PD98059 enhances C2 myoblast differentiation through p38 MAPK activation: a novel role for PD98059

Nasser Al-Shanti and Claire E Stewart

Institute for Biomedical Research into Human Movement and Health, Manchester Metropolitan University, Hassall Road, Alsager, Cheshire ST7 2HL, England, UK

(Correspondence should be addressed to N Al-Shanti; Email: n.al-shanti@mmu.ac.uk)

Abstract

Cell differentiation is usually accompanied by irreversible cell cycle exit, which is a critical step for skeletal muscle differentiation. We therefore hypothesise that PD98059 that blocks the MAP kinase kinase (MEK) pathway (proliferation pathway) when administered to murine C2 skeletal myoblasts will arrest cell cycle and, consequently, enhances differentiation relative to untreated controls. In this study, we aimed to examine this hypothesis using phenotypic differentiation, biochemical assays, flow cytometry and real-time PCR in C2 cells cultured for 48 h in differentiation media only (untreated) or supplemented with either a single dose of 10 ng/ml IGF-I or 20 μ M PD98059 for 48 h. Creatine kinase (CK) activity was increased by 7.5-fold ($P < 0.05$) in the presence of PD98059, whereas untreated and IGF-I-treated cells induced 4.5- and 4-fold increase respectively when compared with baseline controls. Increased CK values in the presence of PD98059 were not only associated with myotube formation but also associated with cell cycle arrest in G1 phase ($86 \pm 3.2\%$; $P < 0.05$). Moreover, the expression of myogenic-specific transcriptional factor mRNAs (MyoD and myogenin) was significantly higher in

PD-treated cells (4.7 ± 0.15 and 314 ± 10.2 ng/reaction respectively; $P < 0.05$) than untreated (2.0 ± 0.2 and 233 ± 11 ng/reaction respectively) or IGF-treated cells (3.2 ± 0.24 and 296 ± 16.2 ng/reaction respectively). Unexpectedly, Id3 mRNA, the potent negative regulator of muscle differentiation, was also expressed at significantly higher levels in PD-treated cells (77 ± 0.346 ng/reaction; $P < 0.05$) than untreated (49 ± 7.7 ng/reaction) or IGF-I-treated cells (47 ± 0.7 ng/reaction). Furthermore, expression of the muscle differentiation-specific genes (IGF-binding protein-5, IGF-II receptor and IGF-II) was also increased significantly in PD-treated cells when compared with untreated cells. Phosflow analysis showed a significant increase in the levels of phosphorylation of p38 mitogen-activated protein kinase ($49.0 \pm 6.7\%$, $P < 0.05$) in PD-treated cells when compared with DM-treated cells ($31.7 \pm 5.7\%$). These findings uncover a previously unconsidered positive effect of PD98059 on C2 myoblast differentiation and identify the pathway(s) underlying PD-induced C2 myoblast differentiation.

Journal of Endocrinology (2008) **198**, 243–252

Introduction

Skeletal muscle is derived from pluripotent mesodermal stem cells through a multistep process including the commitment to myogenic precursors and their subsequent proliferation and differentiation (Rawls & Olson 1997). Skeletal muscle differentiation is a highly ordered process and elucidates the temporal relationships among the events that govern the transition from the proliferation to the terminally differentiated myoblast. These events are 1) entry of myoblasts into the differentiation pathway and activation of muscle-specific genes (muscle contractile protein) by myogenic proteins, 2) irreversible cell cycle exit (indicated by expression of p21), 3) phenotypic differentiation and 4) myoblast fusion resulting in myotube formation (Buckingham 1992, Edmondson & Olson 1993, Weintraub 1993, Andres & Walsh 1996). In addition, skeletal muscle differentiation requires several growth factors, such as insulin-like growth factor-I (IGF-I),

IGF-II, hepatocyte growth factor and basic fibroblast growth factor, that mediate their biological effects through various intracellular signalling cascades, such as Janus kinase/signal transducers and activators of transcription, Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (Coolican *et al.* 1997, Rommel *et al.* 1999, Wu *et al.* 2000). The extracellular signal-regulated kinase (ERK) pathway is the cascade most often associated with signalling mechanisms involved in cell proliferation and cell cycle progression but more recently also in apoptosis (Johnson & Lapadat 2002). A potent method to determine the biological effects of cellular signalling pathways is to use selective inhibitors to block specific pathways. Generally, protein kinase inhibitors are competitive with ATP and are believed to interact within ATP-binding sites of their target protein kinases, which makes these inhibitors less specific (Davies *et al.* 2000). By contrast, PD98059 agent blocks ERK activity by inhibiting activation (phosphorylation) of MEK by Raf-1,

conferring some specificity to PD98059 (Alessi *et al.* 1995). Since cell cycle arrest is a critical step for skeletal muscle differentiation and PD98059 induces G1 cell cycle arrest in many cell types (Hoshino *et al.* 2001, Moon *et al.* 2007), we therefore hypothesise that administration of PD98059 to C2 skeletal myoblast cultures may enhance cell differentiation relative to controls. The purpose of the present study described herein was to examine a potential positive role for PD98059 in myoblast differentiation. The results from this study demonstrate that PD98059 induces increased cell differentiation compared with IGF-I, a potent myogenic factor (Florini *et al.* 1996), in untreated cells. This novel finding was confirmed by increased creatine kinase (CK) activity and morphological differentiation, which was indicated by myotube formation. We have also examined the effect of PD98059 not only on the expression levels of myogenic transcriptional factor mRNAs (myogenin and MyoD) but also on mRNAs for IGF system components, which are vital for C2 differentiation. These mRNAs include IGF-II, IGF-II receptor (IGF-IIR), IGF-binding protein-5 (IGFBP-5) and IGF-IR. Furthermore, the involvement of p38 MAPK in these settings was also investigated. These interesting data begin to examine and address the molecular mechanisms by which PD98059 enhances myoblast differentiation and the signalling pathways involved in this process.

