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Imatinib Targets PDGF Signaling in Melanoma and Host Smooth Muscle Neighboring Cells

Ana Pirraco, Pedro Coelho, Ana Rocha, Raquel Costa, Luísa Vasques, and Raquel Soares*

Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, Porto, Portugal

ABSTRACT

In previous in vitro studies, we showed that imatinib abrogated platelet-derived growth factor receptor α (PDGFRα) signaling, disrupting both breast cancer and smooth muscle cells (SMC). PDGF is also a powerful mitogen for neural crest origin cells like melanocytes. The purpose of the present study was to evaluate the effect of imatinib on melanoma growth and in angiogenesis, with emphasis to the involvement in PDGF signaling. B16 melanoma cells incubation with 5 μM (IC50) imatinib resulted in a significant reduction in cell proliferation and migration. Apoptosis, however, was not significantly affected. Phosphorylated-PDGFRα expression was decreased in B16 lysates. In a mouse model of B16 melanoma, intraperitoneal administration of imatinib at early day light significantly decreased tumor growth. These findings were corroborated by a highly significant reduction in cell proliferation and increase in apoptosis in melanoma tumors. This was accompanied by a decrease in microvessel density and in the number of SMC-presenting vessels. Imatinib further inhibited PDGFRα expression and activity, as confirmed by the down-regulation of downstream Erk signaling pathway. Altogether, this study demonstrates that besides targeting tumor cells, imatinib also prevents vascular integrity. The current study provides evidence that the paracrine crosstalk between tumor cells and host neighboring cells is crucial for the elucidation of imatinib effects. In addition, the fact that this molecule targets vascular support cells further enlarges its therapeutic purpose to a wide range of vasculoproliferative pathologies. J. Cell. Biochem. 111: 433–441, 2010.

KEY WORDS: ANGIOGENESIS; GLIVEC; MELANOMA; SMOOTH MUSCLE CELLS

Melanoma, the most aggressive type of skin cancer, has drastically increased in Caucasian population during the last decades [Boyle et al., 2004; Giblin and Thomas, 2007]. Its high malignancy potential makes it responsible for more than 75% of skin cancer deaths [Jemal et al., 2008]. Melanoma cells express several tyrosine kinases, such as platelet-derived growth factor receptors (PDGFR) and c-kit [Mayorga et al., 2006]. Upon malignant transformation, c-Kit expression progressively decreases with increasing malignancy [Lassam and Bickford, 1992]. Conversely, PDGFR expression correlates with the metastatic potential, implying a role in malignancy [McGary et al., 2004]. PDGF is a four-member family of growth factors, which activate two tyrosine kinase receptors: PDGFR-α and PDGFR-β [Pietras et al., 2003a]. PDGF stimulation influences melanoma cells in an autocrine manner, being also involved in tumor angiogenesis and stroma formation [Lazar-Molnar et al., 2000]. Moreover, PDGFR is present in a large diversity of cells, and is overexpressed in several disorders [Ostman, 2004; Andrae et al., 2008]. PDGF secretion by angiogenic endothelial cells (EC) leads to vascular mural cells recruitment, playing thus, a crucial role in vessels stabilization and maturation [Heldin and Westermark, 1999; Jain and Booth, 2003; Armulik et al., 2005].

Imatinib mesylate (Glivec® or STI571; Novartis Pharmaceuticals) is a specific and potent tyrosine kinase receptor inhibitor approved by FDA for chronic myeloid leukemia and GIST treatment. Presently, imatinib effect is being studied on several PDGFR-expressing tumor types, including ovarian, colon cancers, and melanoma [Ivan et al., 2006]. Recent studies also demonstrate PDGFR expression on vascular support cells [Furuhashi et al., 2004; Rocha et al., 2007], rendering them putative targets for this agent as well. Accordingly, our group showed that imatinib incubation led to PDGFR-α expression and activity prevention, resulting in decreased SMC proliferation, migration and enhanced apoptosis in vitro [Rocha et al., 2007].

Altogether, we hypothesize that imatinib exerts a dual effect by inhibiting tyrosine kinase receptors both in melanoma cells growth and host neighboring cells. In this study we employed cell cultures and a mouse model to investigate the putative action of imatinib in melanoma.

*Correspondence to: Raquel Soares, MSc, PhD, Department of Biochemistry, Faculty of Medicine of the University of Porto, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. E-mail: raqsoa@med.up.pt

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MATERIALS AND METHODS

CELL CULTURE AND IN VITRO TREATMENTS

B16F10 melanoma cell line was kindly provided by Dr São José Nascimento (Pharmacy Faculty, Porto University, Portugal). B16 cells were cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Portugal), 1% penicillin/streptomicyn (Gibco) and 1% non-essential aminoacids (Gibco). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Imatinib mesylate was provided by Novartis Farma (Portugal). A sterile water-diluted working solution was made and added to culture medium at the final concentrations of 2.5, 5, and 7.5 μM, in agreement with previous studies [Rocha et al., 2007]. The recombinant PDGF-AA and the anti-PDGF neutralising antibody (Upstate, NY) were used at a final concentration of 10 ng/mL and 10 μg/mL respectively, in accordance with the manufacturer's instructions. Cells were starved during 16 h before incubation with each treatment for every experiment. Treatments were carried out in serum-free medium for 24 h. Controls were maintained in serum-free conditions.

