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ROLE OF IMATINIB IN HORMONE-DEPENDENT BREAST CANCER ANGIOGENESIS

ANA SOFIA ROCHA

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PAPEL DO IMATINIB NA ANGIOGÉNESE DE CANCRO DA MAMA HORMONO-DEPENDENTE

MESTRADO EM MEDICINA E ONCOLOGIA MOLECULAR

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ABREVIATIONS

BrdU	-	bromodeoxyuridine
CML	-	chronic myeloid leukaemia
CO_2	-	carbon dioxide
DAPI	-	4',6-diamidino-2-phenylindole
DMEM	-	Dulbecco's modified eagle's medium
EC	-	endothelial cells
ECGS	-	endothelial cell growth supplement
ECL	-	enhanced chemiluminescence
EtOH	-	ethanol
FBS	-	fetal bovine serum
GIST	-	gastrointestinal stromal tumors
HAoSMC	-	human aortic smooth muscle cells
HUVEC	-	human umbilical vein endothelial cells
IMAT	-	imatinib
MCF7	-	human breast adenocarcinoma cells
MEM	-	minimum essential medium
MTT	-	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
P4	-	progesterone
PBS	-	phosphate buffered saline (pH 7,4)
PDGF	-	platelet derived growth factor
PDGFR	-	platelet derived growth factor receptor
PR	-	progesterone receptor
SCF	-	stem cell factor
SDS-PAGE	-	polyacrilamide gel electrophoresis
SMC	-	smooth muscle cells
TUNEL	-	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling
VEGF	-	vascular endothelial growth factor

SUMMARY

In previous studies, we found that progesterone was able to induce the expression of platelet-derived growth factor A (PDGF-A) in human breast cancer MCF7 cells. Knowing that PDGF plays a relevant role in angiogenesis, and that imatinib mesylate targets PDGF receptor tyrosine kinase activity, the aim of the present study was to examine the effects of imatinib on progesterone-treated MCF7 cells, Human Aortic Smooth Muscle Cells (HAoSMC) and Human Umbilical Vein Endothelial Cells (HUVEC). Expression of phosphorylated (activated) PDGFR-α was detected in MCF7 cells and HAoSMC, but in a very low extent in HUVEC. The effects of imatinib on cell growth, apoptosis and migration were then analyzed. In MCF7 cells imatinib effectively inhibited anchorage-dependent colony formation, and cell viability as evaluated by MTT assay. Corroborating these findings, a significant increase in the percentage of apoptotic cells was observed when MCF7 cells were treated with imatinib. Surprisingly, these inhibitory effects were all enhanced by the presence of progesterone. Cell migration assays in MCF7 cells did also show a reduction in the migratory capacity after incubation with imatinib. In agreement with the lack of active PDGFR-α, imatinib was unable to prevent HUVEC growth, survival or migration ability. In contrast, HAoSMC viability and proliferation were effectively inhibited by imatinib, as evaluated by MTT and BrdU assay, respectively. Supporting these findings, a significant increase in the percentage of apoptotic HAoSMC was also observed after treatment with imatinib. Cell migration assays did also show a reduction in the migratory ability after incubation with imatinib.

Altogether, these facts reveal that imatinib is able to affect MCF7 and HAoSMC survival, growth and migration. Furthermore, incubation with progesterone seems to sustain PDGFRα activity, prompting these cells to the inhibitory action of imatinib. Our findings indicate that imatinib might be a good therapeutic agent in progesterone-dependent breast cancer and, possibly, against other vascular-associated disorders, such as atherosclerosis, that carry in common smooth muscle cells abnormal growth.

INTRODUCTION

BREAST CANCER

Breast cancer continues to be the most common cancer and the second leading cause of cancer death and morbidity among women in the western world (Soares et al. [41]), being accounted for thirty percent of those deaths. Therefore, the constant search for new therapeutic targets which may result in better disease-free and overall survival becomes of primary relevance (Carvalho et al. [8]; Gonzales-Angulo et al. [18]).

Breast cancer is a heterogeneous disease that can be hormone-dependent or independent. Seventy five percent of breast cancers are hormone-dependent and sixty-five percent of those types of cancers are progesterone-dependent. A profound understanding of the mechanisms of action of progesterone in breast cancer is, thus, an important subject (Cianfrocca and Goldstein [10]).

ANGIOGENESIS

Blood vessels are the first organ to be formed in the human body and constitute a wide network crucial to our organism. Formation of blood vessels can occur, during embryogenesis, by two different processes, vasculogenesis and angiogenesis. Assembly of a primitive network involves vasculogenesis. This primary capillary plexus is then differentiated by angiogenesis where new blood vessels arise from sprouting, branching and intussusceptive growth from pre-existing capillaries (Carmeliet [6]). Angiogenesis comprises extracellular matrix degradation, proliferation, survival, migration and anastomosis of endothelial cells (EC), and it ends up by the adhesion of smooth muscle cells (SMC), resulting in vessel stabilization.

In adults, continuing physiological angiogenesis is only required in a few circumstances that include wound healing and female reproductive organs, otherwise vasculature is usually quiescent (Bergers and Benjamin [4]). The process of angiogenesis is regulated by a fine interplay of angiogenic stimulatory and inhibitory factors. The imbalance of this process has major impact on health, contributing for several pathologies. Abnormal neovascularization is, indeed, associated with many disorders such as ischemia, atherosclerosis, diabetic retinopathy, rheumatoid arthritis and development of tumors (Folkman [15]; Carmeliet [7]; Distler et al. [12]).

STEROID HORMONES AND CANCER

Ovarian steroid hormones, estrogens and progesterone, are essential for cell growth and development of the breast. However, in contrast to estrogens, which are well recognized as breast cancer growth promoters, the role of progesterone remains controversial (Dabrosin [11])

Progesterone receptors are present in vascular mural cells, namely human and rat aortic smooth muscle cells *in vivo* and *in vitro* (Wen-Sen et al. [48]; Nakamura Y et al. [32]) and in endothelial cells (Vásquez et al. [46]). Some studies report the role of progesterone in endometrial angiogenesis, in which this sex steroid seems to stimulate endometrial endothelial cells proliferation and induce endometrial angiogenesis (Walter et al. [47]; Mirkin et al. [29]). The effects of progesterone in tumor angiogenesis, particularly in breast cancer angiogenesis, are poorly understood. However some studies show that progesterone induce vascular endothelial growth factor (VEGF), a potent angiogenic factor, in breast cancer cells, implying that progesterone promotes angiogenesis (Hyder et al. [20]; Liang et al. [26]; Hyder et al. [19]).

