Suppression of PDGF-induced PI3 kinase activity by imatinib promotes adipogenesis and adiponectin secretion

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Abstract

Improved glucose and lipid metabolism is a unique side effect of imatinib therapy in some chronic myeloid leukaemia (CML) patients. We recently reported that plasma levels of adiponectin, an important regulator of insulin sensitivity, are elevated following imatinib therapy in CML patients, which could account for these improved metabolic outcomes. Adiponectin is secreted exclusively from adipocytes, suggesting that imatinib modulates adiponectin levels directly, by transcriptional upregulation of adiponectin in pre-existing adipocytes, and/or indirectly, by stimulating adipogenesis. In this report, we have demonstrated that imatinib promotes adipogenic differentiation of human mesenchymal stromal cells (MSCs), which in turn secrete high-molecular-weight adiponectin. Conversely, imatinib does not stimulate adiponectin secretion from mature adipocytes. We hypothesise that inhibition of PDGFRα (PDGFRa) and PDGFRβ (PDGFRβ) is the mechanism by which imatinib promotes adipogenesis. Supporting this, functional blocking antibodies to PDGFR promote adipogenesis and adiponectin secretion in MSC cultures. We have shown that imatinib is a potent inhibitor of PDGF-induced PI3 kinase activation and, using a PI3 kinase p110α-specific inhibitor (PIK-75), we have demonstrated that suppression of this pathway recapitulates the effects of imatinib on MSC differentiation. Furthermore, using mitogens that activate the PI3 kinase pathway, or MSCs expressing constitutively activated Akt, we have shown that activation of the PI3 kinase pathway negates the pro-adipogenic effects of imatinib. Taken together, our results suggest that imatinib increases plasma adiponectin levels by promoting adipogenesis through the suppression of PI3 kinase signalling downstream of PDGFR.

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Introduction

Imatinib mesylate is a rationally designed chemotherapeutic agent that was developed to target the kinase domain of the BCR–ABL fusion protein, the causative molecular abnormality of chronic myeloid leukaemia (CML). Imatinib binds to the inactive ATP-binding pocket of BCR–ABL, preventing ATP binding and, subsequently, catalytic activity (Druker 2004). Imatinib has also been shown to inhibit the function of a number of off-target kinases, resulting in a range of unique side effects that are observed in some imatinib-treated patients (Vandyke et al. 2010a). For example, hyperphosphataemia and secondary hyperparathyroidism, observed in over 50% of imatinib-treated CML patients, are thought to relate to changes in normal bone remodelling resulting from inhibition of the macrophage colony-stimulating factor (CSF1) receptor, c-fms (CSF1R) and the platelet-derived growth factor receptor (PDGFR). At therapeutically achievable concentrations, imatinib inhibits c-fms, thereby suppressing osteoclast differentiation and function (Dewar et al. 2005, 2006). Conversely, imatinib is thought to stimulate osteogenesis by inhibiting PDGFR signalling in pluripotent mesenchymal stromal cells (MSCs; Fierro et al. 2007, Fitter et al. 2008, Jonsson et al. 2011). Assessment of skeletal elements in CML patients, before and after imatinib treatment, has revealed an increase in trabecular bone volume and increased bone mineral density consistent with an anti-osteoclastic and/or pro-osteogenic effect (Fitter et al. 2008, Jonsson et al. 2008, O’Sullivan et al. 2009).

Improved glucose metabolism has also emerged as an off-target effect of imatinib therapy in some CML patients. This side effect appears to be limited to CML patients with concurrent type 2 diabetes or those who are insulin resistant (Tsapas et al. 2008), as no improvement in fasting glucose levels was observed in non-diabetic CML patients with normoglycaemic profiles at diagnosis (Mariani et al. 2010). Typically, responsive patients experience a significant increase in insulin sensitivity within 3 months of commencing imatinib therapy (Breccia et al. 2004, 2005, Veneri et al. 2005, Gologan et al. 2009, Mariani et al. 2010). This improvement occurs without significant dietary or
lifestyle changes, suggesting that imatinib modulates systemic insulin sensitivity. In support of this, improved glycemic control has also been observed following imatinib treatment in animal models of diabetes. In streptozotocin-induced and spontaneous (non-obese diabetic and db/db) mouse models of diabetes, imatinib has been shown to preserve islet β-cell function, in part through the suppression of c-abl-mediated islet cell apoptosis (Hagerkvist et al. 2007, Han et al. 2009). Imatinib and other inhibitors of PDGFR have also been shown to prevent and even reverse diabetes in non-obese diabetic mice through a mechanism that may involve suppression of a PDGF-induced inflammatory response (Louvet et al. 2008). More recently, we have demonstrated that plasma levels of an adipokine, adiponectin, are elevated two- to three-fold in CML patients after 3 months of imatinib therapy (Fitter et al. 2010). Adiponectin is known to play an important role in glucose metabolism, suggesting that this increase in adiponectin may be a mechanism whereby imatinib causes improvements in insulin sensitivity. In support of this, the increase in plasma adiponectin levels observed following imatinib treatment occurs within a similar time frame as the improvements in insulin sensitivity observed in imatinib-treated CML patients (Breccia et al. 2004, 2005, Veneri et al. 2005, Gologan et al. 2009, Mariani et al. 2010).