Materials and Methods

Materials

All chemicals and reagents, unless otherwise stated, were purchased from Sigma. All cell and tissue culture media and supplements were purchased as sterile or were filter sterilised through a 0.20 µm filter. Heat-inactivated (hi) fetal bovine serum (FBS) and hi new born calf serum (NCS) were purchased from Gibco, hi horse serum (HS) was from TCS Biosciences (Corby, England), Penstrep (penicillin and streptomycin) and trypsin from Bio Whittaker (Wokingham, England), L-glutamine from BDH (Poole, England) and gelatine and phorbol-12-myristate-13-acetate (PMA) from Sigma. Recombinant human IGF-I was obtained from GroPep (Adelaide, Australia), PD98059 and SB202190 inhibitor – dimethyl sulphoxide (DMSO) dissolved – were purchased from Calbiochem (Nottingham, UK) and CK assay kits from Catachem (Bridgeport, CT, USA). Phosflow reagents, anti-phospho-ERK1/2 (T202/Y204) PE-conjugated anti-phospho-p38 MAPK (T180/Y182), PE-conjugated BD Phosflow fix buffer and BD Phosflow Perm Buffer III were ordered from Becton Dickinson (BD, Oxford, UK).

Cell culture

C2 mouse skeletal myoblasts (Yaffe & Saxel 1977) were grown in a humidified 5% CO₂ atmosphere at 37 °C in a growth medium (GM) composed of Dulbecco's modified Engle's medium

(DMEM) with GlutaMAX supplemented with 10% FBS, 10% NCS, penstrep and L-glutamine, at final concentrations of 10 000 U/ml and 2 mM respectively, until 80% confluence was attained. Experiments and differentiation were initiated following washing with PBS, by transferring to low serum-containing differentiation medium (DM; DMEM plus GlutaMAX, supplemented with 2% HS, penstrep and L-glutamine (supplemented as above)) in the presence or absence of specific triggers. These cells are able to undergo spontaneous differentiation into myotubes on serum withdrawal and do not require growth factor addition to stimulate the process.

Cell treatments

Six-well plates were pre-coated with 0.2% gelatine for 5 min at RT and the cells were plated at a density of 1×10^5 cells/ml in the GM to attain confluence the following day. Then the cells were washed twice with PBS and DM was added to the cells that were incubated for 30 min at 37 °C (this time point was denoted time 0, T_0) prior to further treatments for up to 48 h. The cells were cultured for 48 h in DM only (untreated) or DM supplemented either with 10 ng/ml IGF-I (potent myogenic factor) or with 20 µM PD98059. The concentrations of PD98059 and IGF-I, which were used in the present study, were optimised by our group previously (Stewart *et al.* 2004).

CK assay

After 48 h with different treatments, cells were lysed in 200 µl TMT buffer (50 mM Tris–MES (pH 7.8), 1% Triton X-100), and CK was measured using a commercially available kit (Catachem). Absorbance was measured at 3, 4 and 5 min at 340 nm and changes in absorbance/minute and CK activity were then calculated and normalised to total protein concentration as determined by BCA protein assay, according to the manufacturer's instruction.

Phenotypic differentiation

Morphological parameters of differentiation, alignment, elongation and fusion were assessed by cell imaging system at 20× magnification at 48 h (Leica Microsystems, Milton Keynes, UK, DMI 6000 B).

Phosflow analysis of ERK and p38 protein phosphorylation levels

Phosphospecific flow cytometry, which is a powerful tool to analyse and measure the levels of intracellular phosphoproteins (Krutzik & Nolan 2003), was used in the present study. At the end of the cell treatments, the cells were rapidly detached using trypsin–EDTA buffer and fixed immediately by adding equal volume of pre-warmed BD Phosflow fix buffer. The cells were then incubated at 37 °C for 15 min followed by washing twice with cold PBS by centrifugation at 250 g for 10 min. Immediately after washing, the cells were

permeabilised by adding cold BD Phosflow Perm Buffer III and incubated for a minimum of 30 min at -20°C . Finally, the cells were washed twice with staining buffer in $1 \times \text{PBS}$, 1% FBS and 0.09% sodium azide for 10 min by centrifugation at 200 *g* and resuspended in staining buffer at a concentration of 1×10^6 cells/ml. To each tube were added 20 μl anti-phospho-ERK1/2 and anti-p38 antibody. The cells were then subjected to Phosflow analysis using FACSCalibur (BD). Percentage of positive cells for phosphorylated ERK protein was calculated by dividing the median fluorescence intensity (MFI) of each sample by MFI of PMA-treated cells $\times 100\%$.

DNA content and cell cycle analysis

After 48-h incubation under the conditions described previously, cells were harvested using trypsin–EDTA buffer. The cells were washed twice with PBS by centrifugation at 200 *g* for 10 min. The pellets obtained were resuspended in 5 ml of 75% ethanol. After overnight incubation at -20°C , the cells were washed twice with ice-cold PBS by centrifugation at 200 *g* for 10 min, and then resuspended in 0.5 ml PBS followed by the addition of 10 μl propidium iodide (50 $\mu\text{g}/\text{ml}$) and 50 μl RNase (60 $\mu\text{g}/\text{ml}$). Following overnight incubation at 4°C , DNA content was measured on FACSCalibur flow cytometer (BD) and analysed using Cell Quest (BD) and FlowJo software packages.