PROGESTERONE AND PDGF SIGNALING PATHWAYS

Platelet-derived growth factor (PDGF) refers to a four-member family of growth factors; PDGF-A, -B, -C and -D, that exert their cellular effect through two tyrosine-kinase receptors: PDGF α receptor (PDGFR-α) and PDGF β receptor (PDGFR-β), whose activation depends on ligand binding (Ostman [33]; Carvalho et al. [8]).

PDGF is a crucial angiogenic factor involved in blood vessel maturation as it is required for recruitment of pericytes and, hence, stabilization of the microvasculature (Distler et al. [12]; Armulik et al. [2]). PDGFR-α and β are involved in crucial cellular mechanisms as proliferation, survival and maintenance of an invasive phenotype in a wide variety of cells (Tallquist and Kazlauskas [42]). Furthermore, PDGFR overexpression already has been associated with several pathologies, such as vasculoproliferative diseases and cancer (Ostman [33]).

Previous studies of our group showed that progesterone (P4) was able to induce the expression of several genes, namely those involved in platelet-derived growth factor A (PDGF-A) signaling pathway, in human breast cancer MCF7 cells (Soares et al. [41]). In our former study, conditioned medium from progesterone (P4)-induced breast cancer MCF7 cells resulted in increased proliferation and migration of SMC, probably mediated through PDGF signaling pathway (Soares et al. [41]). Thus, this crosstalk between P4 and PDGF signaling pathway might be crucial for the steady angiogenesis that is observed in progesterone-dependent breast cancers.

IMATINIB

Imatinib is a phenylaminopyrimidine analogue drug that competes with ATP for its specific binding site in the kinase domain of specific tyrosine-kinase receptors such as c-Kit and the platelet-derived growth factor receptor (PDGFR) (Buchdunger et al. [5]; Mauro and Druker [28]). Imatinib has only minor side effects, being well tolerated by the human body.

Imatinib mesylate was first developed as an inhibitor of platelet-derived growth factor receptor-α (PDGFR-α) tyrosine kinase activity. It is also able to inhibit Abl tyrosine kinase variants being thus successfully used to treat chronic myeloid leukaemia (CML) (Radford [37]; Mauro and Druker [28]; Panigrahi et al. [34]; Kantarjian and Cortes [23]). Clinical evidence indicates that imatinib does also target gastrointestinal stromal tumours (GIST), which are characterized by the presence of the cell surface transmembrane receptor c-kit , the receptor of stem cell factor (SCF) (Radford [37]; Tuveson et al. [45]). GIST frequently present mutations in c-kit gene. This leads to constitutive activation of this membrane receptor, mediating uncontrolled cell growth and survival. Although changes in the PDGF signalling pathway have been largely described in breast cancer, the effect of imatinib in this type of tumours has only barely been examined (Modi et al. [31]; Roussidis et al. [39]). PDGFR-α is expressed in nearly 40% of invasive breast cancer, and correlates with poor prognostic parameters (Carvalho et al. [8]), rendering imatinib a potential therapeutic agent against these tumours.

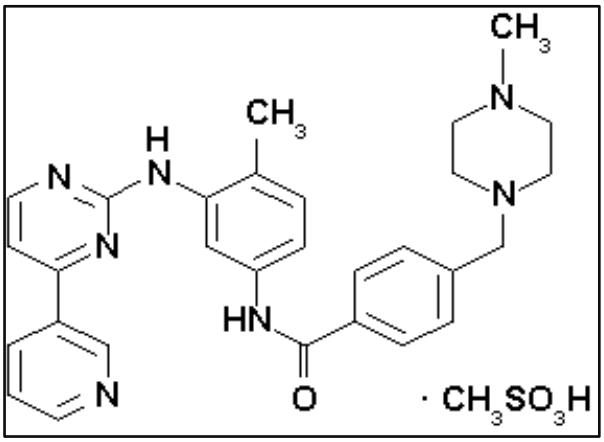

Figure 1. Chemical structure of imatinib

CHAPTER I

PROGESTERONE SENSITIZES BREAST CANCER MCF7 CELLS TO IMATINIB INHIBITORY EFFECTS

MATERIALS AND METHODS

CHEMICALS

Progesterone (P4) was obtained from Sigma Aldrich (Portugal). Imatinib was kindly provided by Novartis. Antibodies against total PDGFR-α, phosphorylated-PDGFR-α and β–actin (Santa Cruz Biotechnology, USA) were used for immunoblotting.

P4 was dissolved in 100% ethanol and added to serum-free medium of cell cultures at a final concentration of 10^{-8}M. Concentration for P4 was chosen from a set of 5 different concentrations ranging from 0.1 to 1000 nM in previous studies (Soares et al. [39]). Ethanol was added to cell culture media at a concentration of 0.01% (v/v). Imatinib was dissolved in distilled water and added to serum-free medium of cell cultures at concentrations ranging from 1 to 10 μM.

CELL CULTURES

Human breast cancer cell line, MCF7, was obtained from the American Type Culture Collection (ATCC, Barcelona, Spain). MCF7 cells were routinely cultured in Eagle MEM medium containing 10% inactivated FBS and 1% penicillin/streptomycin.

Cell culture media, FBS and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Paisley, Scotland, UK). Cells were grown at 37^{o} C in a humidified 5% CO_2 atmosphere. In agreement with previous studies, incubations with the distinct agents were performed for 24h in serum-free conditions (Soares et al. [41]).

Because P4 receptor (PR) is an estrogen-dependent gene product, cells were maintained in 10% FBS and 1% penicillin/streptomycin until 70% confluence, then washed twice with PBS and immediately incubated in serum-free conditions, with no prior serum-free medium incubation, as previously described (Soares et al. [41]).

