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Inhibition of platelet-derived growth factor receptorβ by imatinib mesylate suppresses proliferation and alters differentiation of human mesenchymal stem cells *in vitro*

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Abstract. *Objectives*: Recent data show that Imatinib mesylate (IM) also affects haematopoietic stem cells (HSC), T lymphocytes and dendritic cells that do not harbour constitutively active tyrosine kinases. *Materials and methods*: We evaluated possible effects of IM on human bone marrow-derived mesenchymal stem cells (MSC) *in vitro*. *Results*: Screening the activity of 42 receptor tyrosine kinases revealed an exclusive inhibition of platelet-derived growth factor receptorβ (PDGFRβ). Analysis of downstream targets of PDGFRβ demonstrated IM-mediated reduction of Akt and Erk1/2 phosphorylation. Culture of MSC with IM led to the reversible development of perinuclear multi-vesicular bodies. The proliferation and clonogenicity of MSC were significantly reduced compared to control cultures. IM favoured adipogenic differentiation of MSC whereas osteogenesis was suppressed. The functional deficits described led to a 50% reduction in the support of clonogenic haematopoietic stem cells, cultured for 1 month on a monolayer of MSC with IM. *Conclusion*: In summary, inhibition of PDGFRβ and downstream Akt and Erk signalling by IM has a significant impact on proliferation and differentiation of human MSC *in vitro*.

INTRODUCTION

Mesenchymal stem cells (MSC) have been identified in the bone marrow (BM) as multi-potent non-haematopoietic progenitor cells that differentiate into osteoblasts, adipocytes, chondrocytes, tenocytes, skeletal myocytes and cells of visceral mesoderm (Pittenger *et al*. 1999; Jiang *et al*. 2002; Smith *et al*. 2004). MSC do not express haematopoietic markers, but a specific pattern of molecules, such as SH2 (CD105), SH3 and SH4 (CD73), VCAM-1 (CD106), THY-1 (CD90) and STRO-1 (Pittenger *et al*. 1999; Oswald *et al*. 2004). MSC interact with haematopoietic stem cells (HSC), influencing their homing and differentiation through cell–cell contact and the

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production of factors including chemokines (Majumdar *et al*. 2000). MSC can be cotransplanted with HSC to improve their engraftment in BM (Koc *et al*. 2000), and they may be used to repair or regenerate damaged or mutated bone or cartilage (Pereira *et al*. 1995; Horwitz *et al*. 1999). Finally, MSC have recently been shown to possess immunoregulatory functions (Le Blanc *et al*. 2004; Aggarwal & Pittenger 2005).

Imatinib mesylate (IM) is routinely used for the treatment of patients with Philadelphia chromosome positive chronic myeloid leukaemia (Druker *et al*. 2001; Kantarjian *et al*. 2002; O'Brien *et al*. 2003). The specific blockade of the tyrosine kinase activity of c-kit and platelet-derived growth factor receptorβ (PDGFRβ) by IM has been well described for leukaemic and other malignant cells (Apperley *et al*. 2002; Demetri *et al*. 2002). Recent data support the hypothesis that IM exerts non-specific inhibitory effects on $CD34^+$ normal haematopoietic progenitor cells and dendritic cells (Appel *et al*. 2004; Bartolovic *et al*. 2004). In addition, IM has been shown to inhibit antigen-specific T cell proliferation, thereby potentially mediating immunosuppressive effects (Seggewiss *et al*. 2005).

Surprisingly, little is known about the effects of IM on benign non-haematopoietic stem cells. Because the interaction of haematopoietic stem cells (HSC) with mesenchymal stem cells (MSC) in the bone marrow environment is of special importance for the maintenance of the quiescent HSC pool and MSC might be of relevance for the regeneration of mesenchymal tissue, we studied the effects of IM on primary cultures of human MSC.

MATERIALS AND METHODS

MSC isolation and culture

Bone marrow samples were obtained from healthy donors who underwent a standard bone marrow harvest under general anaesthesia. The volunteer donor's age ranged between 25 and 48 years and all gave informed consent to the research protocol, which had been approved by the institutional review board of the University Hospital of Dresden. MSC were isolated by density gradient (mononuclear fraction) and adherence capacity, based on previously described methods (Majumdar *et al*. 1998; Oswald *et al*. 2004) and cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA; www.invitrogen.com) supplemented with 10% foetal calf serum (FCS) (Biochrom, Cambridge, UK; www.biochrom.de). The immunophenotype (characteristically $CD73^+$, $CD90^+$, $CD105^+$, $CD34^-$, $CD45^-$) and ability to form fibroblastoid colony-forming units (CFU-F) were checked in all samples. All experiments were performed using Imatinib mesylate (IM) at the indicated concentrations dissolved in dimethyl sulfoxide (DMSO) or with 0.5 μl/ml DMSO as control.

