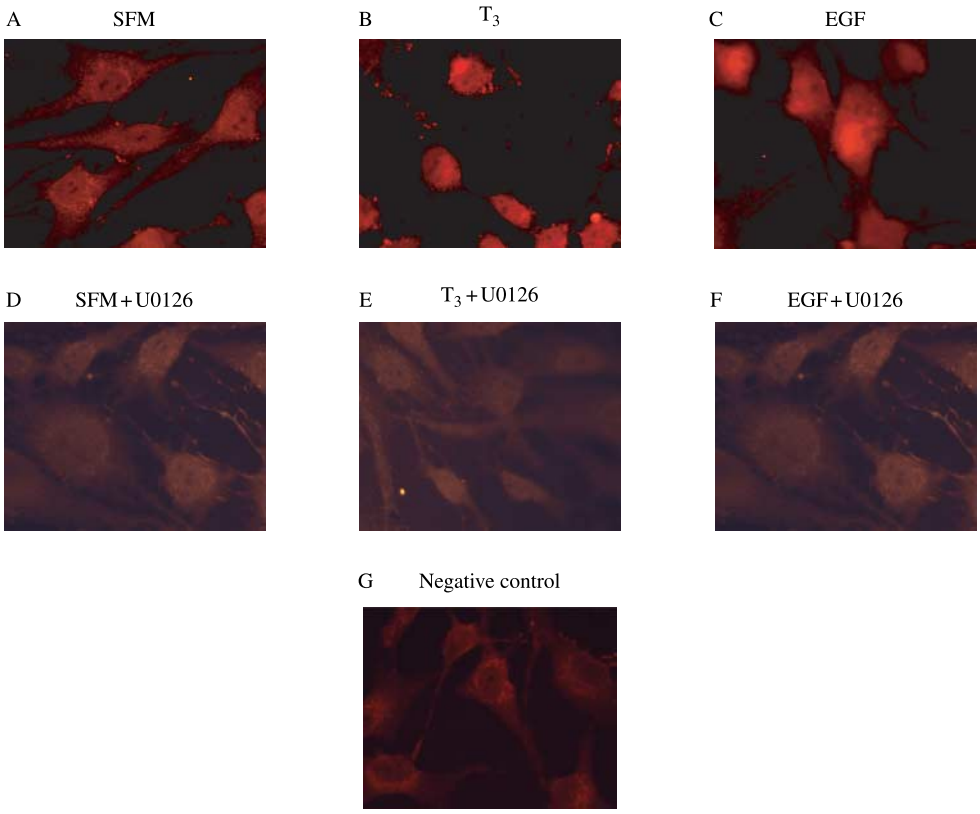

Figure 3 Fluorescent immunocytochemistry to demonstrate the effect of T_3 on pERK in MG-63 cells. MG-63 cells were treated for 10 min with either (A) serum-free media alone (SFM), (B) T_3 (10 nM; T_3) or (C) EGF (10 nM; EGF) as a positive control. MG-63 cells were pretreated for 30 min with U0126 (1 μM), a MEK inhibitor and then for 10 min with either (D) serum-free media alone (SFM+U0126), (E) T_3 (10 nM; T_3+U0126) or (F) EGF (10 nM; EGF+U0126). Following treatment, the cells were stained for pERK and subsequently examined using a fluorescent microscope at 100× magnification. Negative control (G) was processed in the absence of primary antibody.