CLONOGENIC ASSAY

Colony formation assay was performed as previously described (Mittal et al., 2004). Briefly, cells were harvested at a concentration of $1x10^3$ cells/ plate in 21 cm plates, incubated for 24 hr in complete medium and then incubated with ethanol (EtOH), 10^{-8}M P4, or combinations of EtOH or P4 with imatinib for 7 days, enabling each cell to proliferate and form colonies. Cell cultures were then washed with PBS, fixed with 70% ethanol and stained with 0.04% trypan blue solution. Colonies with more than 20 cells were counted on a phase contrast microscope (Nikon, UK). Results were expressed in percentage of the colonies formed by the control cells (ethanol-treated).

MTT PROLIFERATION ASSAY

MCF7 cells were cultured following standard conditions or the treatment procedures for 24 hours. Cells were then washed twice with PBS and subjected to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, an index of cell viability and cell growth. Cells were incubated with MTT solution at a final concentration of 0.5 mg/mL for 3 hours and then lysed in DMSO. Optical density was measured at 540nm. The background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was calculated. The results are given as mean ± SD and are expressed as percentage of control, which was considered to be 100%.

WESTERN BLOTTING

Proteins were isolated from MCF7 cell lysates using Tripure (Roche Diagnostics, Basel, Switzerland). Proteins were quantified using a spectrophotometer (Jenway, 6405 UV/vis, Essex, UK) and equal amounts of protein were subjected to 8% SDS-PAGE with a 5% stacking gel. After electrophoresis proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington, USA), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences, USA). Immunodetection for total PDGFR-α, phosphorylated (activated) PDGFR-α (Pi-PDGFR-α) and β-actin was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences, USA). The relative intensity of each protein blotting analysis was measured using a computerized software program (Biorad, California, USA) and normalized with β-actin bands to compare the expression of proteins in different treatment groups. Experiments were repeated three times.

BRDU PROLIFERATION ASSAY

MCF7 cells ($1x10^4$) were cultured following standard conditions or the treatment procedures for 24 hours. Cells were then washed twice with PBS and subjected to *in situ* detection with BrdU (Bromodeoxyuridine), an index of DNA synthesis and cell proliferation. Cells were incubated with BrdU solution at a final concentration of 0.01 mM for 24 hours and then the *in situ* detection was performed using BrdU *In-Situ* Detection Kit (BD Biosciences Pharmingen, USA), according to the manufacturer's instructions. The results are given as mean ± SD and are expressed as percentage of proliferating cells. The percentage of proliferating cells was evaluated at a x200 magnification field. One thousand nuclei were evaluated. Three independent experiments were performed.

TUNEL ASSAY

Cells grown until 70% confluence onto glass coverslips were incubated with the different referred treatments for 24 hours. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics, Basel, Switzerland). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a x200 magnification field. One thousand nuclei were evaluated. Three independent experiments were performed.

MIGRATION ANALYSIS

Injury Assay

Injury assay was performed as previously described (Soares et al, 2004). Cells were grown to 90% confluence. Using a pipette tip, cells were scrapped from the culture dish leaving a void space. Cells were then incubated for 24 hours following the standard treatments. After incubation cells were washed with PBS and cell migration to the damaged area was visualized and photographed on a phase contrast microscope (Nikon, UK). Magnification ×200.

Double-Chamber Assay

The migration capacity of MCF7 cells was then quantified by counting the number of cells that migrated through matrigel-coated transwell BD-matrigel basement membrane matrix inserts (BD-Biosciences, Belgium). Transwell inserts containing an 8µm pore-size PET membrane coated with a uniform layer of Matrigel basement membrane were used. MCF7 cells (5×10^4) were harvested on inserts in serum-free medium, and placed on wells containing Eagle MEM medium complemented with FBS (10%), penicillin/streptomycin (1%) and EtOH, P4 or a combination of EtOH or P4 with imatinib. After incubation for 24 hr at 37ºC and 5% CO_2, membranes were removed from inserts, stained with DAPI-methanol (Roche Diagnostics, Basel, Switzerland) for 5 minutes and visualized under a fluorescence microscope (Olympus, BH-2, UK). Twenty-five random fields of each membrane were counted on the microscope. Magnification X200.

STATISTICAL ANALYSES

All experiments were performed in triplicate. Quantifications are expressed in mean ± SD. Samples were evaluated by the analysis of variance test. A difference between experimental groups was analyzed by Student's t-test, and was considered statistically significant whenever the p value was less than 5%.

RESULTS

Imatinib reduced cell proliferation and increased apoptosis in a concentration-dependent manner

We first determined the concentration-response effect of imatinib on cell apoptosis and proliferation by TUNEL and BrdU incorporation assays respectively. Treatment of MCF7 cells with 1-10 μM imatinib for 24h resulted in a concentration-dependent increase in the percentage of apoptotic cells (Figure 1). This increase was statistically significant for 5 μM or higher. The percentage of proliferative cells did also decrease in a concentration-dependent manner, reaching significant reduction for concentrations equal or higher than 2.5 μM (Figure 2). According to these findings and in agreement with the literature (Roussidis et al, 2004), imatinib was used at a concentration of 5 μM in subsequent studies.

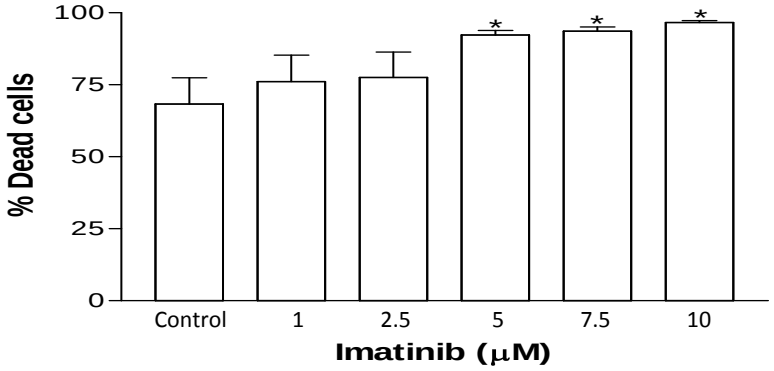

Figure 1. Concentration response of imatinib in MCF7 cells. Percentage of apoptotic cells assessed by TUNEL assay. A significant increase in cell apoptosis relative to controls (0) was found whenever cells were incubated with 5, 7.5 and 10 μM imatinib (*p<0.01 vs control). Cell apoptosis is reported as mean ± SD. Bars represent the percentage of apoptotic cells evaluated by the ratio apoptotic cells/total cells.