Receptor tyrosine kinases (RTK)-phospho array and Western Blots

Nearly (70–80%) confluent MSC cultures were incubated for 24 h in DMEM with 10% FCS. Subsequently, reagents were added in concentrations and for times as indicated in the legend of Fig. 1. After incubation, cells were trypsinized, washed and lysed with extraction buffer (Illmer *et al*. 2004). Protein concentration was measured using the Bio-Rad protein assay kit (Richmond, CA, USA; www.bio-rad.com). The Proteome Profiler™ array – human phospho-RTK array kit was used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA; www.rndsystems.com) loading 250 μg protein/membrane. For immunoblotting, 30 μg total proteins were loaded in each lane. Electrophoresis was performed in 10% acrylamide gels. Nitrocellulose membranes (www.amershambiosciences.com) were incubated with primary

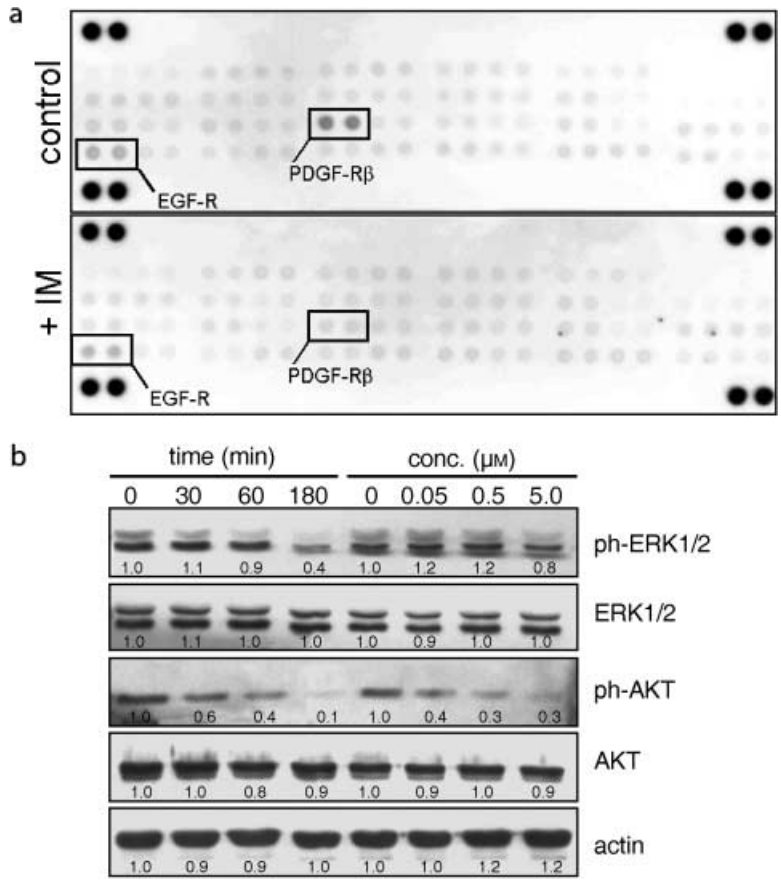

Figure 1. Imatinib mesylate blocks phosphorylation of PDGFRβ, Akt and Erk. (a) Phospho-RTK array: MSC were cultured for 24 h in order to induce autophosphorylation of RTKs. Then, cells were incubated for 1 h with 0.1 μl/ml DMSO (control) or 1 μM IM. Phosphorylation of EGFR and PDGFRβ can be noticed, but only PDGFRβ is inhibited by IM ($n = 2$). (b) Phosphorylation of Akt and Erk1/2 were evaluated in MSC ($n = 5$). In order to evaluate the time-dependent effect, samples were treated with 1 μM IM for 0, 30, 60 and 180 min, respectively. To test the concentration dependency, samples were incubated for 1 h with the respective concentrations of IM before cell lysis and protein extraction. Quantitative densitometric analyses relative to control (time 0 = 1.0; concentration 0 = 1.0) are provided.

antibodies at 4 °C overnight. Antibodies against phosphorylated and total p44/42 (Erk) and Akt were purchased from Cell Signalling (www.cellsignal.com; Danvers, MA, USA) and anti-actin antibody from Sigma (Sigma, St. Louis, MO, USA; www.sigmaaldrich.com). The secondary antibodies used were horseradish peroxidase goat antimouse or horseradish peroxidase goat antirabbit (Dako, Carpinteria, CA, USA; www.dakocytomation.de). Blots were developed with the chemiluminescence detection kit ECL-Plus (Amersham biosciences, Arlington Heights, IL, USA; www.amershambiosciences.com).