Adiponectin is secreted exclusively from peripheral, omental and bone marrow adipose, where its expression is regulated in response to metabolic effectors. Intramedullary adipose is derived from pluripotent MSCs that reside within the bone marrow microenvironment. Differentiation occurs in response to a variety of extrinsic factors that act to initiate or repress the transcriptional programs that govern lineage determination (Gimble 1998). Examination of the bone marrow cellularity of CML patients before and after imatinib therapy revealed a significant increase in intramedullary adipose after 6 months of therapy (Fitter et al. 2010). Furthermore, we and others have observed increased adipocyte numbers in MSC cultures treated with therapeutically relevant doses of imatinib (Fierro et al. 2007, Fitter et al. 2008). Taken together, these findings suggest that imatinib promotes adipogenic differentiation of human MSCs.

In this study, we have shown that imatinib promotes adipogenic differentiation and adiponectin secretion from human MSCs and have identified the PI3 kinase pathway, downstream of PDGFR, as an important mechanism.

**Materials and methods**

**Reagents**

Imatinib mesylate and NVP-BEZ235 were provided by Novartis International. PI3 kinase isoform-specific inhibitors PIK-75 (p110α), TGX-221 (p110β) and IC87114 (p110β) were a kind gift from Prof. Peter Shepherd (Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, New Zealand). Pioglitazone was purchased from Cayman (Ann Arbor, MI, USA). Stock solutions of inhibitors were prepared in 100% DMSO and subsequent dilutions made in media. Unless otherwise specified, all reagents were purchased from Sigma.

**MSC isolation and differentiation**

MSCs were grown from bone chips recovered from posterior iliac crest bone marrow aspirates from haematologically normal human donors as described previously (Fitter et al. 2008) and in accordance with procedures approved by the Royal Adelaide Hospital Ethics Committee. To stimulate adipogenesis, cells were cultured in media with reduced foetal bovine serum (5% v/v) and supplemented with 100 nmol/l dexamethasone sodium phosphate (induction media) for 35 days. Conditioned media were collected from control and imatinib-treated cultures 4 days post-media change and stored at −80°C. To identify and enumerate lipid-laden fat cells, formalin-fixed cells in 96-well plates were stained with nile red (25 ng/ml) and DAPI (300 nmol/l) for 15 min. Nile red-labelled adipocytes and DAPI-stained cell nuclei were visualised using an inverted fluorescence microscope (CKX41; Olympus, Tokyo, Japan), images were captured using a colour CCD camera (4X magnification; DP20, Olympus) and adipocytes and cell nuclei were enumerated using Image J software. For mitogen experiments, induction cultures were treated with rhPDGF-BB (10 ng/ml; Prospec, Rehovot, Israel) or rhEGF (20 ng/ml; Prospec) twice weekly for 4 weeks and adipocytes were enumerated as described.

**Real-time PCR**

Total RNA was isolated using Trizol (Invitrogen) and real-time PCR was performed as described previously (Fitter et al. 2008). Changes in gene expression were calculated relative to β-actin using the 2−ΔΔCt method. Primer pairs (forward and reverse) were as follows: β-actin (ACTB), 5‘-gatcattgtcctcttgac-3‘ and 5‘-gctatggtgccttctaga-3‘; CCAAT/enhancer binding protein, alpha (CEBPα), 5‘-gggcaaggccaaagtct-3‘ and 5‘-gggcaaggccaaagtct-3‘; peroxisome proliferator-activated receptor gamma 2 (PPARG), 5‘-ctcctaggcagcaagac-3‘ and 5‘-ttaaagagttgttttg-3‘; leptin (LEP), 5‘-ggctttgtggctttgttctttg-3‘ and 5‘-ggctttgtggctttgttctttg-3‘; complement factor D (CFD), 5‘-gacaccatgcagcaagac-3‘ and 5‘-ccacgcgctgtagtgc-3‘; retinoic acid receptor responder (tazarotene induced) 2 (RARRES2), 5‘-aagctggcagctggtc-3‘ and 5‘-aagctggcagctggtc-3‘; and adiponectin (ADIPOQ), 5‘-gccctatcttttc-3‘ and 5‘-gccctatcttttc-3‘.
Immunoblotting