Primer design and synthesis

Optimal primer designs are essential to ensure that only a single PCR product is amplified in particular in real-time PCR-based SYBR Green. We therefore used web-based software from Invitrogen to design our primers (Table 1) that were further analysed by Sigma-Genosys software. The primers were designed to yield products spanning exon–intron boundaries to prevent any possible genomic DNA

contamination from total RNA isolation. Sequence homology searches against the database of GenBank showed that these primers matched only the sequence to which they were designed. The primers were synthesised by Sigma-Genosys, which are compatible with real-time PCR without further purification.

Quantitative real-time PCR and data analysis

RNA was extracted using the TRIzol method, according to the manufacturer's instructions (Invitrogen) and 30 ng RNA was used per reaction. Real-time PCR amplifications were carried out using SYBR Green One-Step qRT-PCR and Chromo4, according to the manufacturer's instructions (Bio-Rad). In brief, real-time PCR was performed by the following cycles: 50°C for 10 min (for cDNA synthesis), 95°C for 5 min (transcriptase inactivation), followed by the cycling parameters of 95°C for 10 s and 60°C for 30 s for 40 cycles. At the end of the 40 cycles, dissociation curve (melting curve) analyses were performed by the following protocol: heat at 55°C for 1 sec and measure fluorescence every 0.5°C until 95°C to confirm specific amplification. Standard curve was generated by a fivefold serial dilution from total RNA at a concentration 500 ng to 5 pg to amplify the housekeeping gene RNA polymerase II α (RPII α). To confirm that changes in the genes of interest following the different culturing time points and treatments are not due to RNA quality or quantity, RPII α was amplified in all samples at each time point (Radonic *et al.* 2004). The Bio-Rad MJ Opticon Monitor software version 3.1 was used to analyse the data of the real-time PCR. Using the data from samples designated as standard points with assigned concentration numbers, an arbitrary threshold level is set and C_t values for all PCR samples are calculated, allowing generation of a standard curve by which the concentrations of all unknown samples were derived and averaged among replicates.

Table 1 Real-time PCR primer sequences

Target gene	Primer sequence (5'–3')	Ref. seq. number	Amplicon length (bp)
IGF-II	F: TCTCATCTCTTTGGCCTTCG R: AAGCAGCACTCTCCACGAT	NM_010514	130
IGF-IR	F: CTACCTCCCTCTCTGGGAATG R: GCCCAACCTGCTGTTATTTCT	NM_010513	150
IGF-IIR	F: TGTCTGTGGTCGAGGATTTATG R: CAGAATCGTTGAGTGGGCTAA	NM_010515	95
IGFBP-5	F: GACCCAGTCCAAGTTTGTGG R: GAATCCTTTGCGGTCAAGT	NM_010518	121
MyoD	F: CTACGACACCGCCTACTAC R: GGCTGTCTGTGGAGATGC	NM_010866	116
Myogenin	F: TGAATGCAACTCCCACAGC R: TCCACCGTGATGCTGTCC	NM_031189	153
Id3	F: AGCGTGTATAGACTACATCCTC R: TCCTCTTGTCCTTGGAGATCAC	NM_008321	135

Total RNA was extracted from T0 cells and from untreated, IGF-I- and DP-treated myoblast cell for 48 h; 30 ng RNA from each sample were used in real-time PCR.

Statistical analysis

Statistical analysis and the significance of the data were determined using SPSS software version 12.0. Statistical significance was determined using one-way ANOVA with multiple *post hoc* analyses. Results are presented as mean $\pm$ S.D. and are considered statistically significant when $P < 0.05$ against appropriate controls.

Results

PD98059 blocks phosphorylation of phospho-ERK protein

The main purpose of the present study is to examine a possible role that PD98059 may play in myoblast differentiation. Since PD98059 is dissolved in DMSO, primary experiments were initiated comparing DM-treated with DM-DMSO-treated cells to rule out any possible effect of DMSO on myoblast differentiation. The data from all the experiments were comparable between the two treatments and confirm that DMSO has no effect on myoblast differentiation (data not shown).

As an essential first step towards evaluating a potential role of PD98059 in myoblast differentiation, we analysed the phosphorylation events of ERK protein in the different cell treatments using phosphoprotein flow cytometry to confirm the blockade of the ERK pathway. To validate the methodology of phosphoprotein flow cytometry, stimulation with PMA, which induces strong phosphorylation of ERK, was included as a positive control. In this experiment, IGF-I, which is a unique potent mitogenic and myogenic factor, was also included (Florini *et al.* 1996). We found that myoblasts under basal or IGF-stimulated conditions were 77 ± 8.6 and $69 \pm 7\%$ positive for phosphorylated ERK protein respectively (Fig. 1A and B), whereas the cells stimulated with PMA for 10 min were $92 \pm 2.5\%$ positive for phosphorylated ERK protein (Fig. 1C). However, PD-treated cells showed insignificant phosphorylation of ERK protein ($2.3 \pm 0.6\%$, Fig. 1D), which indicates that the ERK pathway was effectively blocked.

PD98059 induces CK activity and morphological differentiation

Morphological and biochemical studies were performed to examine our hypothesis that PD98059 may induce C2 cell differentiation. DM and IGF-I were included in this study as positive controls for promoting cell differentiation. Photomicrographs were taken prior to harvesting the cells at 48 h for CK assay. CK activity increased significantly ($P < 0.05$) and variably by 4-, 4.5- and 7.5-fold in the presence of DM, IGF-I and PD98059 respectively, when compared with baseline levels of T_0 control cells (Fig. 2A). Furthermore, when PD98059 treatment was compared with DM at 48 h, PD98059 elicited a significant ($P < 0.05$) twofold increase in CK activity. These biochemical findings were supported morphologically whereby the most prominent differentiation and myotube formation are evident in the PD-treated cultures at 48 h (Fig. 2B).