Phase contrast and electron microscopy

For phase contrast imaging, 10 000 MSC/cm^2 were seeded in 12-well plates and cultured for 24 h to reach maximal attachment. The medium was changed and cells were incubated for 24 h then photographed using an Axiovert 25 phase contrast microscope (Carl Zeiss, Oberkochen,

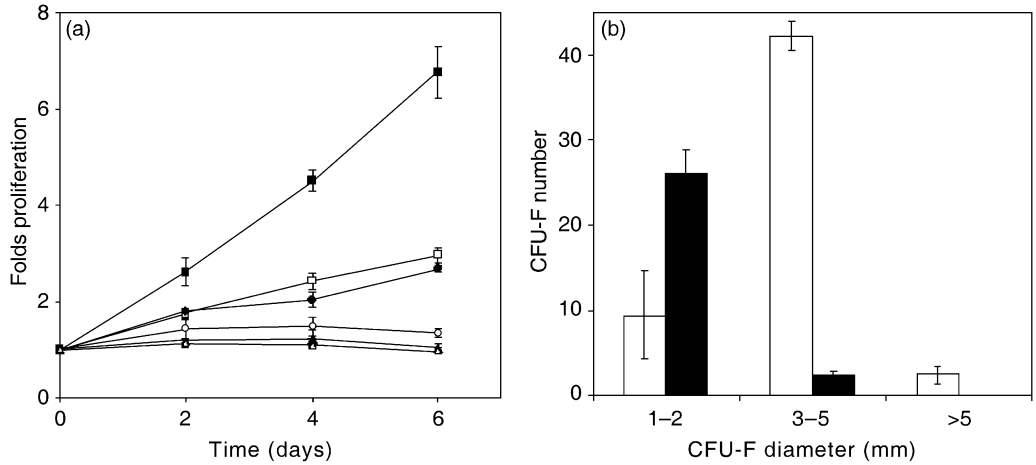

Figure 2. Proliferation (a) and clonogenicity (b) of MSC are negatively affected by Imatinib mesylate. (a) PDGFβ (10 ng/ml; ■, $n = 3$) has a positive effect on cell proliferation as compared to controls (□, $n = 14$), while 20 μM UO126 (△, $n = 3$) completely inhibits cell growth. IM inhibits cell proliferation in a concentration-dependent manner [0.05 μM (●, $n = 3$), 0.5 μM (○, $n = 6$) and 5.0 μM (▲, $n = 4$)]. (b) Colony-forming units assays denote a decrease of total CFU-F in presence of 5 μM IM (black bars) as compared to controls (open bars). Specifically, IM favoured the presence of small colonies, while intermediate and large size CFU-F were strongly inhibited as compared to controls ($n = 4$).

Germany). For transmission electron microscopy, MSC were cultured for 6 days with 10 μM IM or 1 μl/ml DMSO (control), fixed with 2.5% glutaraldehyde buffer for 1 h, washed and post-fixed with 1% OsO_4 for 1 additional hour. Samples were then dehydrated with increasing concentrations of ethanol and embedded in Epon™ (Vantico GmbH, Bad Sackingen, Germany). Finally, ultra-thin slides (65 nm) were set in copper grids and stained with uranyl acetate and lead citrate. Samples were examined with a Zeiss EM 906 microscope (Zeiss, Oberkochen, Germany).

Proliferation and fibroblastoid colony-forming units (CFU-F) assays

For all proliferation curves, 6000 MSC/cm^2 were seeded in 12-well plates and allowed to attach for 24 h. Medium was subsequently replaced with DMEM/10% FCS containing 0.5 μl/ml DMSO (control), variable concentrations of IM, 10 ng/ml PDGFβ (human recombinant, R&D Systems; www.rndsystems.com) or 20 μM UO126 (www.cellsignal.com). Living cells were counted with trypan-blue exclusion dye in a Neubauer chamber. Medium containing the respective supplements was changed at day 3. CFU-F were determined by seeding 2.5×10^6 bone marrow-derived mononuclear cells in one 25-cm^2 flask and cultured for 14 days with stem cell medium (NH-Medium, Miltenyi Biotec, Auburn, CA, USA; www.miltenyibiotec.com), without any medium change. At day 14, samples were fixed with 4% *P*-formaldehyde and stained with Azur-eosin-methylene blue solution (Giemsa, Merck, Darmstadt, Germany). Visible colonies (more than 50 cells) were counted and grouped according to size.