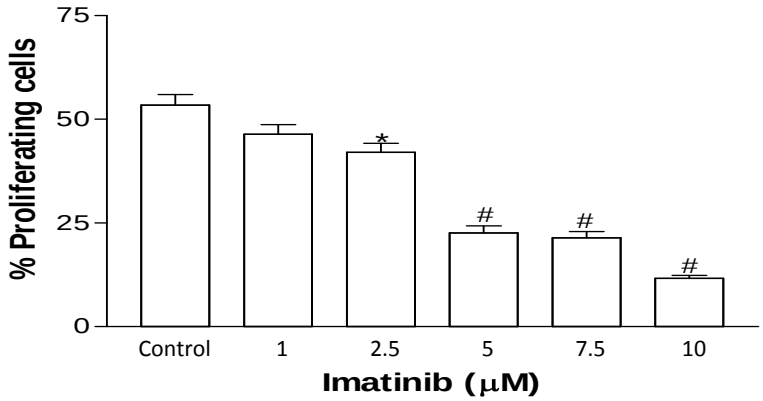

Figure 2. Concentration response of imatinib in MCF7 cells. Percentage of proliferating cells assessed by BrdU incorporation assay. The percentage of BrdU staining cells was down-regulated by imatinib in a concentration-dependent manner, reaching statistical significant levels upon incubation with 2.5 μM (*p<0.01 vs control) or higher (#p<0.001 vs control). Cell proliferation is reported as mean ± SD. Bars represent the percentage of proliferating cells evaluated by the number of BrdU-stained cells in 1000 hematoxylin-satined cells.

Presence of PDGF receptor in MCF7 cells

To ensure that imatinib could be effective in MCF7 cells, we first confirmed the expression of PDGF receptors (PDGFR) in MCF7 cells by Western blotting. The expression of phosphorylated PDGF receptor-α was observed in MCF7 cell lysates after each treatment. However, in the presence of imatinib both total and phosphorylated PDGFR-α expression were down-regulated. This reduction in the expression and activation of PDGFR-α was more evident in the presence of progesterone (P4) (Figure 3).

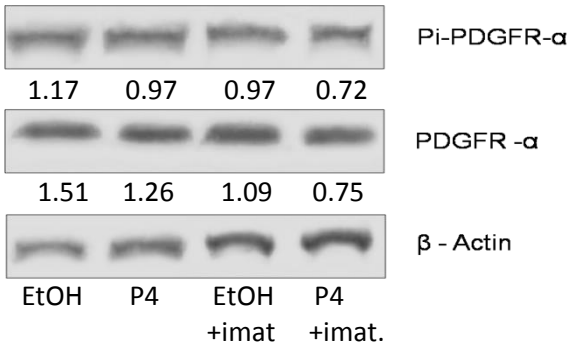

Figure 3. Expression of total and activated (phosphorylated)-PDGFR-α (Pi-PDGFR-α in MCF7 cell lysates after incubation with ethanol (EtOH), progesterone (P4), ethanol+imatinib (EtOH+Imat) or progesterone+imatinib (P4+Imat) during 24 h. PDGFR-α expression was found in every cell lysate. Pi-PDGFR-α was slightly decreased in imatinib-treated cultures as compared to controls. The relative intensity of each band, after normalization to β-actin is shown under each blot, expressed as mean value. A representative Western blotting is shown from 3 independent experiments.

Imatinib decreased MCF7 cell viability and proliferation and increased apoptosis

To determine the effect of imatinib on breast cancer MCF7 cells behaviour, the MTT assay was used. Incubation of MCF7 cells with P4 did not significantly affect cell viability in comparison to controls. A slight decrease in cell viability was found upon exposure to imatinib in the presence of vehicle (EtOH + imat), though not reaching statistical significance. However, treatment with imatinib together with P4 resulted in a significant decrease in MCF7 cells viability (Figure 4) (#p<0.01).

The effect of imatinib in cell viability was further investigated by anchorage-dependent colony formation assay. In comparison to ethanol, treatment of MCF7 cells with P4 resulted in a slight increase in colony formation potential. Incubation of cells with imatinib either in the presence or in the absence of P4 led to a significant reduction in the number of colonies formed (Figure 5).

We then addressed the role of imatinib in the apoptotic levels of MCF7 cells using TUNEL analysis. As illustrated in Figure 6, apoptosis was strongly reduced whenever cells were incubated with P4. The presence of imatinib either alone or in combination with P4 potently increased the percentage of apoptotic cells (Figure 6A) (*p<0.05 vs. control). Interestingly, imatinib completely reversed the survival effects of P4, leading to a statistically significant increase in the percentage of dead cells (Figure 6A and B) (#p<0.01 vs. P4).

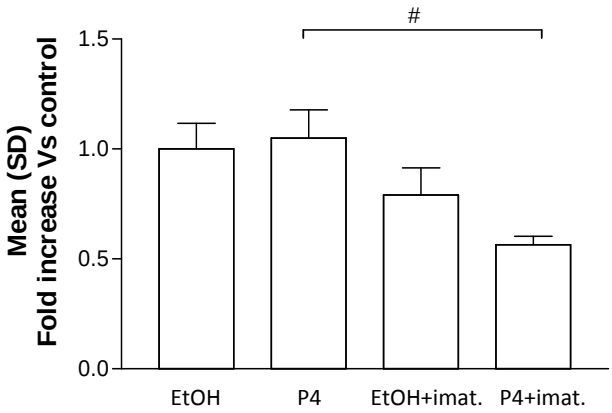

Figure 4. MCF7 cells viability by MTT assay. A slight increase in cell viability was found after incubation with P4 for 24 h. Incubation with imatinib led to a decrease in cell viability, this decrease being significant whenever cells were treated with imatinib combined with P4 (P4+Imat) (#p<0.01 vs. control). Cell viability is expressed in terms of percentage of control cells (EtOH), and is reported as mean ± SD.