Figure 1 Phosflow analysis of ERK protein phosphorylation levels. C2 myoblasts were cultured in (A) DM only or (B) DM supplemented with either 10 ng/ml IGF-I or (D) 20 μ M PD98059 for 48 h. (C) PMA-treated cells (40 nM) for 10 min were used as positive stimuli that produces strong induction ERK protein phosphorylation. At the end of the cell treatments, the cells were detached using trypsin, and immediately fixed in BD Phosflow fix buffer and permeabilised by BD Phosflow Perm Buffer III. The cell pellets were stained with anti-phospho-ERK1/2 and subjected to flow cytometry analysis. Percentage of positive cells for phosphorylated ERK protein was calculated by dividing the MFI of each sample by MFI of PMA-treated cells $\times 100\%$. These data present the mean $\pm$ S.D. of the treatments performed four times.

PD98059 does not enhance DM-induced cell cycle arrest at 48 h

G1 cell cycle arrest is a critical step for skeletal muscle cell differentiation; to investigate whether PD98059 arrested the cell cycle in G1 phase to promote cell differentiation, flow cytometry was used. T_0 cells were included in this experiment as a control for highly proliferative cells. Myoblasts at T_0 show a typical cell cycle of highly proliferative cells with 53% ($\pm 5.9\%$) in G1 and 44% ($\pm 7.4\%$, Fig. 3A) in S-G2/M phase. However, when the cells were cultured for 48 h with DM or IGF-I (differentiation conditions), the proportion of proliferating cells in S-G2/M phases decreased in both cultures to $11 \pm 2.9\%$ (Fig. 3B) and $16 \pm 1.69\%$ (Fig. 3C) respectively. Similarly, PD-treated cells showed a typical cell cycle phase of differentiating cells where the majority of the cells were arrested in G1 ($86 \pm 3.2\%$) with only small numbers present in S-G2/M ($12.5 \pm 3.4\%$ (Fig. 3D)).

PD98059 activates transcription of myogenic regulatory factors

Myogenic regulatory factors (MRFs) (myogenin and MyoD) are known to control myoblast differentiation (Molkentin & Olson 1996, Venuti & Cserjesi 1996); therefore to examine

Figure 2 Biochemical and morphological effects of PD98059 on C2 myoblasts. C2 myoblasts were cultured with DM only or DM supplemented with either 10 ng/ml IGF-I or 20 μ M PD98059. At the end of the cell treatments (48 h), the cells were subjected either to (A) CK assay or to morphological studies to observe myoblast fusion and myotube formation in (B) T0 control, (BII) DM-, (BIII) IGF- and (BIV) DP-treated cells. T0 was also included as control for highly proliferating cells. Calculated CK activity was normalised to total protein concentration as determined by BCA protein assay. CK data present the mean $\pm$ s.d. of the treatments performed four times. * $P < 0.05$ versus T0. (B) Representative photomicrograph of random fields from four independent experiments.

whether PD98059 upregulates the transcription of these factors to enhance cell differentiation, real-time PCR was performed. Our real-time PCR data were validated using individual standard curve that showed correlation coefficients of ~ 1.0 (0.990) and the melting (dissociation) curves of these data exhibit a sharp clean peak. This result indicates that the products are specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest (data not shown).

The expression levels of the control housekeeping gene (mRNA RPII α) in all treatment groups was between 6.8 ± 0.05 ng/reaction ($P = \text{NS}$; data not shown). Real-time PCR further showed that treatment of myoblasts with IGF-I for 48 h elicited $60 \pm 21\%$ increase in MyoD and $19 \pm 9\%$ increase in myogenin expression, compared with untreated controls (Fig. 4). PD98059 led to a significant increase in MyoD (4.7 ± 0.2 ng/reaction, $P < 0.05$; Fig. 4, white bars) and myogenin (314 ± 10.2 ng/reaction, $P < 0.05$; Fig. 4, grey bars) mRNA levels compared with T_0 control cells (2.0 ± 0.2 and 233 ± 11.4 ng/reaction, respectively; Fig. 4, white and

Figure 3 Effects of PD98059 on C2 myoblast cell cycle and DNA content. C2 myoblasts were cultured and prepared as detailed in Fig. 2. DNA content and cell cycle phase of (A) T0, (B) untreated, (C) IGF-I-treated and (D) PD-treated cells were examined by propidium iodide staining using flow cytometry. The proportions of cells in S-G2/M phase are indicated. These data present the mean $\pm$ s.d. of the treatments performed four times.

grey bars). PD98059 still elicited significant increases in each of these mRNAs ($135 \pm 8\%$ in MyoD and $16 \pm 7\%$ in myogenin) when compared with DM.

Myoblast differentiation is also regulated by an inhibitor of differentiation gene 3 (Atherton *et al.* 1996, Arnold & Winter 1998, Wu & Lim 2005). We therefore tested whether PD98059 blocks the expression of Id3 mRNA to enhance differentiation. As expected, the expression level of Id3

Figure 4 Absolute mRNA levels of transcriptional factors with regulatory roles in muscle differentiation. C2 myoblasts were cultured in DM only or DM supplemented with either 10 ng/ml IGF-I or 20 μ M PD98059 for 48 h. T0 was also included as control for highly proliferating cells. Total RNA was extracted from cells, and 30 ng from each sample was used to perform real-time RT-PCR. Standard curve was used to calculate the absolute concentration and expression of target genes MyoD (white bars), myogenin (grey bars) and Id3 (black bars) based on ΔC_t values. These data present the mean $\pm$ s.d. of the treatments performed three times in triplicate. * $P < 0.05$ versus T0.

mRNA was reduced significantly ($P<0.05$) to 36 ± 0.4 and 40 ± 0.7 ng/reaction in DM- and IGF-I-treated myoblasts respectively, when compared with T_0 (49.8 ± 7.4 ng/reaction, black bars). Unexpectedly, PD-treated myoblasts showed significantly increased levels of Id3 mRNA (77 ± 0.4 ng/reaction) when compared not only with T_0 control cells but also with DM or IGF-I treatments (Fig. 4, black bars).