Osteogenic and adipogenic MSC differentiation

Osteogenic differentiation of MSC was induced by incubating 70–80% confluent cultures in standard medium containing 0.2 mM ascorbic acid (www.fagron.de; Fagron GmbH & Co., Barsbüttel, Germany), 0.1 μM dexamethasone and 10 mM β-glycerophosphate (Sigma; both www.sigmaaldrich.com). Medium was replaced every 3–4 days. Alkaline phosphatase (ALP) activity was measured at day 11. Briefly, cells were trypsinized and lysed with 1.5 M Tris-HCl

solution containing 1.0 mM $ZnCl_2$, 1.0 mM $MgCl_2$ and 1% Triton X-100 for 10 min. Lysates were centrifuged at 20 800 ***g*** for 30 min and incubated with 3.7 mM *P*-nitrophenylphosphate solution containing 0.1% Triton X-100 for 30 min. Released *P*-nitrophenolate was determined spectrophotometrically at 405 nm. Protein concentration was measured using the Bio-Rad protein assay kit (www.bio-rad.com). At day 14, calcium phosphate precipitates were stained for Kossa. Briefly, cells were fixed with a fresh solution of 10% formalin for 30 min, stained with 2% silver nitrate solution and developed with 1% pyrogallol. In order to induce MSC adipogenesis, cells were seeded in 6-well plates and grown to 70–80% confluency. Then, medium was changed to MesenCult™ containing adipogenic stimulatory supplements (www.stemcell.com), changed every 3–4 days. At day 14, adipocytes were counted under phase contrast on a defined central area. Adipocytes were arbitrarily defined as cells containing five or more visible lipid vacuoles. The total number of living cells per well was determined as described above. The number of adipocytes was extrapolated to total well area (9.62 cm^2) allowing the determination of MSC differentiated into adipocytes.

Four-week culture-initiating assays

Haematopoietic stem cells (HSC) were isolated from G-CSF-mobilized leukapheresis products and selected for $CD34^+$ using a magnetic cell-sorting system according to manufacturer's recommendations (www.miltenyibiotec.com). 3000 $CD34^+$ HSC/cm^2 were co-cultured over a confluent MSC monolayer with long-term culture medium (MyeloCult H 5100, www.stemcell.com) supplemented with 1 μM hydrocortisone for 4 weeks and with or without 5 μM IM. Afterwards cells were trypsinized, washed and counted. Approximately 50 000 recovered cells were plated in triplicate in 24-well plates in CFU-GM medium (Methocult GF H4435, www.stemcell.com) and assessed at day 14 for the presence of colony forming units. The number of CFU-GM was used to calculate the frequency of colony-forming cells per number of HSC originally seeded. Differential progenitor growth was detected by scoring granulocyte-macrophage (CFU-GM), macrophage (CFU-M), mixed (CFU-GEMM) and burst-forming erythroid colonies (BFU-E).

Statistical analysis

The number of MSC-donors used for each experiment is indicated in the legend of the respective figure. Averages are shown with standard error of the mean as error bars. Statistical comparison was performed using a paired student *t*-test. Observed differences were assumed as significant when the calculated two-sided *P*-value was < 0.05.

RESULTS

IM blocks the MAPK/ERK- and Akt/PI3K-signalling pathways by specific inhibition of PDGFRβ in MSC

In order to determine the molecular target(s) of IM in MSC, 42 different receptor tyrosine kinases (RTK) were screened for auto-phosphorylation and subsequent inhibition by 1 h of treatment with1 μm IM. As seen in Fig. 1(a), epidermal growth-factor receptor (EGFR) and PDGFRβ were activated by phosphorylation after 24 h of culture, but only PDGFRβ phosphorylation was inhibited by IM. RTKs are the initial step for activation of several signalling pathways, including MAPK/Erk, Akt/PI3K and JNK/MAPK. We evaluated critical components of these pathways, finding inhibition of Erk1/2 and Akt in a concentration and time-dependent manner (Fig. 1b), while JNK1 phosphorylation was not affected (not shown).