Analysis of adiponectin multimers was performed as described previously (Lara-Castro et al. 2006, Fitter et al. 2010). Briefly, conditioned media were electrophoresed on 8–13% non-denaturing sodium acetate PAGEs (Invitrogen) and protein complexes transferred to PVDF membrane, blocked for 2 h in 2.5% (w/v) blocking buffer (GE Healthcare, Little Chalfont, UK) and probed for 16 h at 4°C using an adiponectin-specific antibody (BD Biosciences, Franklin Lakes, NJ, USA). Bound antibody was detected using an alkaline phosphatase-conjugated secondary antibody and then visualised on a Typhoon 9410 imager (GE Healthcare) in the presence of an enhanced chemiluminescence substrate. To assess total adiponectin levels, an equivalent amount of culture medium was resolved by denaturing SDS–PAGE (10% v/v), transferred to PVDF membranes and immunoblotted as described earlier. To measure cellular proteins, cell lysates were obtained as described previously (Fitter et al. 2008) and equivalent amounts (50 μg) of protein separated on 10% SDS–PAGE gels and transferred to PVDF membranes. Target proteins were detected using antigen-specific antibodies (C/EBPα and PPARγ2, Millipore, Billerica, MA, USA; β-actin, Sigma; HSP-90, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

PDGF pulse experiments were carried out as described previously (Fitter et al. 2008). Briefly, MSCs were grown to 80–90% confluence in 6 cm dishes and serum deprived for 16 h. Cells were exposed to drugs (at indicated concentrations) for 1 h and then pulse stimulated with rhPDGF-BB (10 ng/ml) for 5 min. Equivalent amounts of protein (50 μg) were separated on 10% SDS–PAGE gels and transferred to PVDF membranes. Target proteins were detected using antigen-specific antibodies (phospho-Akt [Thr 308] and phospho-Erk1/2 [Thr 202/Tyr 204]; Cell Signaling). Immunoblotting using a phspho-specific p70S6K antibody (Cell Signaling) and a total p70S6K antibody (Cell Signaling).

ELISA

Adiponectin levels in cell culture conditioned media were measured using a commercial ELISA kit (R&D Systems, Minneapolis, MA, USA). Frozen conditioned media were thawed at 4°C, centrifuged at 16 000 g for 15 min at 4°C and adiponectin levels were determined in triplicate according to the manufacturer’s instructions. The inter-plate coefficient of variation was calculated as 2.5–7.5 (%).

Cell proliferation assay

The effect of functional blocking antibodies to PDGFRα (PDGFRα) and PDGFRβ (PDGFRB; 10 μg/ml; Millipore), control IgG (10 μg/ml) and imatinib (3 μmol/l) on cell survival/proliferation was assessed using WST-1 as described previously (Vandyke et al. 2010a).

Generation of MSCs expressing myristoylated Akt

A cDNA encoding a 5'-src myristoylation signal in frame with mouse Akt1 (myr-Akt; a gift from Dr Naheed N Ahmed, Fox Chase Cancer Center, Philadelphia, PA, USA) was sub-cloned into pRUF-IREScGFP and then introduced into primary human MSCs by retroviral transduction as described previously (Isenmann et al. 2009). Infected cells (eGFP+) were isolated by preparative flow cytometry and used in differentiation assays. Constitutive Akt activity was confirmed by immunoblotting using a phspho-specific p70S6K antibody (Cell Signaling) and a total p70S6K antibody (Cell Signaling).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 5; GraphPad Software, La Jolla, CA, USA). t-Tests or one-way ANOVA with Dunnett’s post-tests were applied where indicated. Two-sided P values <0.05 were considered statistically significant.

Results

Imatinib stimulates adipogenesis

Differentiation of human MSCs into adipocytes in vitro is typically performed using a drug cocktail containing PPARγ2 agonists, phosphodiesterase inhibitors and glucocorticoid, the latter being essential to drive differentiation (Gimble 1998). To examine the effect of imatinib, we chose to use only glucocorticoid (100 nmol/l dexamethasone) to more closely emulate physiological conditions.