Discussion

The results reported here provide novel evidence that T_3 and T_4 stimulate ERK activation in human osteoblast-like cells as revealed by fluorescent immunocytochemistry for pERK or by phosphorylation of the ERK-regulated transcription factor, Elk-1. These findings are consistent with previous studies in HeLa cells, CV-1 monkey fibroblasts and chick embryo hepatocytes that have demonstrated the ability of thyroid hormones to activate the MAPK signalling cascade and promote the phosphorylation of ERK within 5–20 min after treatment (Lin *et al.* 1999, Alisi *et al.* 2004, Bergh *et al.* 2005). We used concentrations of T_3 and T_4 that were reported previously to produce the maximal ERK activation response in other cell types. The concentration of T_3 needed (10 nM) to achieve ERK activation is in the supraphysiological circulating range (lower concentrations failed to achieve a significant effect (data not shown)), while that of T_4 (100 nM) is within the physiological circulating range. The MAPK signalling pathway has been demonstrated previously in normal human osteoblasts and bone marrow stromal cells (Chaudhary & Avioli 1998) but activation of this pathway in bone cells is generally reported to involve growth factor receptors such as platelet-derived growth factor or fibroblast growth factor (FGF). A 6-h pretreatment with T_3 was shown to enhance FGF2-stimulated MAPK (Stevens *et al.* 2003) although these authors failed to show a stimulation of ERK phosphorylation with T_3 alone. However, these studies were performed in rat osteoblast cell lines and used only T_3 (and not T_4) at a very high concentration of 100 nM for 30 min. Thus, there may be species differences in response, or as reported previously (Lin *et al.* 1999, Alisi *et al.* 2004, Bergh *et al.* 2005), thyroid hormone effects on ERK activation are rapid and transient with activation returning to control levels by 30 min.

The early reports of rapid thyroid hormone activation of ERK in HeLa and CV-1 cells (which lack functional TRs) suggested that this effect of T_3 and T_4 was cell surface initiated and not mediated via TRs. The existence of binding sites for thyroid hormone on the cell surface has been known for many years in the red blood cell membrane (Yoshida & Davis 1981) and in the synaptosome (Giguere *et al.* 1996) and indeed, the

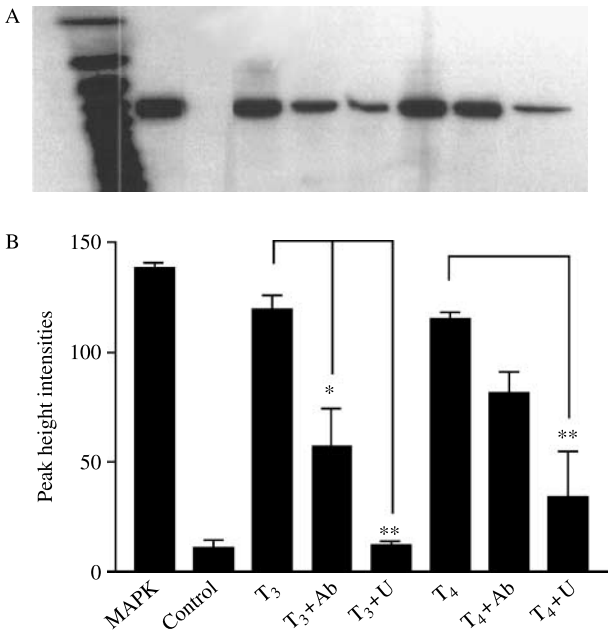

Figure 4 Elk-1 phosphorylation in MG-63 cells by T_3 and T_4. MG-63 cells were pretreated for 30 min with serum-free media alone or U0126 (1 μM) or anti-integrin $\alpha_V\beta_3$ antibody (1:500) and then treated for 10 min with serum-free media, T_3 (10 nM) or T_4 (100 nM) in the continued presence or absence of U0126 (U) or anti-integrin $\alpha_V\beta_3$ antibody (Ab). ERK activation was determined by an immunoprecipitation kinase assay. An autoradiograph of pElk-1 is shown (upper panel) with accompanying scanning densitometry data from three independent experiments (lower panel). *$P<0{\cdot}05$, **$P<0{\cdot}01$ compared with T_3- or T_4-treated cells. A positive control for the kinase assay (MAPK) is also shown where active MAPK (20 ng) was added to untreated cell extract.