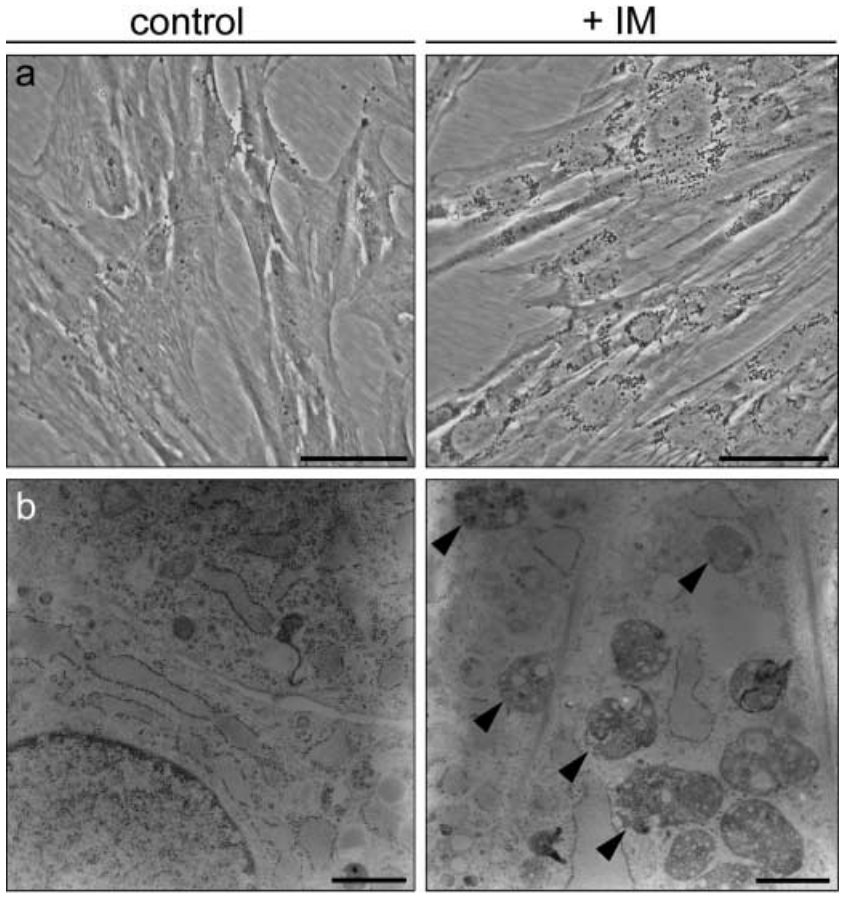

Figure 3. Imatinib mesylate alters the morphology of MSC. (a) Phase contrast microscopy of MSC cultured for 24 h with or without IM ($n = 3$). (b) Perinuclear granula resembled multi-vesicular bodies (highlighted with arrowheads) as observed in MSC after 6 days with or without IM using transmission electron microscopy ($n = 2$).

IM changes MSC morphology

Characteristic morphological changes could be observed in MSC incubated with IM: spindle-shaped cells flattened, lost confluence and developed perinuclear granules (Fig. 3a). After 24 h of incubation, perinuclear granules were evident only at concentrations of 5 μM or higher. In addition, time-lapse analyses showed the appearance of granula gradually in the cytosol within the first 12 h with a pronounced migration to the perinuclear region after 18–20 h. These granules resembled multi-vesicular bodies as suggested by transmission electron microscopy (Fig. 3b). After removal of IM, the granules disappeared completely within 2 days.

IM decreased proliferation and clonogenicity of MSC

Addition of IM inhibited growth of MSCs in a concentration-dependent manner. As shown in (Fig. 2a), the negative effect of IM on MSC proliferation was detectable after short times of culture, but seemed not to be due to toxicity because the number of living cells did not decrease

below the original number seeded. Annexin V/PI staining after 3 days of culture with 5 μM IM showed no increase in apoptotic cells compared to controls (FACS measurements, data not shown). Because the lower proliferation rate was expected to be due to the inactivation of PDGFRβ in MSC leading to inhibition of the MAPK/Erk signalling pathway, we evaluated the effect of human recombinant PDGFβ and the MAPK kinase (MEK1/2)-specific inhibitor UO126 on MSC proliferation. As expected, addition of 10 ng/ml PDGFβ-favoured cell proliferation enormously while 20 μM UO126 inhibited the cell growth to a similar degree as did IM at 5 μM. To investigate effects of prolonged IM exposition, we kept MSC in medium containing 5 μM IM for up to 21 days with medium changes twice a week. Cell proliferation was blocked leading to constant cell numbers up to day 21. No signs of cell death or apoptosis could be detected.

In accordance with these observations, IM also affected the clonogenicity of MSC, evaluated by the number and size of CFU-F after 14 days of culture. The absolute number was drastically lower in IM-treated samples compared to controls (Fig. 2b). Specifically, colonies of intermediate and large size (3 mm to 5 mm, and bigger than 5 mm diameter, respectively) were significantly reduced in IM-treated samples ($P = 0.0005$), favouring the growth of small colonies (1–2 mm diameter). Therefore, the effect of IM on CFU-F seemed to be predominantly inhibition of cell proliferation, rather than the induction of apoptosis.