PD98059 stimulates mRNA expression of the IGF-I system

To further explore the specificity of PD98059 in myoblast differentiation, we examined the mRNA expression profiles of the IGF system components (IGFBP-5, IGF-IIR, IGF-IR and IGF-II), known to have regulatory roles in muscle cell growth and differentiation. Once again the expression levels of the housekeeping gene RPII α mRNA were comparable among all treatments (6.8 ± 0.3 – 0.5 ng/reaction ($P=NS$; data not shown). Real-time PCR data analyses showed that all treatments (DM, IGF-I and PD) led to approximately two- and threefold increases ($P<0.05$) in expression levels of IGFBP-5 and IGF-IIR mRNAs respectively compared with T_0 control cells (Fig. 5, grey and white bars). There was no significant difference between DM, IGF or PD98059. Similarly, there was no significant difference in IGF-IR mRNA expression between all cell treatments including T_0 (data not shown). However, dramatic increases of five-, six- and tenfold were obtained in IGF-II mRNA expression in DM-, IGF-I- and PD-treated cells respectively, when compared with the expression levels of T_0 (Fig. 5, black bars). Furthermore, when compared with DM or IGF-I, PD98059 still elicited significant increases ($P<0.05$) in IGF-II mRNA expression.

Figure 5 Absolute mRNA levels for muscle-specific genes with regulatory roles during muscle differentiation. C2 myoblasts were cultured in DM only or DM supplemented with either 10 ng/ml IGF-I or 20 μ M PD98059 for 48 h. T_0 was also included as a control for highly proliferating cells. Total RNA was extracted from cells, and 30 ng from each sample was used to perform real-time RT-PCR. Standard curve was used to calculate the absolute concentration and expression of target genes IGFBP-5 (grey bars), IGF-IIR (white bars) and IGF-II (black bars) based on ΔC_t values. These data present the mean $\pm$ s.d. of the treatments performed thrice in triplicate. * $P<0.05$ versus T_0 .

PD98059 induces myoblast differentiation through p38 MAPK activation

The involvement of MAPKs in osteoclast differentiation has recently been studied using specific inhibitors of MAPK-ERK (MEK) and p38 (Matsumoto *et al.* 2000, Lee *et al.* 2002, Wei *et al.* 2002). Moreover, it was reported that PD98059 may promote and accelerate osteoclast differentiation through the inhibition of ERK and activation of p38 MAP kinase pathway (Hotokezaka *et al.* 2002). To examine this proposed mechanism, p38 MAPK phosphorylation levels were determined in all cell treatments using anti-phospho-p38. PMA-treated and T_0 cells (unstimulated and unstained cells) were used as positive and negative controls respectively. The analysis of p38 phosphorylation by flow cytometry revealed that myoblasts under basal conditions (DM-treated cells) were $31.7\pm5.7\%$ positive for phosphorylated p38 protein, whereas 88.7 ± 3.4 and $0.7\pm0.3\%$ of the cells stimulated with PMA for 10 min and the negative control respectively were positive for phosphorylated p38 protein (Fig. 6A). Interestingly, PD-treated cells showed a significant increase in the levels of phosphorylation of p38 protein, by $49.0\pm6.7\%$ ($P<0.05$), when compared with basal control ($31.7\pm5.7\%$, Fig. 6A). To ascertain whether p38 plays an important role in PD-induced myoblast differentiation, the cells were treated with an inhibitor to p38 MAPK (SB202190) in association with PD98059 and compared with basal control (DM-treated cells). An initial dose-response experiment (0.5 – 16 μ M) for SB202190 was performed to ascertain an ideal working concentration; 8 μ M SB202190 was determined as the optimal concentration based on cell morphology, CK assay and flow cytometry data (data not shown). Morphological and biochemical studies were performed on these treatments to further confirm the involvement of p38 in our model. Photomicrographs were taken prior to harvesting the cells at 48 h for CK assay. CK activity decreased significantly ($P<0.05$) by 30 ± 42 and $50\pm9\%$ in the presence of the combined inhibitors and p38 inhibitor respectively, when compared with baseline control levels of cells DM-treated cells (Fig. 6B). These biochemical findings were supported morphologically whereby the differentiation and myotube formation were blocked in the presence of either SB202190 or combined MEK and p38 inhibitors when compared with DM-treated cells (Fig. 6C), with the most prominent differentiation and myotube formation being evident in the PD-treated cultures at 48 h (Fig. 6C).

Discussion

Skeletal muscle differentiation is a highly ordered process requiring myoblast proliferation followed by cell cycle arrest, expression of muscle-specific regulatory factors and the synthesis of muscle contractile proteins, resulting in fusion of mononucleated myoblasts into terminally differentiated multinucleated myotubes (Andres & Walsh 1996).

The pharmacological inhibitor PD98059 has been widely utilised to infer a physiological role for MEK1/2 MAP kinase and its downstream effector MAP kinase (ERK1/2) and also routinely used to implicate ERK1/2 signalling in many different settings (Favata *et al.* 1998, Kultz *et al.* 1998, Davies *et al.* 2000, Galvin *et al.* 2003). The results presented here provide important evidence that long-term experiments (48 h) with PD98059 promote extensive myoblast differentiation and support previous data that showed PD98059 enhanced differentiation of mouse cultured primary skeletal myoblasts (Galvin *et al.* 2003). These studies thus uncover a

novel role of this widely used MEK inhibitor with important physiological consequences.