ANIMAL STUDIES

Six-week-old C57Bl/6 female mice (Charles River Laboratories, Inc., USA) were housed in lab cages at 23 ± 5°C and humidity of 35 ± 5% under a 12 h dark/light cycle in accredited animal facilities. All animals were maintained on standard rodent chow with water ad libitum. Mice were subcutaneously inoculated into the dorsal skinfold with 1×10^6 B16 cells suspended in 100 μL PBS. Mice were randomly divided into two groups, receiving intraperitoneally 100 μL of saline solution (control group; n = 6) or 100 mg/kg/day of imatinib (treatment group; n = 10). Treatment was administered during the early light phase for 14 consecutive days. Tumor volume growth was assessed according to the formula: $0.52 \times \text{length} \times (\text{width})^2$. Tumors were used for molecular assays and were paraffin-embedded for histological analysis. All handling of animals were performed according to the Portuguese Act 1005/92 (number 3, iii) and European Community guidelines (86/609/EEC) for the use of experimental animals.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded B16 tumors were immunostained against vWF (1:200) (Chemicon, Hofheim, Germany) for capillary endothelial cells (EC) and against α-SMA (1:50) (Santa Cruz Biotechnology, CA) for vessel support cells. Signal was developed using DAB (Vector Laboratories, USA) as peroxidase substrate. ABC-complex method (Vector ABC kit) was used according to the manufacturer's instructions. Sections were counterstained with hematoxylin (Sigma–Aldrich, Portugal). Clusters of stained cells with or without lumen were considered an individual vessel. The entire area of each slide was covered at 200× magnified field. Areas were measured by Image-J software (NIH, USA). Immunostaining was quantified in serial sections.

APOPTOSIS ASSAYS

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed in B16 cells (1×10^4 cells/ml), after 24 h incubation with the different treatments in serum-free conditions, and in paraffin-embedded B16 tumors, using the In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland). The manufacturer's instructions was followed for each sample material. Nuclei were counter-stained with DAPI (Roche Diagnostics) and immunofluorescence was visualized under a fluorescence microscope (Nikon, UK). The percentage of TUNEL-stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of DAPI-stained nuclei at a 200× magnification field. One thousand nuclei were evaluated. The results are presented as mean ± SEM.

BrdU PROLIFERATION ASSAY

B16 cell (1×10^4 cells/ml) cultures were incubated with standard treatments in serum-free conditions and also with bromodeoxyuridine solution (BrdU), at a final concentration of 0.01 mM, for 24 h. For animal studies, mice were injected intraperitoneally with 1 mg/mL BrdU solution, 24 h before sacrifice. The in situ detection was performed using the BrdU In Situ Detection Kit (BD Biosciences Pharmingen, USA), according to the manufacturer's instructions. BrdU immunostaining was evaluated on an inverted phase-contrast microscope (Nikon) at a magnification of 200×. One thousand nuclei were evaluated. The results are given as mean ± SEM.

MOTILITY ANALYSIS BY INJURY ASSAY

To perform the injury assay, B16 cells at 90% confluence were scrapped from the culture dish using a pipette tip, which left a void space. Cells were then incubated for 24 h following the standard treatments. Damage recovery was then visualized and photographed on a phase-contrast microscope (Nikon) at a 200× magnification.

DOUBLE CHAMBER ASSAY

Migration capacity was quantified on a transwell BD-Matrigel basement membrane matrix inserts (BD Biosciences Pharmingen, USA), according to manufacturer's instructions. B16 cells (5×10^4 cells/ml) were harvested on inserts in serum-free medium and placed on wells containing the standard treatments in complete medium for 24 h. Membranes were then removed, stained with DAPI-methanol (Roche Diagnostics) and visualized under a fluorescence microscope (Nikon). Twenty-five random fields of each membrane were counted on the microscope at a 200× magnification.

WESTERN BLOTTING ANALYSIS

Proteins were isolated using Tripure (Roche Diagnostics) either from B16 cell lysates or B16 melanoma tumor tissue lysates. Equal amounts of protein extracts were loaded onto a 8–12% SDS-PAGE with 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham Biosciences, Arlington), using a mini-transblot electrophoresis transfer cell (Amersham Biosciences). Immunodetection for total PDGFR-α, phosphorylated c-Kit, total c-Kit and β-actin (all Santa Cruz Biotechnology); and phosphorylated PDGFR-α, total ERK, phosphorylated ERK, total AKT and phosphorylated AKT (Cell Signaling, MA) was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences). The relative intensity of each protein

blotting analysis was measured using Vision WorksLs Analysis Software (UVP, UK) and normalized to β-actin.

STATISTICAL ANALYSIS

All experiments were performed at least three times in duplicates. Results are expressed as mean ± SEM. Differences between samples and parameters between the two experimental groups were evaluated by Student's *t*-test. Differences in tumor growth between groups were assessed by repeated measures ANOVA followed by Bonferroni post hoc test. Significance was set at $P < 0.05$.

RESULTS

IMATINIB REDUCES B16 CELL PROLIFERATION AND INVASION THROUGH PDGF SIGNALING ABROGATION, BUT HAS NO EFFECT ON APOPTOSIS.

Based on concentration values used in previous studies of our group [Rocha et al., 2007; Soares et al., 2007], cells were incubated with 2.5, 5, and 7.5 μM imatinib in order to determine the best concentration for this agent in B16 cell cultures. We first established the concentration response curve to imatinib by evaluating its effect on apoptosis and proliferation of B16 melanoma cells. Imatinib did not statistically increase the percentage of apoptotic cells although a slight dose-dependent increase was observed (Fig. 1A). Conversely, the percentage of dividing cells decreased in a concentration-dependent manner, being significant for 5 μM (14.5 ± 2.5 vs. 31.9 ± 5.2 in controls) or higher (Fig. 1B). According to these results and in agreement with the literature [Roussidis et al., 2004; Rocha et al., 2007], imatinib was used at a concentration of 5 μM (corresponding to its IC50) for the subsequent in vitro studies.