IM affected osteogenic and adipogenic differentiation of MSCs

The negative effect of IM on MSC osteogenesis was apparent from reduced calcium phosphate precipitates compared to controls, after 14 days of culture under osteogenic stimuli. Although a typical disruption of the cell layer and loss of confluence were observed, differentiation was not fully inhibited (Fig. 4, upper left panels). In order to quantify the effect of IM on osteogenesis, we evaluated the induction of ALP activity on day 11 (Fig. 4, upper right panel). IM decreased ALP activity significantly from 4.4 fold induction (control) to 3.4 fold, while the specific MEK inhibitor UO126 led to only a 2.0 fold induction of ALP (data not shown). On the other hand, differentiation of MSC into adipocytes was favoured in the presence of IM (Fig. 4, lower panels). Adipogenic differentiation was induced to a similar degree by the specific MEK inhibitor UO126 supporting the role of Erk1/2 signalling for osteogenic differentiation (data not shown).

IM affects MSC-mediated support of HSC growth

A characteristic function attributed to MSCs is their ability to support long-term growth of haematopoietic stem cells (HSC). In order to evaluate this feature, we performed a 4-week co-culture of $CD34^+$ selected HSC over a monolayer of MSCs, followed by a CFU-GM assay. Incubation of HSC over MSC in the presence of IM halved the number of haematopoietic cells with clonogenic potential, compared to control co-cultures ($P < 0.01$) (Fig. 5). Nevertheless, this effect was mainly due to a reduced recovery of HSCs after 4 weeks of co-culture, rather than to a direct negative effect on the clonogenic capacity of the recovered HSC. In fact, the number of CFU-GM per dish was higher when HSCs harvested after 4 weeks of culture with IM were seeded at the same concentration as the controls (205.9% higher, $P < 0.01$) (data not shown), suggesting that early stem and progenitor cells are resistant to IM. Limiting the exposure of MSC to a 72-h preincubation with 5 μM IM did not reduce the yield of CFU-GM after 4 weeks arguing for a reversible effect of IM. In addition, we saw no significant difference in the proportion of CFU-GM, CFU-M, CFU-GEMM and BFU-E derived from HSC after 4 weeks between IM-treated co-cultures and controls. Therefore, we propose that the major effect of IM was the impairment of the MSC feeder-layer's quality by inducing adipogenic differentiation thereby reducing the support of HSC, rather than by directly suppressing HSC proliferation.