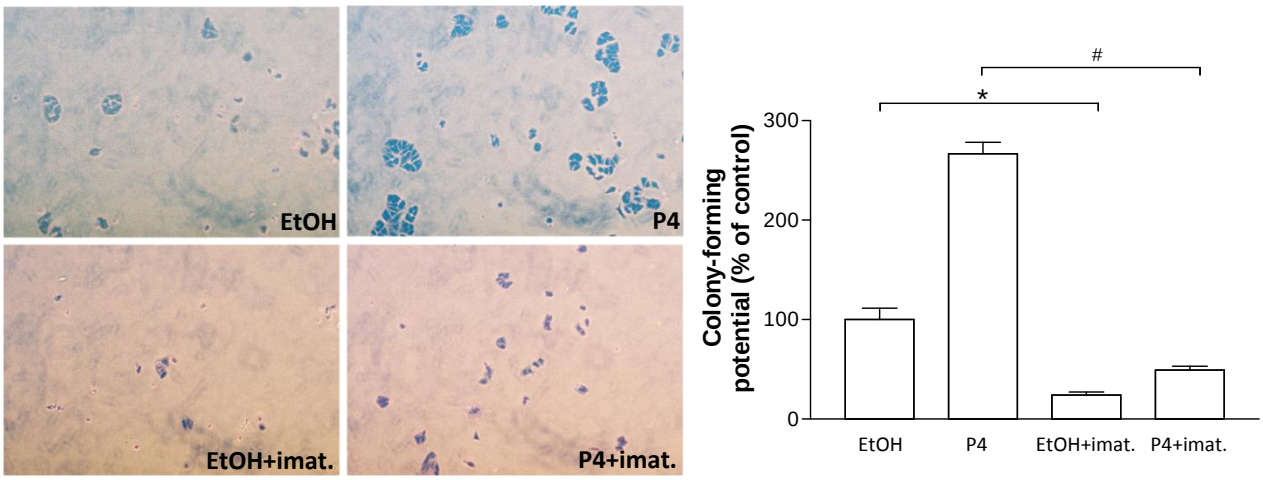

Figure 5. Cell proliferation assessed using the clonogenic assay in MCF7 cells. Treatment of cells with 10^{-8}M P4 increased colony-forming potential recorded after 7-day treatment. Incubation of MCF7 cells with imatinib in the absence or in the presence of P4 (EtOH+imat. or P4+imat.) resulted in a significant reduction in the number of colonies. Experiments were repeated three times with identical results. Magnification (×200)

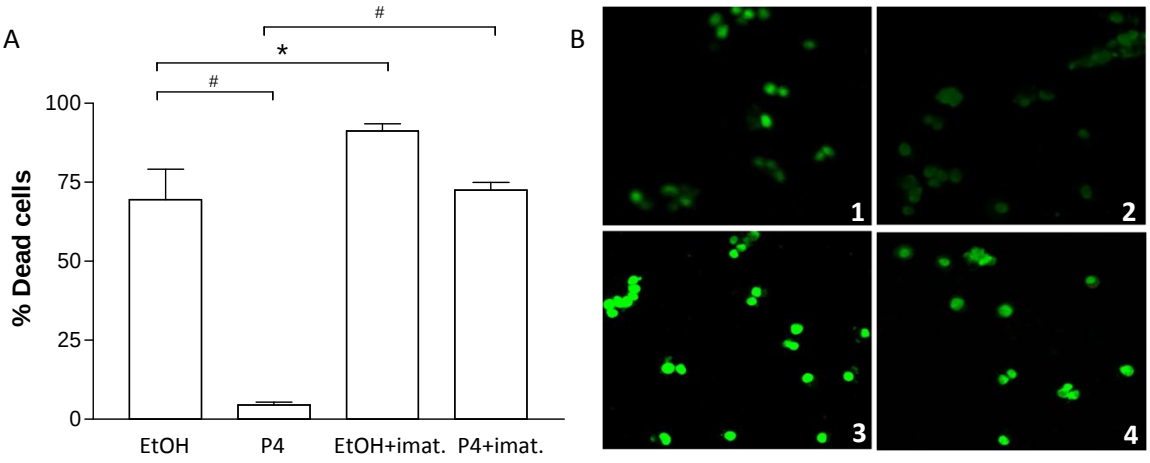

Figure 6. Apoptosis was evaluated in MCF7 cells by TUNEL assay. **(A)** P4 resulted in a strong reduction in the percentage of apoptotic cells (#p<0.01 vs. EtOH). Treatment with imatinib significantly increased apoptosis (*p<0.05). Imatinib significantly reversed the effect of P4 (*p<0.01 vs. P4 alone). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results. **(B)** TUNEL assay images. 1, MCF-7 cells incubated with Ethanol. 2, MCF-7 cells incubated with Progesterone. 3, MCF-7 cells incubated with Ethanol and Imatinib. 4, MCF-7 cells incubated with Progesterone and imatinib. Green fluorescent spots correspond to apoptotic cells.

Imatinib prevented migration of MCF7 cells

The migration ability was first examined using injury assay. Incubation with ethanol (EtOH) for 24h resulted in the migration of a few cells into the damaged areas of cell culture. Migration capacity was intense upon cell incubation with P4, practically covering the whole injured area. Incubation with imatinib either in the presence or in the absence of P4 resulted in much less migration ability to the injured areas as compared to EtOH-treated and to P4-treated cell cultures. Remarkably, in contrast to imatinib in the presence of vehicle only (EtOH+imat), the presence of imatinib combined with P4 resulted in an increase in the number of dead cells (P4+imat) (Figure 7).

To confirm that these findings were due to inhibition of cell migration by imatinib, and not just caused by increased apoptosis, the number of migrating cells was then quantified by double-chamber assay. In comparison to ethanol, P4 led to a strong increase in migration capacity of MCF7 cells (#p<0.01 vs EtOH). A significant decrease in the number of migrating cells was found after incubation with imatinib independently of the presence of P4 (*p<0.05 vs EtOH; #p<0.01 vs P4) (Figure 8).