To investigate if imatinib interfered with PDGF signaling in B16 melanoma cell cultures, immunoblotting for pPDGFR was performed in these cells. Incubation with imatinib significantly down-regulated the active (phosphorylated) form of PDGFRα ($^{*}P < 0.05$ vs. control) (Fig. 2A,B). This reduction was more evident when imatinib was incubated in the presence of recombinant PDGF-A ($P = 0.015$ for Imat + PDGFA vs. control; $P = 0.027$ for Imat + PDGFA vs. Imat). Moreover, incubation with recPDGF-A alone showed a tendency towards significant increase of pPDGFRα expression ($P = 0.067$ for PDGFA vs. control). The effect of recPDGF-A was abrogated when anti-PDGF neutralizing antibody was added to the culture ($^{*}P < 0.05$ for PDGFA+ Anti-Ab vs. PDGFA). Furthermore, these values showed a tendency towards significant decrease when compared to control ($P = 0.059$ for PDGFA+ Anti-Ab vs. control). Since imatinib also abrogates c-kit activity, another tyrosine kinase activity associated with melanoma progression, we also examined the expression of the phosphorylated form of this receptor (p-cKit). We found no significant differences in p-c-Kit receptor expression upon incubation with imatinib either alone or in combination with recPDGF-A. These data indicate that the putative effects of this agent in B16 melanoma cells target PDGF signaling pathway.

Our earlier data showed that PDGF signaling plays a role in proliferation, apoptosis and invasion of smooth muscle cells. Therefore, we next examined the effects of the agent in B16 melanoma cells. Interestingly, co-administration of PDGF neutralizing antibody and recombinant PDGF-A resulted in a cell growth decrease of 27% (Fig. 2C), implying a significant role of PDGFRα on B16 cell proliferation. The percentage of proliferating cells was also decreased in the presence of imatinib ($^{*}P < 0.05$ vs. control), being this effect enhanced by co-incubation with recPDGF-A ($^{\#}P < 0.01$ Imat + PDGF-A vs. control) as illustrated in Figure 2C.

Then, we further examined the action of imatinib in cell and invasion. The ability of cells to invade is associated with cell motility. Therefore, we first attempted to visualize cell migration capacity in damaged cultures. Cell motility was significantly inhibited by incubation with imatinib, either alone or in the presence of recPDGF-A, in contrast to control cells or cells treated with recPDGF-A (Fig. 2D). Surprisingly, the inhibitory effect of imatinib treatment was more evident than the one exerted by the anti-PDGF antibody. These preliminary findings suggest that imatinib is able to affect B16 cell invasive capacity. Accordingly, imatinib decreased

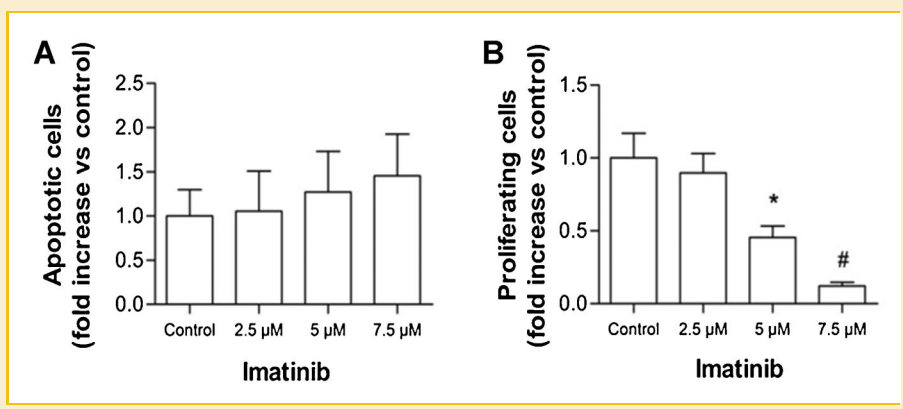

Fig. 1. Imatinib reduces B16 melanoma cells viability in a dose-dependent manner. A: B16 cells were incubated with three different concentrations of imatinib or untreated (control) for 24 h and a gradual increase in the percentage of apoptotic cells was observed when compared with control although not reaching statistical significance. Results represent the percentage of apoptotic cells evaluated by the ratios of TUNEL-stained cells versus total DAPI-stained nuclei. B: The number of growing cells, incorporating BrdU, decreased with increasing concentrations of imatinib, reaching statistical significance at concentrations of 5 μM ($^{*}P < 0.05$ vs. control) and 7.5 μM ($^{\#}P < 0.01$ vs. control). Cell proliferation is evaluated by the number of BrdU-stained cells in 1,000 hematoxylin-stained nuclei, and is reported as mean ± SEM. Experiments were repeated three times.