At therapeutically relevant doses of imatinib (le Coutre et al. 2004), a dose-dependent increase in adipocyte numbers was observed (Fig. 1A and B). Real-time PCR was utilised to investigate transcriptional changes in genes involved in adipogenic differentiation and function. In response to imatinib, the expression of key adipogenic transcription factors CEBPA and PPARG was elevated at all time points examined (Fig. 1C). Imatinib treatment also stimulated the expression of several adipokines including LEP, CFD and RARRES2. Most profoundly, adiponectin gene expression (ADIPOQ) was elevated up to 70-fold in imatinib-treated cultures at day 32, when compared with the vehicle control (Fig. 1B). Consistent with the transcriptional increases seen in response to imatinib, an increase in C/EBPα, PPARγ2 and adiponectin protein levels were detected by immunoblotting (Fig. 1D).
Adipocytes in imatinib-treated cultures secrete high-molecular-weight adiponectin

To investigate whether adiponectin was secreted from imatinib-treated MSCs, an adiponectin-specific ELISA was used. Consistent with the gene expression studies, increased levels of secreted adiponectin were detectable in the conditioned media of MSCs cultured in the presence of imatinib for 21 days or longer (Fig. 2A).

To determine the nature of adiponectin complexes secreted from adipocytes in imatinib-treated MSC cultures, conditioned media were analysed by non-denaturing PAGE and western blotting. The predominant secreted form is the high-molecular-weight (HMW) complex that was evident from 17 to 21 days of culture (Fig. 2B).

To determine whether imatinib promotes adiponectin secretion from mature adipocytes, MSCs were cultured under induction conditions in the presence of imatinib for 28 days to promote adipogenesis. The induction medium was then replaced with normal growth medium for 4 days after which time differentiated adipocyte cultures were stimulated with imatinib or the known PPARγ2 agonists pioglitazone or indomethacin for 4 days in induction media. Conditioned media were collected and adiponectin levels were determined by ELISA. No significant increase in adiponectin secretion was observed in differentiated cultures treated with imatinib, whereas both pioglitazone and indomethacin strongly promoted adiponectin secretion when compared with the vehicle control (Fig. 2C).

Blocking antibodies to PDGFR promote adipogenesis and adiponectin secretion

Imatinib inhibits a number of non-receptor and receptor tyrosine kinases. These include PDGFR, which is highly expressed on mesenchymal cells. PDGF acts as a potent MSC mitogen and an inhibitor of osteogenic and adipogenic differentiation (Kratchmarova et al. 2005, Tokunaga et al. 2008). In light of this,
we hypothesised that imatinib mediates its effects through inhibition of PDGFR function. To test this, functional blocking antibodies to PDGFRα and PDGFRβ were included in MSC cultures under induction conditions. An antibody concentration (10 μg/ml) that inhibited cell proliferation and PDGF-induced activation of Akt equivalent to that observed following treatment with 3 μM imatinib was selected (Fig. 3A and B). Consistent with a role for PDGF as an inhibitor of differentiation, blocking antibodies to PDGFR promoted the formation of adipocytes in MSC cultures (Fig. 3C and D), and this was associated with an up-regulation of PPARγ2 and adiponectin gene expression (Fig. 3E). Adipocytes formed in the presence of functional blocking antibodies to PDGFRα and PDGFRβ were shown to secrete the HMW form of adiponectin, consistent with the effects of imatinib (Fig. 3F and G).

Inhibition of p110α promotes adipogenesis and adiponectin secretion

In mesenchymal cells, stimulation with PDGF has been shown to strongly activate the PI3 kinase pathway, as evidenced by the phosphorylation of the regulatory and catalytic subunits of class I PI3 kinase family members and downstream effector molecules such as Akt (Kratchmarova et al. 2005). Suppression of PI3 kinase using pharmacological inhibitors has been shown to promote MSC differentiation (Kratchmarova et al. 2005, Fitter et al. 2008, Martin et al. 2010), suggesting that this pathway is important for transducing the inhibitory effects of PDGF on MSC differentiation. Consistent with this hypothesis, imatinib is a potent inhibitor of PDGF-induced PI3 kinase activation, as evidenced by a dose-dependent decrease in the phosphorylation of Akt (Fig. 4A). By contrast, PDGF-induced Erk activation, a measure of MAPK activity, is not affected by imatinib treatment (Fig. 4A).