The effectiveness of PD98059 in blocking the ERK pathway was confirmed using phosphoprotein flow cytometry (Krutzik & Nolan 2003) and a phospho-specific antibody against ERK1/2 protein. In association with this blockade in ERK phosphorylation and consistent with results of previous studies reporting that CK is strongly induced in differentiating myoblasts (Meadows *et al.* 2000, Foulstone *et al.* 2001), we also observed increased CK values in all treatments when compared with T_0 cells. However, the maximal CK activity was obtained in PD-treated cells, which indicates extensive cell differentiation over and above that observed with DM or IGF-I treatment. These biochemical findings support the morphological studies where myotube formation was seen in the presence of PD98059 but to a lesser extent in the presence of IGF-I at 48 h. IGF-I is known as a unique proliferation and differentiation promoter of myoblasts (Ewton *et al.* 1994, Florini *et al.* 1996, Coolican *et al.* 1997, Miller *et al.* 2000, Singleton & Feldman 2001). Therefore, the enhanced differentiation (both biochemical and morphological) in the presence of PD98059 versus IGF-I is potentially highly relevant when studying the regulators of enhanced myotube formation.

Recent studies have reported that PD98059 induces cell cycle arrest in G1 phase (Yamaguchi *et al.* 2002, Koyama *et al.* 2007, Moon *et al.* 2007, Zheng *et al.* 2007), which is a critical step for skeletal muscle differentiation to progress (Halevy *et al.* 1995, de la Serna *et al.* 2001). Although G1 cell cycle arrest, a prerequisite for differentiation, was comparable among treatment groups within our study, it does not define the subsequent mechanisms of actual differentiation, which could be different between treatments. This was indeed the case, as was confirmed by determining the expression levels of myogenin, MyoD, Id3 (key MRFs), IGF system components (key survival and differentiation factors) and p38 phosphorylation.

To this end, we wished to establish whether PD98059 was capable of altering the expression of the MRFs and thus increasing differentiation. We therefore investigated the effect

Figure 6 Phosflow analysis of p38 phosphorylation levels and biochemical and morphological effects of combined MEK and p38 MAPK inhibitors on C2 myoblasts. C2 myoblasts were cultured with DM only or DM supplemented with either 20 μ M PD98059, 8 μ M SB202190 or 20 μ M PD98059/8 μ M SB202190 (combined inhibitors). At the end of the cell treatments (48 h), the cells were subjected either to (A) Phosflow analysis, (B) CK assay and (C) morphological studies. Phosflow analysis of p38 protein phosphorylation was carried out as described in Fig. 1, and the proportions of positive cells for phosphorylated p38 are indicated in histograms. The CK assay and the morphological studies to observe myoblast fusion was performed as described in Fig. 2. p38 protein phosphorylation data present the mean $\pm$ s.d. of the treatments performed four times. * $P < 0.05$ versus DM-treated cells. Representative photomicrograph of random fields from four independent experiments, CK data present the mean $\pm$ s.d. of the treatments performed four times. * $P < 0.05$ versus DM.

of PD98059 on the expression levels of muscle-specific transcription markers, myogenin and MyoD (Isobe *et al.* 1998, de la Serna *et al.* 2001, Zhu *et al.* 2007), as well as the negative regulator of differentiation, Id3 (Wu & Lim 2005). Myogenin and MyoD expression levels were increased, but were not significantly increased in the presence of IGF-I when compared with baseline levels. By contrast, the expression levels of their mRNA were significantly increased in the presence of PD98059 suggesting a link between PD98059, MEK, MRFs and C2 cell differentiation.

Under conditions of differentiation, one would expect a decline in Id3 expression. Indeed, as expected, the expression of Id3 mRNA in DM-treated cells and in the presence of IGF-I was reduced significantly. In contrast to untreated and IGF-I-treated cells, PD-treated cells significantly upregulated the expression levels of Id3 mRNA, which is controversial relative to what has been reported in C2C12 cells (Atherton *et al.* 1996, Melnikova & Christy 1996, Wu & Lim 2005). Extensive cell differentiation, which was promoted following the administration of PD98059, may ultimately deplete the myoblast pool; therefore cells upregulate the expression of Id3 mRNA as a negative regulatory mechanism to slow down the rate of differentiation.

Indeed, studies by Navarro *et al.* (2001) have suggested that IGF causes upregulation of Id2; therefore in our model, Id3 may be increased as a consequence of increased IGF-II expression as a mechanism of controlling the rate of differentiation in a feed-forward loop. In addition, utilising the non-differentiating myoblast cell line NFB4, Sarbassov *et al.* has shown that myogenin and IGF-II are capable of activating each other's expression. Together, these studies suggest crosstalk between IGF system and both positive and negative regulators of differentiation. Given our MRF data, we therefore also wished to assess whether PD98059 influenced the expression of IGF system components in our model (Sarbassov *et al.* 1995). In order to provide additional lines of evidence for the proposed role of PD98059 in C2 myoblast differentiation, we examined changes in mRNA expression patterns of IGF-II and its receptor, which are upregulated in C2 cells and other myoblast lines to facilitate their differentiation (Tollefsen *et al.* 1989, Rosenthal *et al.* 1991, Stewart & Rotwein 1996). Moreover, IGF-II and its receptor are produced in fusion differentiating skeletal muscle cells, suggesting that IGF-II may be an autocrine differentiation factor for muscle (Tollefsen *et al.* 1989). Consistent with the findings described above, the expression levels of IGF-II and IGF-IIR mRNAs were induced in all treatments, including PD98059, which had a profound impact particularly on IGF-II expression. This responsiveness of IGF-II to PD treatment may therefore indeed underpin the increased expression levels of the MRFs. Finally, in agreement with several studies which reported that IGFBP-5 is upregulated during the differentiation of several cell lines including myoblast cell lines (James *et al.* 1993, Cobb *et al.* 2004), and that IGFBP-5 is upregulated in response to MyoD (Bergstrom *et al.* 2002), our real-time PCR data also showed increased

expression levels of IGFBP-5 mRNA, which were accompanied by cell differentiation regardless of the cell treatments, importantly, again linking the IGF system to the MRFs. Further studies are, however, needed to confirm this potential link between PD98059, IGF-II and MRFs.