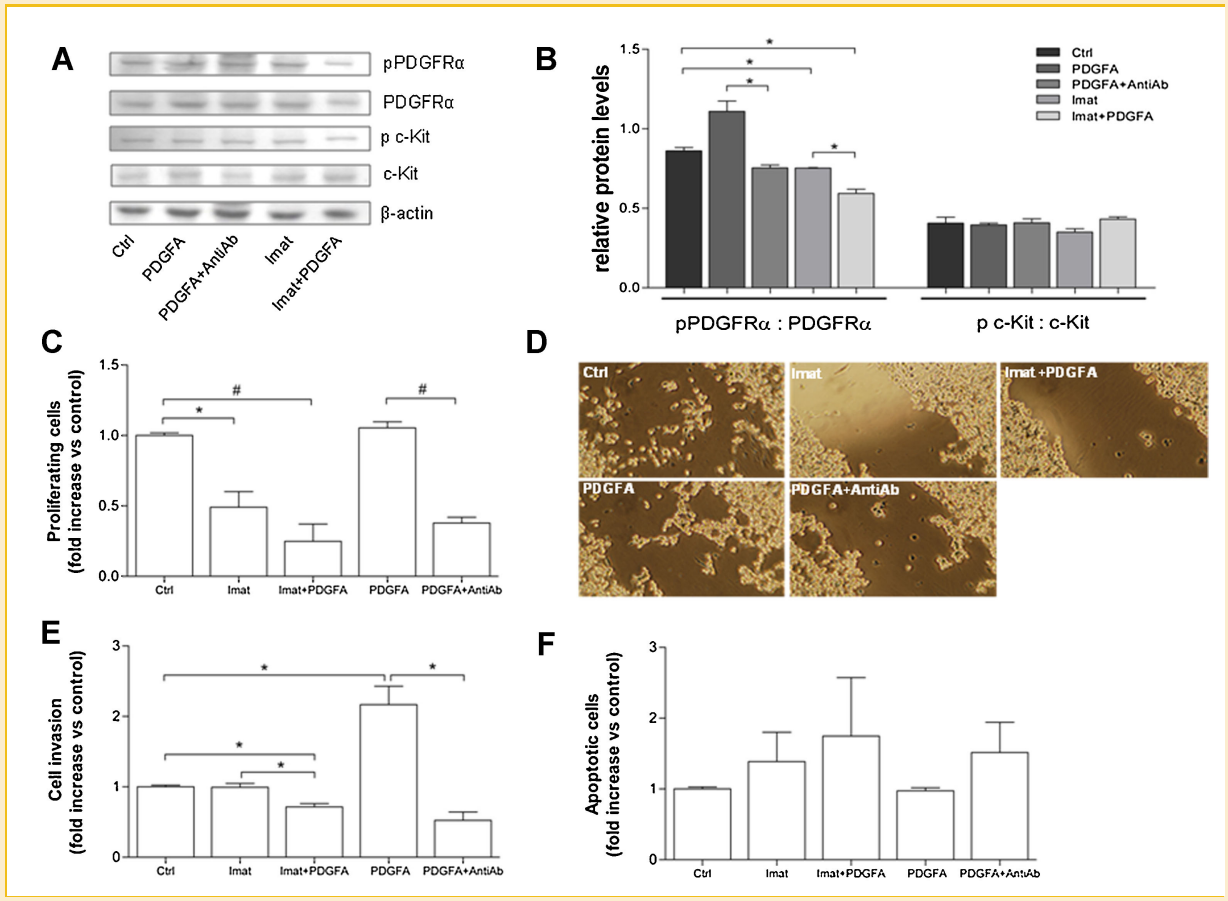

Fig. 2. Imatinib acts on B16 cell viability and invasive capacity by inhibiting PDGF signaling. A: Immunoblots showing imatinib effects on expression of phosphorylated (p)PDGFRα but not in phosphorylated (p)-c-Kit in B16 cell lysates. B: pPDGFR-α expression was significantly reduced in cultures treated with imatinib alone or in combination with recPDGF-A ($^{*}P < 0.05$ imat vs. crtl and, $^{*}P < 0.05$ imat + PDGFA vs. control). The presence of recPDGF-A enhanced this effect ($^{*}P < 0.05$ imat vs. imat + PDGF-A). A representative Western blot from three independent experiments is shown. C: The percentage of proliferating cells was reduced upon imatinib treatments ($^{*}P < 0.05$ vs. control), being more evident in the presence of recPDGF-A ($^{\#}P < 0.01$ vs. control), highlighting the PDGF signaling involvement. Anti-PDGF neutralizing antibody was used as a negative control (n = 6/treatment). D: Scratch assay. B16 cell were allowed to migrate into a cell culture damaged area for 24 h. Cell cultures were then visualized under an inverted microscope. E: Transwell migration assay. B16 cells were added to the upper compartment of a Boyden chamber and treatments were added to the lower chamber in medium complemented with 10% FBS as a chemoattractant. Cells that migrated through the matrigel membrane were counted. Results are presented as mean ± SEM. F: Apoptosis was evaluated in B16 cells subjected to the different treatments by TUNEL assay. Bars represent the percentage of apoptotic cells relative to total DAPI-stained nuclei. Values are expressed as mean ± SEM. Independent experiments were performed three times. $^{*}P < 0.05$; $^{\#}P < 0.01$. Ctrl = Control; PDGFA = recPDGF-A; Anti-Ab = Anti-PDGF; Imat = Imatinib. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

B16 invasion only in the presence of recPDGF-A as examined by double chamber assays (Fig. 2E), implying that this agent exerts anti-migrating effects by preventing PDGF signaling ($^{*}P < 0.05$ Imat + PDGFA vs. control). Corroborating these findings, recPDGF-A led to a significant increase of cell invasion in comparison to controls ($^{*}P < 0.05$ PDGFA vs. control), which was confirmed by a considerable reduction after co-incubation with PDGF neutralizing antibody ($^{*}P < 0.05$ PDGFA + AntiAb vs. PDGFA).