In light of the inhibitory effect of imatinib on PDGF-induced PI3 kinase activation, we hypothesised that an inhibitor of the catalytic subunit of PI3 kinase would promote adipogenesis. As MSCs express PI3 kinase subunits p110α, p110β and p110δ (data not shown), the isoform-specific inhibitors BEZ235, PIK-75, TGX-221 and IC87114 were tested initially to assess the role of each subunit in PDGF-induced Akt activation. Only PIK-75, an inhibitor of p110α (Chaussade et al. 2007) and BEZ235, a pan class I inhibitor (Serra et al. 2008), were found to inhibit PDGF-induced Akt phosphorylation (Fig. 4B). PIK-75 inhibits PDGF-induced Akt phosphorylation dose dependently (Fig. 4C) with an IC50 of 10 nM. Consistent with the results obtained for imatinib and functional blocking PDGFR antibodies, PIK-75 was shown to dose dependently promote adipocyte formation under induction conditions (Fig. 4D and E). Similarly, high levels of secreted HMW and LMW
adiponectin complexes were detected in cultures treated with PIK-75 for 18 days or more, when compared with vehicle-treated control cultures (Fig. 4F and G).

Mitogens that activate PI3 kinase negate the pro-adipogenic effects of imatinib but not PIK-75

To further investigate the role of the PI3 kinase pathway in MSC differentiation, we examined the effects of adding exogenous mitogens to imatinib or PIK-75-treated MSC cultures under induction conditions. Both PDGF and EGF activate the PI3 kinase pathway in human MSCs (Kratchmarova et al. 2005). Consistent with previous reports (Artemenko et al. 2005, Gagnon et al. 2009), we found that addition of growth factors alone completely inhibited glucocorticoid-induced adipogenesis (Fig. 5A and B). In the presence of imatinib, PDGF had no effect on imatinib-mediated adipogenesis whereas EGF inhibited the imatinib response (Fig. 5A and B). In contrast, PIK-75 promoted adipogenesis, albeit to a lesser extent, in the presence of either PDGF or EGF (Fig. 5A and B), suggesting that the PI3 kinase pathway is important for transducing the inhibitory effects of mitogens on MSC differentiation.

Constitutive activation of Akt negates the effects of imatinib on MSC differentiation

Having established a signalling pathway that links imatinib with MSC differentiation, we sought to confirm our findings using a genetic approach. Akt is the primary effector molecule of the PI3 kinase pathway, and its activation is suppressed by imatinib in response to PDGF stimulation. It therefore follows that constitutive activation of Akt would negate the pro-adipogenic effects of imatinib. To test this, MSC cultures that express constitutively activated Akt (myr-Akt) or vector only were treated with imatinib and their ability to differentiate into adipocytes was measured. To confirm activation of Akt, the phosphorylation status of p70S6K, an Akt effector protein that inhibits mTOR, was determined using a phospho-specific antibody.

Figure 3 Functional blocking antibodies to PDGFR promote adipogenesis. (A) MSC cultures were starved overnight, treated with functional blocking anti-PDGFR antibodies or imatinib and then stimulated with rhPDGF-BB (10 ng/ml) for 5 min. Cell proteins were harvested, resolved by SDS–PAGE and then transferred to PVDF membranes. The activation status of Akt was determined using a phospho-specific antibody. (B) MSCs were cultured in normal growth medium supplemented with functional blocking PDGFRα and PDGFRβ antibodies (mAbs, 10 μg/ml, black dashed line), isotype control antibodies (cIgG, 10 μg/ml, grey dashed line), imatinib (3 μmol/l, black solid line) or vehicle (PBS, grey solid line) for 6 days. The relative number of viable, metabolically active cells per well was then detected using WST-1 reagent read at 540 nm. (C) MSC cultures were treated with mAbs, cIgG, imatinib (3 μmol/l) or vehicle (PBS) under induction conditions for 28 days and adipocytes were visualised using nile red. (D) The number of adipocytes was enumerated by counting nile red-labelled cells and DAPI-labelled cell nuclei (mean ± s.d. of triplicate measurements). (E) RNA harvested from treated cultures was reverse transcribed into cDNA and comparative gene expression studies performed using real-time PCR. Bar graphs show changes in gene expression for indicated genes (mean ± s.d. of triplicate measurements). (F) An ELISA was used to determine the amount of adiponectin secreted from MSC cultures treated as indicated. (G) Secreted adiponectin was analysed by non-denaturing PAGE and immunoblotting. Molecular weights: HMW, ~360 kDa; LMW, ~180 kDa (* P<0.05, t-test, relative to controls). Bar = 500 μm.
molecule (Hay & Sonenberg 2004), was assessed. Hyperphosphorylated p70S6K was detected in immunoblots of proteins isolated from cells expressing myr-Akt, when compared with vector-only control cells lysates (Fig. 6A). Under induction conditions, imatinib promoted adipogenic differentiation of vector control cells but failed to promote adipogenesis in hMSCs expressing myr-Akt (Fig. 6B). By contrast, indomethacin, a PPAR agonist (Lehmann et al. 1997), promoted adipogenesis in both control and myr-Akt-expressing cells.