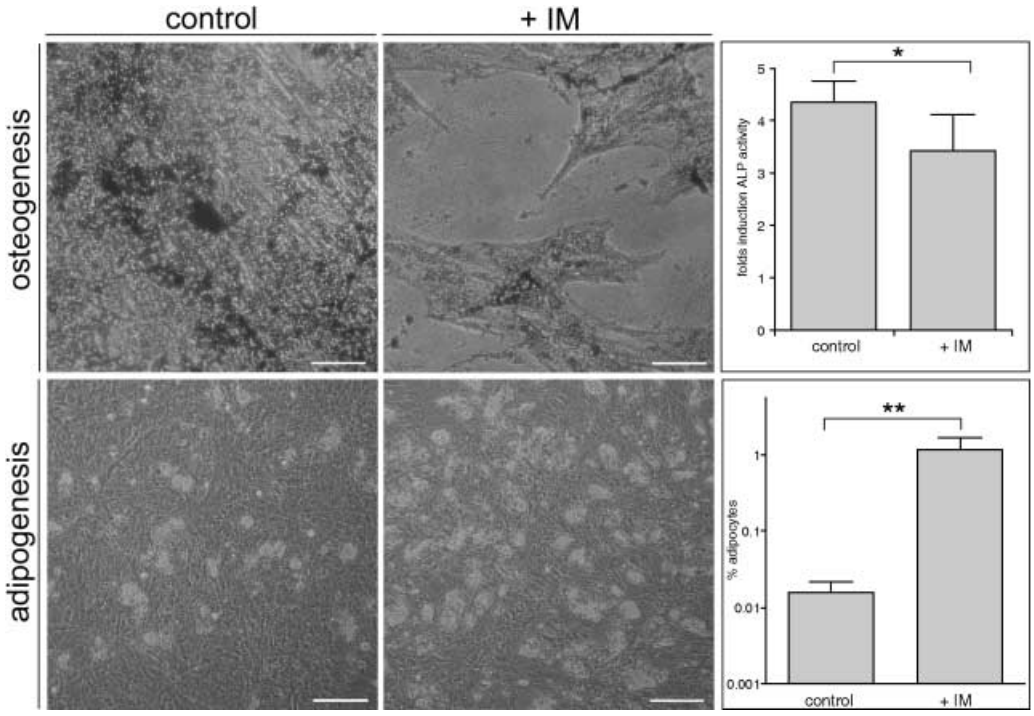

Figure 4. Imatinib mesylate affects osteogenic and adipogenic differentiation of MSC. Upper panels: Calcium phosphate precipitates stained with von Kossa at day 12 were diminished in presence of 5 μM IM as compared to control cultures. In addition, disruption of the cell layer and loss of confluence were detected. Upper right panel, effect of 5 μM IM on ALP activity of MSC after 11 days of osteogenic differentiation ($n = 4$). Lower panels: MSC cultured for 14 days with adipogenic medium containing 0.5 μl/ml DMSO (control) or 5 μM IM, representative regions were directly photographed. This increase of adipocytes was quantified as described in materials and methods (lower left panel, $n = 4$). Scale bars = 100 μm *$P = 0.02$, **$P = 0.12$.

DISCUSSION

This is the first detailed report describing the effects of IM on bone marrow-derived MSC. We evaluated the effect of IM on morphology, clonogenicity, differentiation potential, support of HSC growth and identified molecular targets that are related to the inhibitory effect of IM on MSC.

The data presented here support in different ways the importance of PDGFRβ and EGFR signalling for MSC proliferation and differentiation. First, the phospho-RTK array revealed exclusive phosphorylation of EGFR and PDGFRβ in *ex vivo*-expanded MSC. Second, IM blocked constitutive PDGFRβ signalling with subsequent inhibition of the PI3K/Akt and Erk pathway. Activation of the MAPK/Erk-signalling pathway influences proliferation and differentiation in various types of mammalian cells (Zhang & Liu 2002), including MSC. Additionally, the MAPK/Erk pathway has been previously linked to osteogeneic differentiation, and inhibition of this pathway induces adipogenesis in MSC (Jaiswal *et al*. 2000). Specifically, the MAPK pathway can activate RUNX-2 and blocks PPAR-gamma, transcription factors that are critical for osteogenic and adipogenic differentiation (Xiao *et al*. 2000; Kim *et al*. 2001).

A recent publication provided evidence for differential signalling in MSC via EGFR or PDGFRβ (Kratchmarova *et al*. 2005). The authors showed that regulatory and catalytic members