The effect of imatinib on apoptosis was investigated using TUNEL analysis. There were no significant changes in cell death either with imatinib or rec-PDGF-A treatment (Fig. 2F). Altogether, these findings indicate that imatinib exerts direct actions in melanoma cells growth and migration, which involves inhibition of PDGF transduction pathway, but no significant effect on apoptosis.

IMATINIB THERAPY DECREASES IN VIVO MELANOMA GROWTH

The in vivo effects of imatinib on melanoma were appraised through subcutaneous inoculation of B16 cells into C57Bl/6 mice. One day after inoculation, mice were randomized to control (vehicle-treated) or imatinib-treated group and received daily i.p. injection at the early hours of day-light, when response to therapy has been established to be enhanced by Nakagawa et al. [2006]. Tumors became palpable from day 7 onwards. Imatinib administration affected tumor volume, reaching statistical significance upon day 11 of treatment ($^{*}P < 0.05$ vs. controls) (Fig. 3A). Consistent with these data, imatinib treatment strongly reduced tumor cell proliferation to 13% of control values as observed by BrdU immunostaining (Fig. 3B,C). Remarkably, apoptosis was highly increased by imatinib administration. We found a threefold increase in the percentage of apoptotic cells after imatinib treatment (Fig. 3D,E). The latter result was not anticipated by the in vitro findings on B16 cells, suggesting