Finally, we investigated the mechanism by which the PD may induce myoblast differentiation and the involvement of p38 MAPK in this process. The phosphorylation levels of p38 protein were significantly increased in PD-treated cells when compared with baseline control levels. These findings support previously proposed contribution of p38 MAPK in cell differentiation (Matsumoto *et al.* 2000, Hotokezaka *et al.* 2002, Lee *et al.* 2002). Additional possible mechanisms may contribute to this novel effect of PD98059 on myoblast differentiation that the PD98059 inhibitor may affect other unknown effector molecules or pathways, culminating in altered IGF-I and MRF expression, which underpin the enhanced differentiation reported.

Taken together, our data suggest that the chemical inhibitor PD98059 could extensively enhance and accelerate myoblast differentiation through the activation of p38 MAPK. These findings uncover an important 'side effect' of PD98059 following a single dose, which was studied for 48 h. The data suggest that PD98059 may function to enhance regulators of differentiation and ultimately myotube production, and thus reveals an important tool for dissecting the precise mechanisms underpinning this process.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

Funding for these studies was provided by the Institute for Biomedical Research into Human Movement and Health (IRM).

References

- Alessi DR, Cuenda A, Cohen P, Dudley DT & Saltiel AR 1995 PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *Journal of Biological Chemistry* **270** 27489–27494.
- Andres V & Walsh K 1996 Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *Journal of Cell Biology* **132** 657–666.
- Arnold HH & Winter B 1998 Muscle differentiation: more complexity to the network of myogenic regulators. *Current Opinion in Genetics and Development* **8** 539–544.
- Atherton GT, Travers H, Deed R & Norton JD 1996 Regulation of cell differentiation in C2C12 myoblasts by the Id3 helix-loop-helix protein. *Cell Growth and Differentiation* **7** 1059–1066.
- Bergstrom DA, Penn BH, Strand A, Perry RL, Rudnicki MA & Tapscott SJ 2002 Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. *Molecular Cell* **9** 587–600.