Figure 4 Suppression of p110α promotes adipogenesis. (A) MSC cultures were starved overnight, treated with imatinib or vehicle (PBS), as indicated, and then stimulated with rhPDGF-BB (10 ng/ml) for 5 min. Cell proteins were harvested, resolved by SDS–PAGE and then transferred to PVDF membranes. The activation status of PDGFR effector molecules was determined using phospho-specific antibodies as indicated. (B) Starved MSC cultures were treated with vehicle (0.1% DMSO), NVP-BEZ235 (100 nmol/l), PIK-75 (50 nmol/l), TGX-221 (200 nmol/l) or IC87114 (2 μmol/l) for the indicated times, pulsed with rhPDGF-BB and the phosphorylation status of Akt was determined by immunoblotting. (C) Starved MSC cultures were treated with PIK-75 (as indicated) or vehicle (0.1% DMSO) for 1 h, pulsed with rhPDGF-BB and the phosphorylation status of Akt was determined by immunoblotting. (D) MSC cultures were treated with PIK-75 (as indicated) or vehicle (0.1% DMSO) under induction conditions for 32 days and adipocytes visualised using nile red. (E) The number of adipocytes was enumerated by counting nile red-labelled cells and DAPI-labelled cell nuclei (mean ± s.d. of triplicate measurements). *P < 0.001. One-way ANOVA with Dunnett’s post test. (F) An ELISA was used to determine the amount of adiponectin secreted from PIK-75 (10 nmol/l)-treated MSC cultures (black bars) and vehicle controls (white bars) at the indicated time points (mean ± s.d. of triplicate measurements). (G) Secreted adiponectin was analysed by non-denaturing PAGE, to identify complexes, and denaturing PAGE, to detect total adiponectin, followed by immunoblotting. Cellular adiponectin was analysed by denaturing PAGE and immunoblotting. As a loading control, heat-shock protein 90 (HSP90) levels are shown. Molecular weights: HMW, ~360 kDa; LMW, ~180 kDa; total, 30 kDa. (*P < 0.005. t-test, relative to controls). Bar=500 μm.
and DAPI-labelled cell nuclei (mean adipocytes were enumerated by counting nile red-labelled cells
A) Adipocytes were labelled using nile red. (B) The number of

ments; * Bar

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Figure 5 Mitogens that activate PI3 kinase negate the pro-
adipogenic effects of imatinib. MSC cultures were stimulated with
rhPDGF-BB (10 μg/ml) or rhEGF (20 ng/ml) in the presence or
absence of imatinib (3 μmol/l) or PIK-75 (10 nmol/l) for 28 days.
(A) Adipocytes were labelled using nile red. (B) The number of
adipocytes were enumerated by counting nile red-labelled cells
and DAPI-labelled cell nuclei (mean ± s.d. of triplicate measure-
mements; *P<0.001. One-way ANOVA with Dunnett’s post-test).
Bar = 500 μm.

Discussion

Here, we demonstrate that therapeutically relevant
concentrations of imatinib promote the differentiation of
human MSCs into adipocytes, which in turn secrete the
HMW form of adiponectin. Imatinib failed to
promote adiponectin secretion from mature adipocytes,
suggesting that imatinib does not promote
adiponectin secretion through a direct effect on
adiponectin gene expression but rather indirectly
through an effect on adipocyte numbers.

MSCs are pluripotent and lineage specificity can be orchestrated in vitro using a combination of ligands
that act directly, or indirectly, to drive lineage-specific
transcription factors (Pittenger et al. 1999). Crucial to
this response is the addition of glucocorticoid that acts
to prime cells to be responsive to differentiation signals
(Gimble 1998). Treatment of MSCs with imatinib alone
is sufficient to stimulate adipogenesis in glucocorticoid-
treated cells, as evidenced by a temporal increase in key
adipogenic transcription factors C/EBPα and PPARγ2,
the accumulation of lipid-laden vacuoles and secretion of the adipokine adiponectin. Glucocorticoid-treated
cells appear highly sensitive to the pro-adipogenic
effects of imatinib, resulting in adipogenic differen-
tiation of MSCs at hydrocortisone concentrations well
below physiological levels (5 nmol/l; data not shown).
As the reductase required to regenerate active cortisone
in vivo, 11β-hydroxysteroid dehydrogenase type I
(11βHSD), is highly expressed in MSCs and bone cells
(Justesen et al. 2004), bone marrow-synthesised steroid
and imatinib could act locally on MSC populations to
drive adipogenic differentiation.