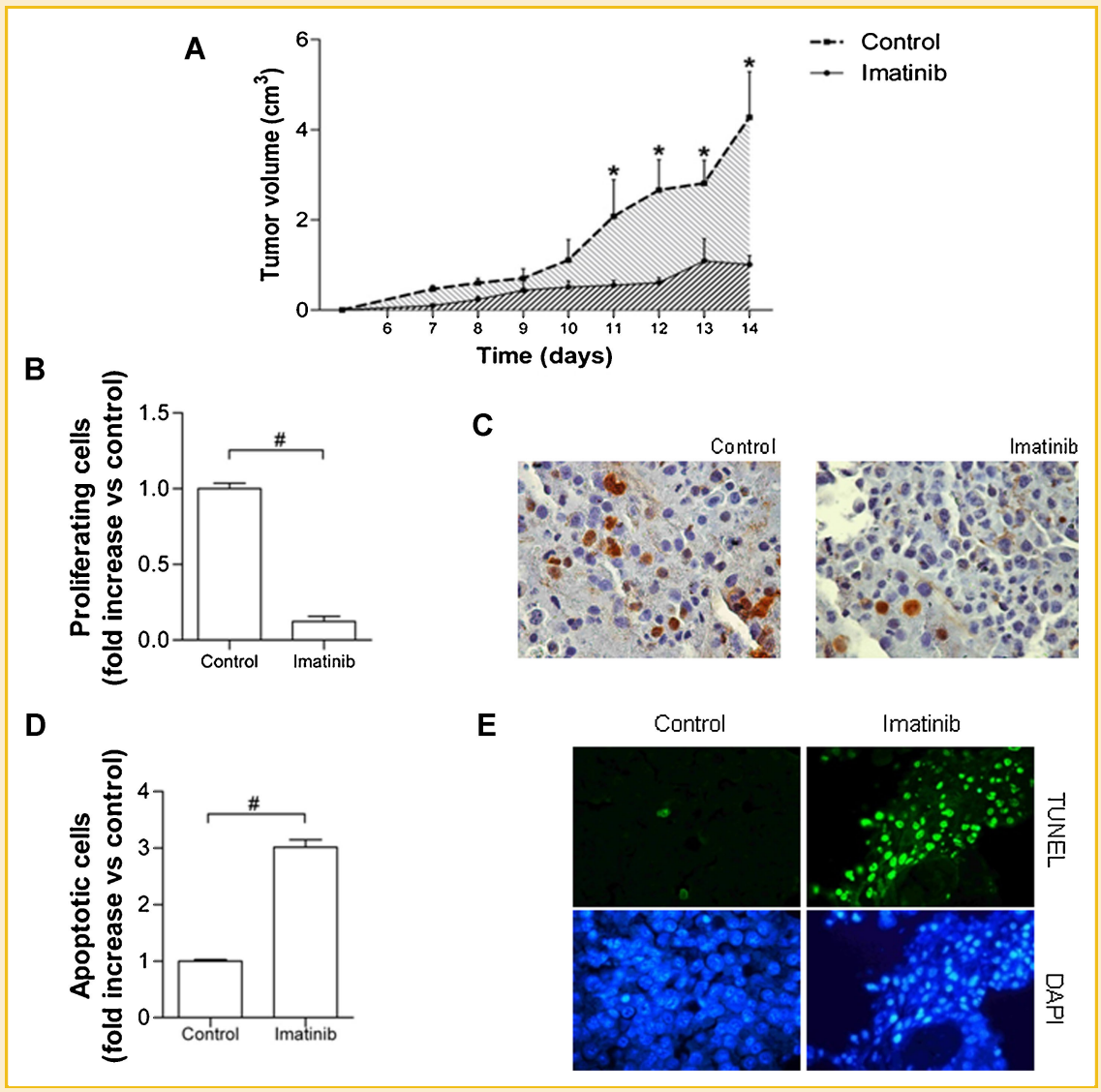

Fig. 3. Imatinib inhibits B16 tumor volume and cellularity. C57Bl/6 mice at 6 weeks of age were inoculated s.c. with B16 melanoma cells and treated with 100 mg/kg/day imatinib ($n = 10$) or saline solution (control) ($n = 6$) for 14 consecutive days. A: Tumor volume (cm^3) was monitored daily in imatinib-treated and control mice. Significant differences were observed after day 11 of treatment. Results are presented as mean ± SEM. B: Mice were injected i.p. with BrdU 24 h before sacrifice. Paraffin-embedded tumor tissue was immunostained against BrdU. Bars represent the percentage of proliferating cells evaluated by the number of BrdU-stained cells in 1,000 hematoxylin-stained B16 tumor cells. C: Immunohistochemistry assay using anti-BrdU antibody (brown) staining of control and imatinib-treated tumors. A representative image of imatinib-treated and control melanomas is shown. Magnification 200×. D: Percentage of apoptotic cells in B16 melanoma tumors assessed by TUNEL assay. Bars represent the percentage of apoptotic cells. E: Apoptotic cells (green fluorescence) relative to total number of DAPI-stained nuclei (blue fluorescence). Note the significant increased number of apoptotic cells in tumors from imatinib-treated mice in comparison to controls. Representative images are shown. Magnification 200×. $^{*}P < 0.05$ versus controls; $^{\#}P < 0.01$ versus controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

that microenvironment host cells, like pericytes, smooth muscle cells, inflammatory cells or fibroblasts, are probably crucial for the imatinib effects in melanoma. Accordingly, several distinct cell types other than EC are known to contribute for the angiogenic process both in an autocrine or paracrine manner [Bergers et al., 2003; Costa et al., 2007; Rocha et al., 2007; Soares et al., 2007].

VASCULAR SUPPORT CELLS ARE INHIBITED BY IMATINIB

To investigate potential structural changes in the tumor vessels of imatinib-treated mice, we performed immunohistochemical analysis in paraffin-embedded tumor tissues, using α-SMA-staining for mural cells and vWF for endothelium. Quantification of EC-staining showed a 45% reduction in microvessel density (MVD) in imatinib-treated mice tumors when compared to control group (Fig. 4A). Then, we further examined the presence of support cells within these vessels. Interestingly enough, we found that imatinib treatment significantly decreased the number of α-SMA-positive mural cells-presenting vessels from 27% in control tumors to 3% upon imatinib treatment (Fig. 4B,C). These results further emphasize the significant anti-angiogenic effects of imatinib in mouse melanoma, highlighting its action in both endothelium and vascular support cells.

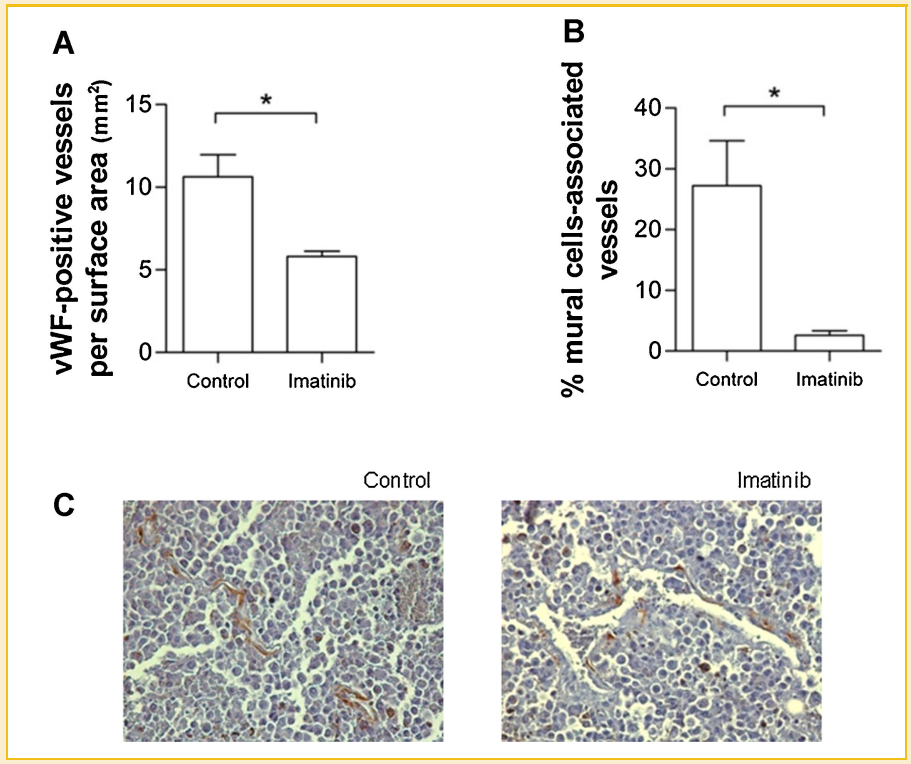

Fig. 4. Imatinib diminishes EC and vascular support cells within the tumor. A: Microvessel density (MVD) evaluated in paraffin-embedded tumor tissues from mice treated with imatinib or control mice using immunohistochemistry against vWF. B: The number of vessels presenting vascular support cells was counted on paraffin-embedded tumors from the two groups of mice, using immunohistochemistry analyses against α-SMA. Bars indicate the mean ± SEM (n = 6/group). C: Immunostaining for α-SMA (support cells) on 5 μm slides paraffin-embedded tumor tissue. Note the reduction of α-SMA staining on imatinib-treated tumor vessel. Magnification 200×. $^{*}P < 0.05$ versus controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