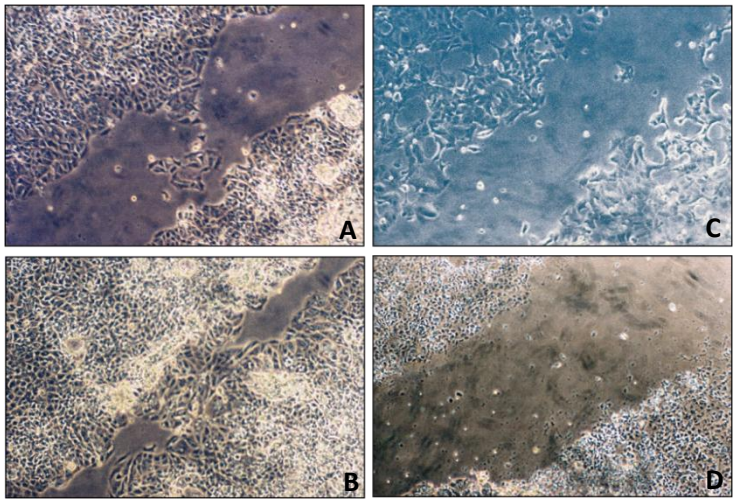

Figure 7. Cell migration was visualized by injury assay after incubation with different treatments during 24 h. P4-treated cells (B) rapidly migrated to the damaged area in comparison to ethanol-treated cells (A). Migration was not observed in cultures incubated with imatinib, either in the absence (C) or in the presence of P4 (D). Note the increased number of dead cells whenever MCF7 cells were incubated with P4+Imat. Pictures are representative of three independent studies. Magnification (X200).

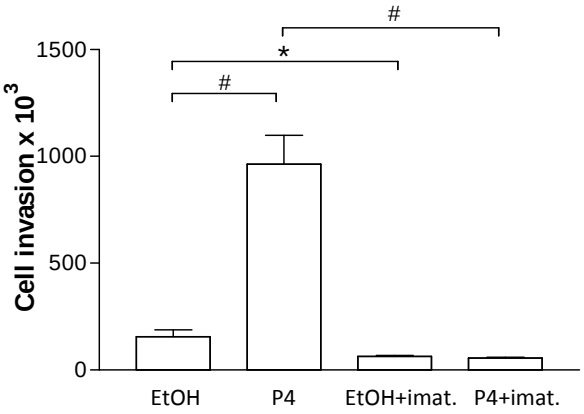

Figure 6. Migration was quantified in a double-chamber assay using medium complemented with 10% FBS as a chemoattractant. Incubation with P4 resulted in effective migration of cells (#p<0.01 vs. EtOH). Treatment with imatinib completely abrogated migration capacity (*p<0.05 vs. EtOH and #p<0.01 vs. P4). Bars represent the number of invasive cells. Assays were repeated twice and performed in triplicate.

DISCUSSION

That breast tumors express PDGF has far been reported (Modi et al. [31]; Roussidis et al. [39]; Carvalho et al. [8]). Nevertheless, only a few studies focused on the presence of PDGFR in the tumor cell membrane (Carvalho et al. [8]; Jechlinger et al. [21]). In previous studies, our group has demonstrated that P4 resulted in increased expression of genes from the PDGF-A signaling pathway in MCF7 cells (Soares et al. [41]). In addition, incubation of these cells with P4 led to increased cell growth and migration ability. These findings were reversed by incubation of cells with PDGF neutralizing antibodies, implying that P4-induced survival and proliferation was accomplished by the interaction with PDGF signaling. The present study focused on the activity of imatinib, an inhibitor of PDGFR-α tyrosine kinase activity, on the behavior of breast cancer MCF7 cells. We first showed that imatinib led to concentration-dependent decreased cell proliferation and increased apoptosis. We also found out that total and active (phosphorylated) PDGFR-α were reduced by imatinib, mainly in the presence of P4. These findings indicate that imatinib is able to both down-regulate the expression and prevent phosphorylation of PDGFR-α. These findings prompted us to examine whether imatinib could exert any effect in the behavior of MCF7 cells.

Moreover, we found that P4 induces proliferation, as regarded by the enlarged number of colonies formed during the seven-day period incubation. In contrast, incubation of cells with imatinib effectively prevented colony formation. This data was corroborated by the MTT viability assays. Accordingly, P4-treated cells presented slightly higher number of viable cells than controls (vehicle-treated cells). Incubation with imatinib efficiently decreased cell viability. In addition, this effect was only significant in the presence of P4. We, therefore, hypothesize that by inducing the activation of PDGFR-α as previously demonstrated (Soares et al. [41]), P4 sensitizes tumor cells against imatinib action. Conversely, in the absence of P4, MCF7 cells are likely promoting other signaling pathways that result in increased proliferation and survival. The effect of P4 on MCF7 cell survival was also denoted by the marked reduction in apoptotic rates. This is not unexpected since P4 has been mainly regarded as a survival factor (Faivre and Lange [14]; Soares et al. [41]). Incubation with imatinib completely abrogated this effect, resulting in significantly increased percentage of apoptotic cells. Imatinib alone also resulted in increased number of apoptotic cells in comparison to ethanol-treated cells. Together with the findings on cell viability, this data emphasize that imatinib might be of primordial interest in hormone-dependent breast cancer.

In agreement with the current data, a recent study by Roussidis et al. ([39]), applying imatinib in breast cancer cell lines, showed that this agent significantly inhibited MCF7 proliferation, by maintaining cells at the G2/M cell cycle phase, which resulted in a concomitant decrease in S-phase. Conversely, they were not able to find an increase in apoptosis in any of the breast cancer cell lines studied upon incubation with imatinib. The current study focused on the role of imatinib in P4 pathway. Our results clearly showed a significant increase in apoptosis after imatinib incubation together with P4. However, and in agreement with Roussidis et al. ([39]), the increase found when imatinib was incubated with

ethanol was much lower, emphasizing again the role of P4 in enhancing imatinib effects (Roussidis et al. [39]).