Previous studies have demonstrated that PDGF is a
potent MSC mitogen that has been shown to inhibit
MSC differentiation (Hock & Canalis 1994, Kubota et al.
Imatinib is a potent PDGFR inhibitor (Buchdunger
et al. 2000), suggesting that imatinib mediates its pro-
adipogenic effects through inhibition of PDGFR. In
support of this, we have shown that functional blocking
antibodies to PDGFR promote adipogenesis and
adiponectin secretion when used at concentrations
that suppress cell proliferation and PDGF-induced Akt
activation similar to that achieved with imatinib. We
therefore hypothesise that, in cultures treated with
physiological levels of glucocorticoid alone, the basal
adipogenic response to glucocorticoid is suppressed by
PDGF, which is present in bovine serum, and is secreted
in an autocrine and/or paracrine fashion by MSCs
(Bonner 2010). Addition of imatinib or functional
blocking antibodies ameliorates this inhibitory effect
allowing differentiation to proceed unabated.

Studies using mouse 3T3-L1 pre-adipocytes and
human subcutaneous adipose-derived stem cells have suggested that PDGF-induced activation of PKCz and
IKKβ mediates the inhibitory effect of PDGF on
adipogenesis (Artemenko et al. 2005, Gagnon et al.
2009). However, we failed to detect activation of these
pathways in response to PDGF in bone marrow-derived
MSCs (data not shown), which may be due to cell-type
differences or the PDGF isoform used. In hMSCs, PDGF
strongly activates the PI3 kinase pathway and previous
studies have suggested that the anti-differentiative
effects of PDGF could be inhibited using PI3 kinase
inhibitors such as Wortmannin and LY94002 (Kratch-
Consistent with this view, we have demonstrated that
imatinib is a potent inhibitor of PDGF-induced Akt
phosphorylation, a major substrate of the PI3 kinase
pathway. Furthermore, the pro-adipogenic effect of
imatinib, but not that of indomethacin, was negated by
constitutive activation of Akt. Addition of exogenous EGF to MSC cultures also inhibited imatinib-induced adipogenesis. EGF activates the PI3 kinase pathway in MSCs, albeit to a lesser extent than PDGF (Kratchmarova et al. 2005) and, unlike PDGFR, EGFR is insensitive to imatinib (Buchdunger et al. 2000). The anti-differentiative effects of PI3 kinase signalling on hMSCs was further demonstrated using PIK-75, a selective inhibitor of p110\(\alpha\), the catalytic subunit of the obligate heterodimer type I PI3 kinase. PIK-75 promotes hMSC adipogenic differentiation and adiponectin secretion even in the presence of exogenous growth factors. Unlike imatinib, PIK-75 inhibits PI3 kinase activity irrespective of the nature of the mitogen. At the concentrations used in this study, PIK-75, imatinib and anti-PDGFR antibodies inhibit proliferation and suppress the PI3 kinase pathway.

While inhibition of proliferation may be important for the pro-adipogenic effects, studies using \(\gamma\)-irradiation show that it is not sufficient to promote differentiation under induction conditions (data not shown). Further analysis of Akt substrates that are suppressed or activated in PIK-75- and imatinib-treated MSCs is required to gain further mechanistic insight.

Our hypothesis that inhibition of PI3 kinase, downstream of PDGFR, promotes adipogenic differentiation of bone marrow-derived hMSCs is in contrast to a large body of data demonstrating an essential role for PI3 kinase signalling in adipogenesis in murine cells. Much of this evidence has been obtained using the well-characterised murine 3T3-L1 cell line (derived from white adipose tissue), which differentiates into adipocytes in response to insulin. This insulin-mediated differentiation process can be blocked using PI3 kinase inhibitors Wortmannin and LY94002 and by over-expression of a dominant-negative p85 (Sakaue et al. 1998, Xu & Liao 2004). Furthermore, over-expression of constitutively active forms of PI3 kinase or Akt has been shown to recapitulate the effects of insulin on Glut4 translocation and adipogenic differentiation in these cells (Kohn et al. 1996, Magun et al. 1996). Insulin and the PI3 kinase pathway has also been demonstrated to be essential for adipogenic differentiation in primary mouse cells, as demonstrated by a complete lack of adipocytes and complete abrogation of PI3 kinase activity in murine embryonic fibroblasts (MEFs) isolated from insulin receptor substrate \((Irs1)\) and \((Irs2)\) knockout mice (Miki et al. 2001). Furthermore, MEFs isolated from \(p110\alpha\) null mice fail to differentiate into adipocytes in response to insulin (Zhao et al. 2006).