IMATINIB TREATMENT REDUCES PDGFR-α EXPRESSION AND SIGNALING IN MOUSE MELANOMA

To investigate whether imatinib effects were due to abrogation of PDGF signaling pathway, immunoblotting for pPDGFRα and its downstream effectors was carried out in B16 tumor lysates. The expression of the active form of PDGFR-α was found to be significantly decreased in imatinib-treated tumors ($^{\#}P < 0.01$ vs. controls) (Fig. 5A,B). Given the established role of Erk and Akt on PDGF-induced signaling pathways, we next examined the involvement of these proteins in the B16 tumors. As illustrated in Figure 5A,B, no significant difference in pAkt expression was found in tumors upon imatinib treatment. However, a tendency towards significant decrease was observed in pErk expression in imatinib-treated tumor lysates ($P = 0.07$ vs. controls). Taken together, our findings suggest that imatinib effects described may reflect the synergistic inhibition of PDGF signaling in melanoma and vascular support cells. This latter effect prevents the vascular outgrowth required for tumor progression.

DISCUSSION

In this study, we have shown that imatinib plays a dual role in preventing melanoma progression. This agent targets both melanoma cells and tumor microenvironment host cells, namely by abrogating vascular wall cells. We found that treating melanoma-inoculated mice with imatinib results in a reduction in tumor volume, which was associated with a decrease in cell division and a prominent apoptosis enhancement. In addition, we were able to see that these effects were caused by a down-regulation of PDGF signaling pathway in tumor and vascular support cells.

Our in vivo results are in line with the ones described by Redondo et al. [2004], although these authors did not observe a significant decrease in tumor growth between imatinib-treated and control tumors. Furthermore, several studies show imatinib effectiveness only when combined with adjuvant therapy such as anti-VEGF agents or to traditional chemotherapy [Pietras et al., 2003b; Vlahovic et al., 2007; Ogawa et al., 2008]. We strongly believe that differences in findings between ours and these previous studies are due to dosing schedule. As recently reported, PDGFR tyrosine kinase activity displays a significant 24 h oscillation, and imatinib therapy presented higher anti-tumor effects when administered in the early light-phase [Nakagawa et al., 2006]. Chronotherapy studies further reveal that therapeutic schedules are determinant for preventing toxicity and tolerability [Ohdo et al., 2001; Levi, 2006; Levi et al., 2007]. Considering these findings, our treatments were administered daily at early light-phase. The current study did not attempt to address the best schedule for imatinib delivery. Nevertheless, in agreement with studies in the literature [Lazar-Molnar et al., 2000; von Mehren, 2005; Soria et al., 2008], we assume that imatinib

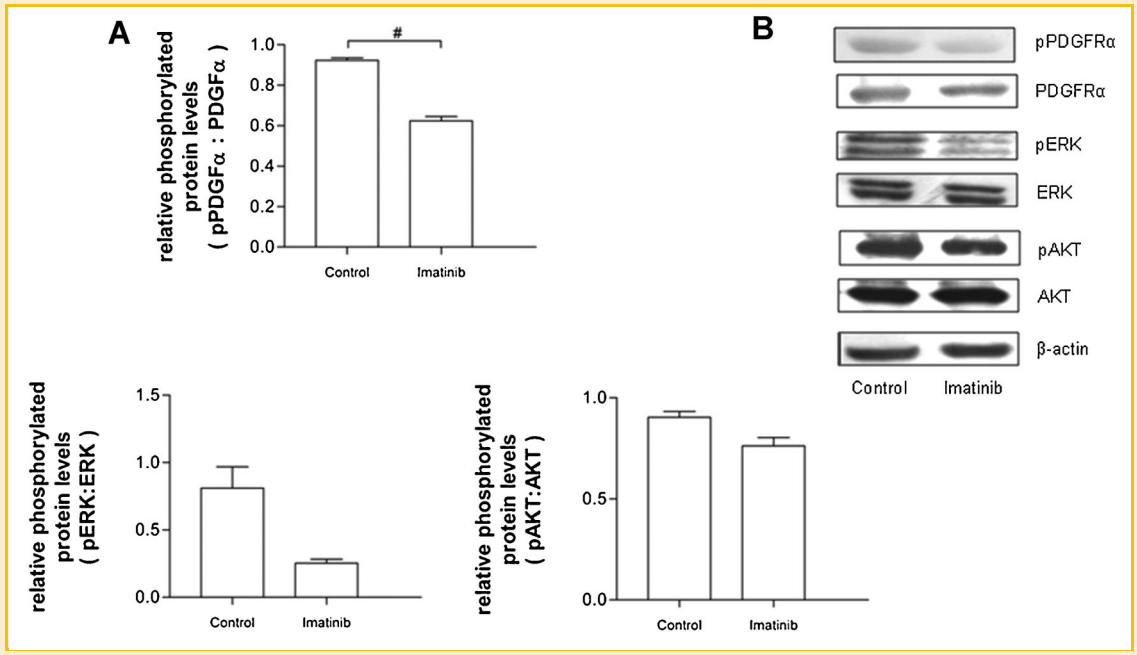

Fig. 5. Imatinib reduces PDGF signaling pathway activity in B16 melanomas. Protein extracts from vehicle-treated and imatinib-treated (100 mg/kg/day) mice melanoma tissue were analyzed by immunoblotting for p-PDGFR-α, p-Erk and p-Akt (A). The relative intensity of each band was normalized with β-actin (B). A representative Western blot is shown from three independent experiments. $^{\#}P < 0.01$.

delivery during the period of highest PDGFR activity is likely to enhance the multi-targeted actions within the tumor. This is actually a commonly forgotten issue that may render therapy more efficacious and definitely requires further study.