A phase II clinical trial showed that imatinib resulted in significant toxicity in patients presenting metastatic breast cancer (Modi et al. [31]). This trial was conducted on progressive metastatic breast cancer independently of prior therapeutic strategies. Despite the findings by Modi et al. ([31]) being disappointing, these authors did definitely not target the cases having PDGF signaling activation. Accordingly, only four cases out of 13 presented kit or PDGFR expression. Given the activation of PDGF signaling pathway by progesterone, the current paper provides new data regarding the use of imatinib as beneficial against a particular type of breast cancer, which is progesterone-dependent.

The current study also addressed the effect of imatinib on cell migration. As previously described (Soares et al. [41]), P4 strongly induced MCF7 migration. Imatinib completely reversed this effect. In accordance, the number of migrating cells strongly declined after incubation with imatinib independently of the presence or absence of P4. Although these findings encourage further studies, they already suggest that other signaling pathways might also be involved in cell migration ability, which are not stimulated by P4.

To our knowledge, the studies using imatinib mesylate in breast cancer clinical settings are rare. Only one study reported a phase II trial of imatinib monotherapy in metastatic breast cancer with ineffective results due to increased toxicity (Modi et al. [31]). Although careful trials are needed in order to test imatinib for safety and efficacy in progesterone-dependent breast cancer, the present data points to the potential interest of imatinib as a therapeutic strategy in specific types of breast tumors, namely the ones depending on progesterone signaling pathways.

CHAPTER II

ANTI-ANGIOGENIC EFFECTS OF IMATINIB TARGET SMOOTH MUSCLE CELLS BUT NOT ENDOTHELIAL CELLS

MATERIALS AND METHODS

CHEMICALS

Progesterone (P4) was obtained from Sigma Aldrich (Portugal). Imatinib was kindly provided by Novartis. Antibodies against total PDGFR-α, phosphorylated-PDGFR-α (Santa Cruz Biotechnology, USA) and β-actin (Neomarker, USA) were used for immunoblotting.

P4 was dissolved in 100% ethanol and added to serum-free medium of cell cultures at a final concentration of 10^{-8}M. Concentration for P4 was chosen from a set of 5 different concentrations ranging from 0.1 to 1000 nM in previous studies (Soares et al. [39]). Ethanol was added to cell culture media at a concentration of 0.01% (v/v). Imatinib was dissolved in distilled water and added to serum-free medium of cell cultures at a final concentration of 5 μM. Concentration for imatinib was chosen from a set of 5 different concentrations between 1 and 10 μM used in previous studies (Data submitted for publication).

CELL CULTURES

Human Aortic Smooth Muscle Cells (HAoSMC) were obtained from ScienceCell Research Labs (SanDiego, USA). HAoSMC were routinely cultured in DMEM high glucose medium (Sigma, USA) containing 10% inactivated FBS and 1% penicillin/streptomycin.

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from ScienceCell Research Labs (SanDiego, USA) and cells were used between passages 3 and 8 in this study. HUVECs were cultured in M199 medium supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen Life Technologies, Scotland, UK), 0.1% heparin (Sigma-Aldrich, Portugal) and 0.03 mg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich, Portugal), and maintained at 37º C in a humidified 5% CO_2 atmosphere. Cells were seeded on plates coated with 0.1% gelatin (Sigma) and allowed to grow until 70-90% confluence before experimental treatment.

In agreement with previous studies, incubations with the distinct agents were performed for 24h in serum-free conditions (Soares et al. [41]). Because P4 receptor (PR) is an estrogen-dependent gene product, cells were maintained in complete medium until 70% confluence, then washed twice with PBS and immediately incubated in serum-free conditions, with no prior serum-free medium incubation, as previously described (Rocha et al. [38]).

WESTERN BLOTTING

Proteins were isolated from HAoSMC and HUVEC lysates using Tripure (Roche Diagnostics, Basel, Switzerland). Proteins were quantified using a spectrophotometer (Jenway, 6405 UV/vis, Essex, UK) and equal amounts of protein were subjected to 8% SDS-PAGE with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington, USA), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences, USA). Immunodetection for total PDGFR-α, phosphorylated (activated) PDGFR-α, Pi-PDGFR-α and β-actin was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences, USA). The relative intensity of each protein blotting analysis was measured using a computerized software program (Biorad, California, USA) and normalized with β-actin bands to compare the expression of proteins in different treatment groups. Experiments were repeated three times.

MTT PROLIFERATION ASSAY

Cells were cultured following standard conditions or the treatment procedures for 24 hours. Cells were then washed twice with PBS and subjected to MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] assay, an index of cell viability and cell growth. Cells were incubated with MTT solution at a final concentration of 0.5 mg/mL for 3 hours and then lysed in DMSO. Optical density was measured at 540 nm. The background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was calculated. The results are given as mean ± SD and are expressed as percentage of control, which was considered to be 100%.

BRDU PROLIFERATION ASSAY

HAoSMC and HUVEC ($1x10^4$) were cultured following standard conditions or the treatment procedures for 24 hours. Cells were then washed twice with PBS and subjected to *in situ* detection with BrdU (Bromodeoxyuridine), an index of DNA synthesis and cell proliferation. Cells were incubated with BrdU solution at a final concentration of 0.01 mM for 24 hours and then the *in situ* detection was performed using BrdU *In-Situ* Detection Kit (BD Biosciences Pharmingen, USA), according to the manufacturer's instructions. The results are given as mean ± SD and are expressed as percentage of proliferating cells. The percentage of proliferating cells was evaluated at a x200 magnification field. One thousand nuclei were evaluated. Three independent experiments were performed.

TUNEL ASSAY

Cells ($1x10^4$) grown on glass coverslips were incubated with the different referred treatments for 24 hours. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed using the *In-Situ* Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics, Basel, Switzerland). The percentage of TUNEL-stained nuclei was evaluated in relation to 1000 DAPI-stained nuclei observed in each field. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a x200 magnification field. Three independent experiments were performed.

MIGRATION ANALYSIS

Double-Chamber Assay

The migration capacity of HAoSMC and HUVEC was quantified by counting the number of cells that migrated through matrigel-coated transwell BD-matrigel basement membrane matrix inserts (BD-Biosciences, Belgium). Transwell inserts containing an 8µm pore-size PET membrane coated with a uniform layer of Matrigel basement membrane were used. Cells (5×10^4) were harvested on inserts in serum-free medium, and placed on wells containing complete medium complemented with EtOH, P4 or a combination of EtOH or P4 with imatinib. After incubation for 24h at 37ºC and 5% CO_2, membranes were removed from inserts, stained with DAPI-methanol (Roche Diagnostics, Basel, Switzerland) for 5 minutes and visualized under a fluorescence microscope (Olympus, BH-2, UK). Twenty-five random fields of each membrane were counted on the microscope (X200).