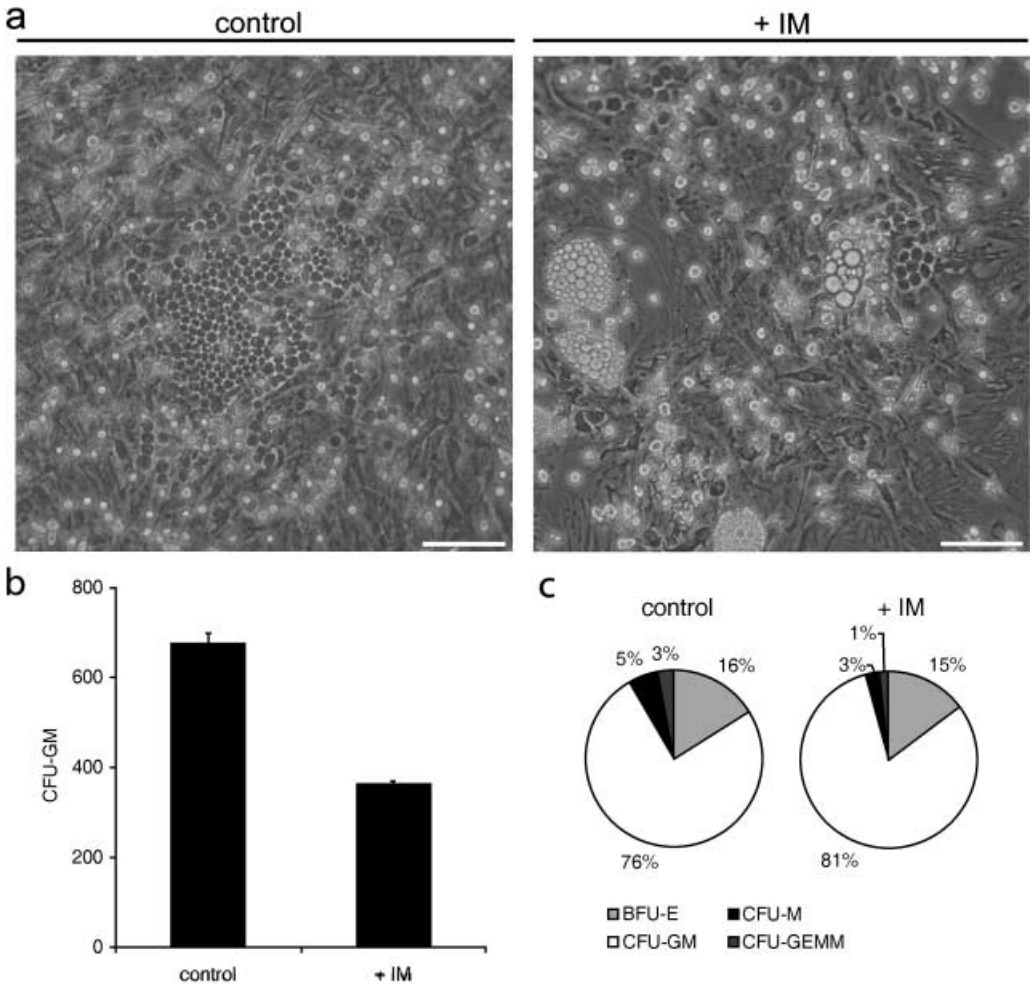

Figure 5. Imatinib mesylate affects MSC-mediated support of HSC growth. (a) Control (left panel) shows $CD34^+$ selected haematopoietic stem cells (HSC) aggregating in large cobblestone forming areas (CFA) over a confluent monolayer of MSC after 4 weeks of co-culture. In contrast, co-cultures of HSC and MSC in the presence of 5 μM IM (right panel) contained smaller and fewer CFA. In addition, a disrupted monolayer of MSC with spontaneous adipogenesis can be observed ($n = 3$). (b) Frequency of CFU-GM, after 4-week coculture ($n = 3$). Scale bar on (a) = 100 μm (c) Proportion of granulocyte-macrophage (CFU-GM), macrophage (CFU-M), mixed (CFU-GEMM) and burst-forming eythroid (BFU-E) colonies derived from HSC cultured for 4 weeks on MSC.

of the PI3Kinase family were critically affected by PDGFRβ stimulation, whereas other MAPKinases like Erk1/2 could be stimulated by either PDGFRβ or EGFR. As shown by us, the MAPKinase inhibitor UO126 had a negative effect on proliferation and osteogenic differentiation of MSC in their report. The set-up of our experiments differed in that we did not add exogenous growth factors but specifically blocked PDGFRβ signalling. In accordance with the findings of Kratchmarova *et al*., we observed significant inhibition of the PI3/Akt pathway after the inhibition of PDGFRβ. Because EGFR was not blocked to any extent, the less pronounced reduction of Erk1/2 phosphorylation may be attributed to the residual activity of EGFR within our system.

Because multi-vesicular bodies could only be observed at the highest concentration of IM, corresponding to maximum plasma concentrations achievable only for short-time periods in patients, the relevance of these findings has to be discussed (Le Coutre *et al*. 2004). Nevertheless, the effects on proliferation and differentiation could be observed at IM concentrations comparable to trough plasma levels.

The inhibitory effects of IM in the co-culture experiments may in part be explained by the direct inhibition of kinase activity in clonogenic haematopoietic progenitors (Bartolovic *et al*. 2004; Dewar *et al*. 2005). But, as mentioned above, the observed reduction in overall CFU-GM was mainly due to a reduced recovery of HSCs after 4 weeks of co-culture. As shown in Fig. 5(a), this was mainly due to the functional loss of the MSC layer incubated with IM leading to a reduced recovery of HSC after 4 weeks. Therefore, we propose that the major effect of IM is the impairment of the MSC feeder-layer's quality by inducing adipogenic differentiation thereby reducing the support of HSC, rather than by directly suppressing HSC proliferation. Given the limitations of our *in vitro* model, we are not able to speculate on the chronic effects of IM on MSC and HSC *in vivo*.

No case of irreversible aplasia has been described after the use of IM, because the most primitive stem cells seem to be spared from the effects of IM. Available data from syngeneic animal transplant models and most clinical studies do not suggest that IM has a negative impact on the speed and durability of engraftment *in vivo* (Hoepfl *et al*. 2002; Bornhauser *et al*. 2006). Several effects observed in our short-term *in vitro* culture model might therefore be compensated during long-term use of IM in patients.

In summary, our experiments show that IM inhibits the capacity of human MSC to proliferate and to differentiate into the osteogenic lineage, favouring adipogenesis. This effect is mainly mediated by an inhibition of PDGFRβ autophosphorylation leading to a more pronounced inhibition of PI3K/Akt compared to Erk1/2 signalling. The selective effects of IM and other small molecule kinase inhibitors might be helpful to further delineate the signalling cascades involved in distinct differentiation pathways of adult mesenchymal stromal cells. Whether the observed *in vitro* effects can become relevant in specific clinical and therapeutic settings remains to be studied (Berman *et al*. 2006).

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