Imatinib is known to inhibit multiple tyrosine kinase receptors activity. Our cell culture experiments addressed the PDGF signaling pathway. PDGF is a family of four distinct ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) that are able to dimerize forming hetero- or homodimers, which in turn exert different signaling properties [Andrae et al., 2008]. The most well described PDGF isoforms (PDGF-A and PDGF-B) frequently display physiologically distinct expression patterns, leading to the assumption that they present nonoverlapping roles [Andrae et al., 2008]. In addition, PDGF-AB heterodimer, for instances, has been observed in human platelets, yet with no clear role described so far. PDGF ligands activate two receptors—PDGFRα and PDGFRβ characterized by an intracellular tyrosine-kinase domain [Heldin and Westermark, 1999]. In vivo evidence revealed that PDGF-AA and PDGF-CC dimers activate PDGFRα, whereas PDGF-AB, PDGF-BB, and PDGF-DD have been shown to engage PDGFRβ. Both receptors are involved in crucial cellular mechanisms as proliferation, survival, and maintenance of an invasive phenotype in a wide variety of cells [Tallquist and Kazlauskas, 2004]. Furthermore, PDGFR overexpression is associated with several pathologies, such as vasculoproliferative diseases [Ostman, 2004]. Interestingly, PDGF-A is implicated in skin homeostasis [Betsholtz, 2004]. Accordingly, knock out mice for PDGF-A, PDGF-C, or PDGFRα present skin defects, implying a relevant role of this receptor in skin.

PDGFRs are extensively studies in cancer. In melanoma both PDGFRs and ligands are expressed [Barnhill et al., 1996; Lazar-Molnar et al., 2000; Shen et al., 2003]. Accordingly, PDGFR expression plays a role in the malignant transformation of melanocytes, and has been suggested to correlate with the metastatic potential [McGary et al., 2004]. Cells with higher metastatic potential expressed increased levels of PDGFRα, whereas melanomas with low metastatic potential presented higher PDGFRβ expression [McGary et al., 2004]. These findings, together with the fact that imatinib is able to prevent both PDGFRs, led us to investigate the effect of this agent in PDGFRα signaling pathway.

Consistent with previous data in the literature [Redondo et al., 2004], our in vitro studies showed that imatinib significantly suppressed B16 cells proliferation but had no effect on apoptosis. B16 cell invasion was significantly disrupted by imatinib only in the presence of recombinant PDGF-A, indicating that PDGF signaling-enhanced activation prompts imatinib to act through this pathway. This was not required in the mice studies, since a wide variety of stromal cells within tumor environment are able to release several factors, which engage autocrine and paracrine roles in melanoma growth, angiogenesis, and stroma formation [Lazar-Molnar et al., 2000]. An intricate coordination between EC and SMC is required upon angiogenesis so that newly formed vessels originate a stable and mature network. PDGF plays a determinant role in mural cell recruitment [Costa et al., 2007; Rocha et al., 2007].

Tumor lysates immunoblotting further revealed PDGFR-α expression impairment by imatinib. Previously, imatinib administration to melanoma xenografts-bearing mice significantly reduced PDGFR-α phosphorylation, but tumorigenicity remained unaffected [McGary et al., 2004]. In the present study, down-regulation of PDGF signaling by imatinib in vivo was corroborated by the decrease in ERK phosphorylation, a downstream effector of

PDGF established to be involved in cell migration [Frost et al., 2009].

Interestingly, a reduction in MVD as well as a decrease in α-SMA-positive mural cells-associated vessels was evident upon treatment with imatinib. In accordance with this, previously we demonstrated that imatinib disturbs SMC behavior through a PDGF signaling pathway but has no effect on endothelial cells in vitro [Rocha et al., 2007]. Pericytes and SMC participate actively in the assembly of blood vessels [Abramsson et al., 2003; Furuhashi et al., 2004; Hasumi et al., 2007]. A recent study, applying a DNA aptamer specifically against PDGF-B, showed a rapid decrease in pericyte coverage of tumor vessel, which led to vessel regression [Sennino et al., 2007]. Consistently, combining imatinib with an anti-VEGF agent reported impaired tumor burden and vessel disruption caused by support cells' detachment [Bergers et al., 2003]. Interestingly, normal tissue vasculature was not affected by the pharmacological agents used in these studies, suggesting a selective sensitivity of tumor vasculature.

Imatinib targets vascular support cells. The absence of support cells renders vessels prone to disruption and regression, leading often to non-functioning endothelial-lined blood vessels. Moreover, vascular impairment considerably contributed for the strong increase in tumor cell's apoptosis. In agreement with the recent established assumption that tumor vascular normalization is a much more accurate procedure than its abrogation, our findings demonstrate that by promoting the development of unstable vessels, imatinib might be used as a vascular remodeling agent, eventually in a concerted action with other molecules. In conclusion, the data obtained herein implicate imatinib on vessels support cells as well, providing a wide novel therapeutic field for this agent. Given the recent concept of angiogenesis as an "organizing principle" [Folkman, 2007], the results obtained in the present study can further be applied to other pathological conditions in which angiogenesis (and particularly SMC proliferation) is exacerbated.

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