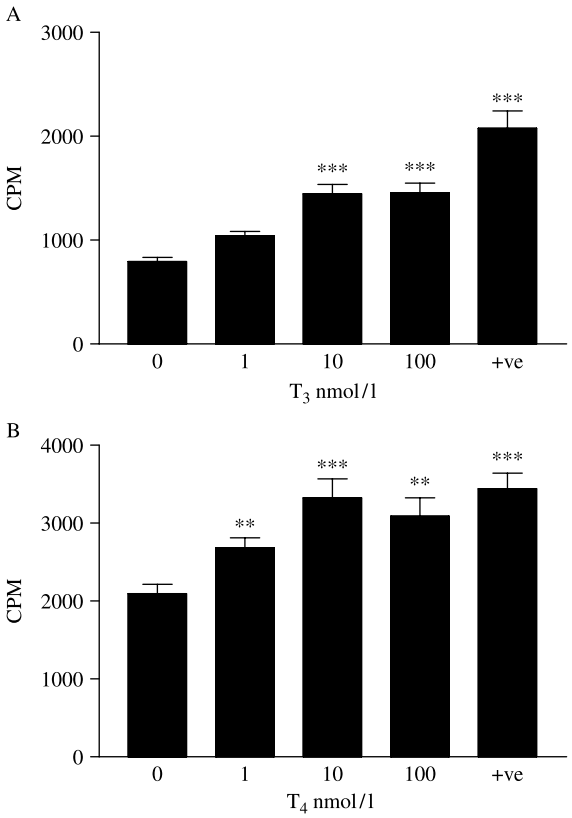

Figure 5 Dose–response effects of T_3 and T_4 on [^{3}H]thymidine incorporation into MG-63 cells. Cells were treated with either serum-free media alone (0), T_3 (1, 10 or 100 nM) or T_4 (1, 10 or 100 nM) for 24 h. A medium containing 10% FCS was used as the positive (+ve) control. [^{3}H]thymidine (1 μCi/well) was added for the last 6 h of culture. Results are expressed as c.p.m. and show means ± S.E.M. of at least 12 individual incubations (**$P<0{\cdot}01$, ***$P<0{\cdot}001$).

existence of plasma membrane-associated receptors for T_3 in bone was proposed in 1991 (Lakatos & Stern 1991). However, it was not until recently that the identity of one cell surface protein capable of initiating non-genomic actions of thyroid hormone was revealed as integrin $\alpha_V\beta_3$ (Bergh *et al.* 2005, Davis *et al.* 2006, Mousa *et al.* 2006). Evidence supporting this concept was provided by the stimulatory effects of thyroid hormone on angiogenesis and MAPK activation being inhibited either in the presence of small interfering RNAs directed against the genes encoding the α_V or β_3 subunits or with the use of a specific integrin $\alpha_V\beta_3$ antagonist or the RGD peptide (Bergh *et al.* 2005, Davis *et al.* 2006, Mousa *et al.* 2006). The presence of integrin $\alpha_V\beta_3$ in human osteoblasts has not been reported, thus as an initial experiment we examined the expression levels of α_V and β_3 subunits in both MG-63 and SaOs-2 cells. We found that α_V and β_3 subunit mRNA and protein were expressed in both the cell types. To function as active receptors, integrins have to be present on the cell surface as dimers, which dissociate when cell lysates are examined by western blotting. Therefore, to examine whether heterodimeric integrin $\alpha_V\beta_3$ was mediating the rapid thyroid hormone activation of ERK in osteoblasts, we used a blocking antibody to the dimer $\alpha_V\beta_3$. Integrin $\alpha_V\beta_3$ is also known to function as an active receptor for latent transforming growth factor-β (TGF-β) and this anti-$\alpha_V\beta_3$ antibody has been used previously to reduce the transcriptional effects of TGF-β in scleroderma fibroblasts (Asano *et al.* 2005). The attenuating effects of the anti-$\alpha_V\beta_3$ antibody were greater on T_3 than T_4-stimulated ERK activation. This was also the case when cells were treated with the specific MEK inhibitor, U0126, and might suggest that molecular mechanisms other than those initiated at the integrin $\alpha_V\beta_3$ receptor exist for T_4 action at the cell surface as proposed by Davis *et al.* (2005). In other cell types, activation of the integrin $\alpha_V\beta_3$ receptor is reported to activate the MAPK pathway via protein kinase C (PKC; Alisi *et al.* 2004) and we have preliminary data using a specific PKC inhibitor (data not shown) that would suggest that this is also the case in osteoblast cells.