Important distinctions that may account for our findings relate to the intrinsic responsiveness of hMSCs to insulin signalling and to the nature of the pro-adipogenic stimuli used in our study. Unlike mouse 3T3-L1 cells, hMSCs do not differentiate into adipocytes in response to insulin or insulin-like growth factor 1 (IGF1) alone, instead requiring glucocorticoid to prime the cells to be responsive to pro-adipogenic stimuli (Greenberger 1979, Scavo et al. 2004). In light of this, we have used a differentiation media solely consisting of physiological levels of glucocorticoid to prime the cells to be responsive to pro-adipogenic stimuli (Greenberger 1979, Scavo et al. 2004). In light of this, we have used a differentiation media solely consisting of physiological levels of glucocorticoid to prime the cells to be responsive to pro-adipogenic stimuli (Greenberger 1979, Scavo et al. 2004).

![Figure 6 Constitutively activated Akt negates the imatinib response. Myristoylated murine Akt (myr-Akt) and empty vector (vector) were introduced into MSCs by retroviral transduction. (A) Cellular proteins from vector control and myr-Akt-infected cells were harvested, resolved by SDS–PAGE and then transferred to PVDF membranes. The activation status of p70S6K was determined by immunoblotting. (B) Vector control and myr-Akt-expressing cells were cultured under induction conditions with vehicle (0.1% DMSO), imatinib (3 \(\mu\)mol/l) or indomethacin (60 \(\mu\)mol/l) for 28 days. The number of adipocytes was enumerated by counting nile red-labelled cells and DAPI-labelled cell nuclei (mean±s.d. of triplicate measurements; *\(P<0.001\). One-way ANOVA with Dunnett’s post-test).](Image 100x584 to 158x642)

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adipogenesis by uncoupling the normal regulation of Akt. However, our results indicate that Akt phosphorylation is decreased during PIK-75 or imatinib-mediated differentiation of hMSCs (Supplementary Figure 1, see section on supplementary data given at the end of this article), indicating that Akt signalling is not hyper-activated in response to these drugs under induction conditions. PI3 kinase-independent mechanisms of adipogenic differentiation have also been described. Suppression of MAPK activity in hMSCs has been shown to promote adipogenesis in the absence of insulin and IGF1 signalling (Jaiswal et al. 2000). However, we failed to detect a decrease in the activation status of the MAPK substrates Erk1/2 in the presence of imatinib or PIK-75 during differentiation (Supplementary Figure 1), suggesting that suppression of the MAPK pathway was not responsible for the pro-adipogenic effect of these inhibitors. Conversely, p38 MAPK signalling has been proposed as a pro-adipogenic stimuli, as inhibition of p38 has been shown to negate adipogenic differentiation in hMSCs (Aouadi et al. 2007). However, imatinib suppresses PDGF-induced p38 activation (Fitter et al. 2008), suggesting that activation of p38 MAPK is an unlikely mechanism by which imatinib or PIK-75 promotes adipogenesis.

In cultures treated with PIK-75 or imatinib, the HMW form of adiponectin is the predominant form secreted. Several lines of evidence suggest that the HMW form is the most active with respect to modulating insulin sensitivity (Waki et al. 2003, Lara-Castro et al. 2006, Wang et al. 2006). Consistent with this, a analysis of plasma adiponectin complexes in CML patients, before and after 3 months of imatinib therapy, revealed a significant increase in the level of HMW complexes (Fitter et al. 2010). In humans, adiponectin levels correlate negatively with insulin resistance and metabolic syndrome, with low adiponectin levels being associated with a higher risk of type 2 diabetes (Li et al. 2009). Thiazolidinedione compounds, a class of anti-diabetic drugs that improve systemic insulin sensitivity, are thought to work, at least in part, by promoting the formation of small adipocytes that secrete high levels of HMW adiponectin (Yamauchi et al. 2001, Phillips et al. 2003, Nawrocki et al. 2006).

In summary, we have identified the PI3 kinase pathway downstream of PDGFR as important in mesenchymal cell differentiation. Inhibition of this pathway, using imatinib or PIK-75, promotes adipogenesis and secretion of HMW adiponectin. In light of this, we hypothesise that inhibition of PDGFR signalling in imatinib-treated CML patients results in increased intramedullary adipogenesis and, subsequently, in an increase in circulating adiponectin levels. These increased adiponectin levels may account for the improvements in glucose and lipid metabolism observed in some imatinib-treated CML patients with concurrent type 2 diabetes.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1550/JME-12-0003.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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