- Buckingham M 1992 Making muscle in mammals. *Trends in Genetics* **8** 144–148.
- Cobb LJ, Salih DA, Gonzalez I, Tripathi G, Carter EJ, Lovett F, Holding C & Pell JM 2004 Partitioning of IGFBP-5 actions in myogenesis: IGF-independent anti-apoptotic function. *Journal of Cell Science* **117** 1737–1746.
- Coolican SA, Samuel DS, Ewton DZ, McWade FJ & Florini JR 1997 The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *Journal of Biological Chemistry* **272** 6653–6662.
- Davies SP, Reddy H, Caivano M & Cohen P 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **351** 95–105.
- Edmondson DG & Olson EN 1993 Helix-loop-helix proteins as regulators of muscle-specific transcription. *Journal of Biological Chemistry* **268** 755–758.
- Ewton DZ, Roof SL, Magri KA, McWade FJ & Florini JR 1994 IGF-II is more active than IGF-I in stimulating L6A1 myogenesis: greater mitogenic actions of IGF-I delay differentiation. *Journal of Cellular Physiology* **161** 277–284.
- Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F *et al.* 1998 Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *Journal of Biological Chemistry* **273** 18623–18632.
- Florini JR, Ewton DZ & Coolican SA 1996 Growth hormone and the insulin-like growth factor system in myogenesis. *Endocrine Reviews* **17** 481–517.
- Foulstone EJ, Meadows KA, Holly JM & Stewart CE 2001 Insulin-like growth factors (IGF-I and IGF-II) inhibit C2 skeletal myoblast differentiation and enhance TNF alpha-induced apoptosis. *Journal of Cellular Physiology* **189** 207–215.
- Galvin CD, Hardiman O & Nolan CM 2003 IGF-1 receptor mediates differentiation of primary cultures of mouse skeletal myoblasts. *Molecular and Cellular Endocrinology* **200** 19–29.
- Halevy O, Novitsch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D & Lassar AB 1995 Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267** 1018–1021.
- Hoshino R, Tanimura S, Watanabe K, Kataoka T & Kohno M 2001 Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27(Kip1). *Journal of Biological Chemistry* **276** 2686–2692.
- Hotokezaka H, Sakai E, Kanaoka K, Saito K, Matsuo K, Kitaura H, Yoshida N & Nakayama K 2002 U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. *Journal of Biological Chemistry* **277** 47366–47372.
- Isobe A, Hirayama E & Kim J 1998 Characterization of myogenin expression in myotubes derived from quail myoblasts transformed with a temperature sensitive mutant of Rous sarcoma virus. *Cell Structure and Function* **23** 57–67.
- James PL, Jones SB, Busby WH Jr, Clemmons DR & Rotwein P 1993 A highly conserved insulin-like growth factor-binding protein (IGFBP-5) is expressed during myoblast differentiation. *Journal of Biological Chemistry* **268** 22305–22312.
- Johnson GL & Lapadat R 2002 Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298** 1911–1912.
- Koyama M, Matsuzaki Y, Yagisawa S, Hitomi T, Kawanaka M & Sakai T 2007 ZD1839 induces p15INK4b and causes G1 arrest by inhibiting the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway. *Molecular Cancer Therapeutics* **6** 1579–1587.
- Krutzik PO & Nolan GP 2003 Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry. Part A* **55** 61–70.
- Kultz D, Madhany S & Burg MB 1998 Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. *Journal of Biological Chemistry* **273** 13645–13651.
- Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH & Kim HH 2002 The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. *Bone* **30** 71–77.
- Matsumoto M, Sudo T, Saito T, Osada H & Tsujimoto M 2000 Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). *Journal of Biological Chemistry* **275** 31155–31161.
- Meadows KA, Holly JM & Stewart CE 2000 Tumor necrosis factor-alpha-induced apoptosis is associated with suppression of insulin-like growth factor binding protein-5 secretion in differentiating murine skeletal myoblasts. *Journal of Cellular Physiology* **183** 330–337.
- Melnikova IN & Christy BA 1996 Muscle cell differentiation is inhibited by the helix-loop-helix protein Id3. *Cell Growth and Differentiation* **7** 1067–1079.
- Miller KJ, Thaloer D, Matteson S & Pavlath GK 2000 Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. *American Journal of Physiology. Cell Physiology* **278** C174–C181.
- Molkentin JD & Olson EN 1996 Defining the regulatory networks for muscle development. *Current Opinion in Genetics and Development* **6** 445–453.
- Moon DO, Park C, Heo MS, Park YM, Choi YH & Kim GY 2007 PD98059 triggers G1 arrest and apoptosis in human leukemic U937 cells through downregulation of Akt signal pathway. *International Immunopharmacology* **7** 36–45.
- Navarro M, Valentinis B, Belletti B, Romano G, Reiss K & Baserga R 2001 Regulation of Id2 gene expression by the type 1 IGF receptor and the insulin receptor substrate-1. *Endocrinology* **142** 5149–5157.
- Radonik A, Thulke S, Mackay IM, Landt O, Siegert W & Nitsche A 2004 Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications* **313** 856–862.
- Rawls A & Olson EN 1997 MyoD meets its maker. *Cell* **89** 5–8.
- Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD & Glass DJ 1999 Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* **286** 1738–1741.
- Rosenthal SM, Brunetti A, Brown EJ, Mamula PW & Goldfine ID 1991 Regulation of insulin-like growth factor (IGF) I receptor expression during muscle cell differentiation. Potential autocrine role of IGF-II. *Journal of Clinical Investigation* **87** 1212–1219.
- Sarbasov DD, Stefanova R, Grigoriev VG & Peterson CA 1995 Role of insulin-like growth factors and myogenin in the altered program of proliferation and differentiation in the NFB4 mutant muscle cell line. *PNAS* **92** 10874–10878.
- de la Serna IL, Roy K, Carlson KA & Imbalzano AN 2001 MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes. *Journal of Biological Chemistry* **276** 41486–41491.
- Singleton JR & Feldman EL 2001 Insulin-like growth factor-I in muscle metabolism and myotherapies. *Neurobiology of Disease* **8** 541–554.
- Stewart CE & Rotwein P 1996 Insulin-like growth factor-II is an autocrine survival factor for differentiating myoblasts. *Journal of Biological Chemistry* **271** 11330–11338.
- Stewart CE, Newcomb PV & Holly JM 2004 Multifaceted roles of TNF- α in myoblast destruction: a multitude of signal transduction pathways. *Journal of Cellular Physiology* **198** 237–247.
- Tollefsen SE, Sadow JL & Rotwein P 1989 Coordinate expression of insulin-like growth factor II and its receptor during muscle differentiation. *PNAS* **86** 1543–1547.
- Venuti JM & Cserjesi P 1996 Molecular embryology of skeletal myogenesis. *Current Topics in Developmental Biology* **34** 169–206.
- Wei S, Wang MW, Teitelbaum SL & Ross FP 2002 Interleukin-4 reversibly inhibits osteoclastogenesis via inhibition of NF-kappa B and mitogen-activated protein kinase signaling. *Journal of Biological Chemistry* **277** 6622–6630.
- Weintraub H 1993 The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75** 1241–1244.

- Wu J & Lim RW 2005 Regulation of inhibitor of differentiation gene 3 (Id3) expression by Sp2-motif binding factor in myogenic C2C12 cells: downregulation of DNA binding activity following skeletal muscle differentiation. *Biochimica et Biophysica Acta* **1731** 13–22.
- Wu Z, Woodring PJ, Bhakta KS, Tamura K, Wen F, Feramisco JR, Karin M, Wang JY & Puri PL 2000 p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Molecular and Cellular Biology* **20** 3951–3964.
- Yaffe D & Saxel O 1977 Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270** 725–727.
- Yamaguchi K, Tomita H, Sugano E, Nakazawa T & Tamai M 2002 Mitogen-activated protein kinase inhibitor, PD98059, inhibits rat retinal pigment epithelial cell replication by cell cycle arrest. *Japanese Journal of Ophthalmology* **46** 634–639.
- Zheng SM, Jiang MD, Zeng WZ, Xu H, Wang YX, Ma HD, Xie FW, Zhang Y, Qin JP & Wu XL 2007 Effects of extracellular signal-regulated kinase on rat cultured hepatic stellate cells stimulated by acetaldehyde. *Journal of Digestive Diseases* **8** 148–153.
- Zhu W, Boachie-Adjei O, Rawlins BA, Frenkel B, Boskey AL, Ivashkiv LB & Blobel CP 2007 A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells. *Journal of Biological Chemistry* **282** 18676–18685.

Received in final form 28 April 2008

Accepted 8 May 2008

Made available online as an Accepted Preprint
8 May 2008