Both T_3 and T_4 are reported to influence proliferation in a number of different cell types (Garcia-Silva *et al.* 2002, Alisi *et al.* 2004, Davis *et al.* 2006). T_3 has been found to stimulate, inhibit or exert no effect on osteoblastic cell proliferation but a consensus

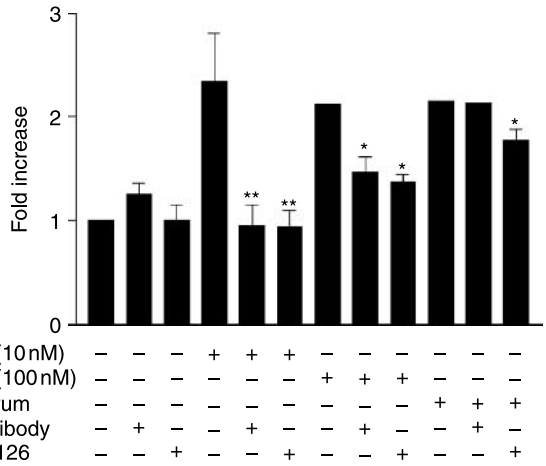

Figure 6 The involvement of integrin $\alpha_V\beta_3$ and ERK in T_3- and T_4-stimulated [^{3}H]thymidine incorporation into MG-63 cells. These cells were pretreated for 30 min with serum-free media alone or U0126 (1 μM) or anti-integrin $\alpha_V\beta_3$ antibody (1:500 dilution), followed by treatment for 24 h with serum-free media, T_3 (10 nM) or T_4 (100 nM) in the continued presence or absence of U0126 or anti-integrin $\alpha_V\beta_3$ antibody. Media containing 10% FCS (serum) was used as a positive control. Results are expressed as fold increase over cells treated with serum-free media alone and show means ± S.E.M. of three independent experiments, each performed in quadruplicate (*$P<0{\cdot}05$, **$P<0{\cdot}01$ compared with T_3, T_4 or serum alone).

suggests that T_3 stimulates osteoblast activity (Harvey *et al.* 2002). There are no reports in the literature of T_4 effects on osteoblast proliferation. Our results demonstrate that both T_3 and T_4 are able to significantly increase DNA synthesis in MG-63 cells although the concentration of T_3 (10 nM) needed to achieve this effect was again supraphysiological. Similarly, in glioma cells, T_4 (1–100 nM) was reported to increase thymidine incorporation while the concentrations of T_3 needed to achieve this effect were again supraphysiological (Davis *et al.* 2006). These authors had previously shown that the affinity of $\alpha_V\beta_3$ for T_3 is substantially lower than the affinity for T_4 (Bergh *et al.* 2005), which may explain these concentration-dependent differences between T_3 and T_4. In glioma cells, the stimulatory effects of T_4 on DNA synthesis were blocked by the RGD peptide (Davis *et al.* 2006). In our study, the stimulatory effects of T_3 were completely abolished in the presence of either anti-$\alpha_V\beta_3$ blocking antibody or U0126, suggesting that T_3 effects are mediated entirely via the integrin $\alpha_V\beta_3$ receptor and subsequent ERK activation. T_4-stimulated thymidine incorporation was only partially inhibited by either the anti-$\alpha_V\beta_3$ blocking antibody or U0126, despite this inhibitor being used at a concentration reported to completely suppress the activation of MKK1 (Davies *et al.* 2000). These results would again suggest that additional mechanisms other than those initiated at the integrin $\alpha_V\beta_3$ receptor might exist for T_4 effects on thymidine incorporation.

In summary, our results suggest that T_3 and T_4 rapidly stimulate ERK activation in MG-63 cells and that one of the functional effects of this ERK activation is increased DNA synthesis and cell proliferation. In addition, this study has demonstrated the presence of integrin $\alpha_V\beta_3$ in human osteoblast-like cells and provides *in vitro* evidence to support a role for integrin $\alpha_V\beta_3$ as the apparent initiation site for T_3- and T_4-induced ERK activation and cell proliferation in human osteoblast-like cells.

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