STATISTICAL ANALYSES

All experiments were performed in triplicate. Quantifications are expressed in mean ± SD. Samples were evaluated by the analysis of variance test. A difference between experimental groups was analyzed by Student's t-test, and was considered statistically significant whenever the p value was less than 5%.

RESULTS

Expression of activated PDGFR-α in HAoSMC and HUVEC

To ensure that imatinib could be effective in HAoSMC and HUVECS, we first confirmed the expression of PDGF receptors (PDGFR) in these cells by Western blotting. The expression of total and active (phosphorylated) PDGFR-α (Pi-PDGFR-α) was observed in HAoSMC lysates after each treatment (Figure 1A). Incubation with P4 resulted in increased activation of PDGFR-α, in comparison to vehicle-treated cells (EtOH). Interestingly, imatinib treatment led to a reduction in PDGFR-α expression, particularly in control cells (intensity band of 0.77). This decrease was also found in the phosphorylated (active) form of this receptor after incubation either in the presence or in the absence of P4 (Figure 1A), implying that imatinib was able to abrogate PDGFR-α signaling pathway in these cells, independently of the presence of P4. In contrast, PDGFR-α expression was hardly detected in HUVECs (Figure 1B). Furthermore, incubation with imatinib led to no significant differences.

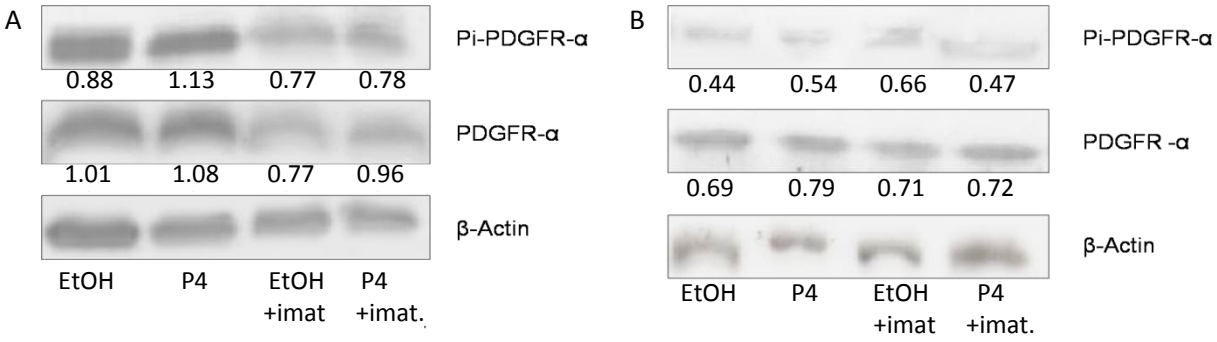

Figure 1. Expression of total PDGFR-α and activated (phosphorylated)-PDGFR-α (Pi-PDGFR-α) in HAoSMC and HUVEC cell lysates after incubation with ethanol (EtOH), progesterone (P4), ethanol+imatinib (EtOH+Imat) or progesterone+imatinib (P4+Imat) during 24 h. (A) In HAoSMC PDGFR-α expression was found in every cell lysate. Pi-PDGFR-α was slightly decreased in imatinib-treated cultures as compared to controls. The relative intensity of each band after normalization to β-Actin is shown under each blot, expressed as mean values. (B) In HUVECs PDGFR-α was found, at a very low extent, in every cells lysate. The relative intensity of each band after normalization to β-Actin is shown under each blot, expressed as mean values (Figure 1B). A representative Western blotting is shown from three independent experiments.

Imatinib decreased cell viability and proliferation and increased apoptosis in HAoSMC

To determine the effect of imatinib on HAoSMC, cell viability was investigated by MTT assay. Incubation of HAoSMC cells with P4 did not significantly affect cell viability in comparison to controls (Figure 2). A significant decrease in cell viability was found upon exposure to imatinib in the presence of vehicle (EtOH + imat) (*p<0.05 vs Ethanol). However, treatment with imatinib together with P4 resulted in a more significant decrease in HAoSMC viability (Figure 2) (#p<0.01 vs P4).

We then addressed the role of imatinib in cell proliferation by BrdU analysis. P4 led to a significant increase in cell growth (Figure 3). Again, the presence of imatinib resulted in a marked decrease in the percentage of proliferating cells (Figure 3). This reduction was more evident after incubation with P4 (Figure 3) (#p<0.01 vs P4).

The effect of imatinib in HAoSMC apoptosis was investigated using TUNEL analysis. As illustrated in Figure 4, apoptosis was strongly reduced whenever cells were incubated with P4. The presence of imatinib significantly increased the percentage of apoptotic cells both in control and in P4-treated cell cultures, reversing the survival effects of P4 (#p<0.01 vs P4) (Figure 4).

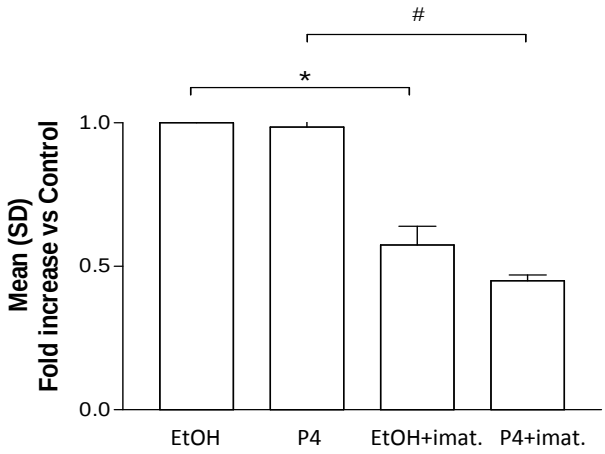

Figure 2. Cell viability in HAoSMC was assessed by MTT assay. A significant increase in cell viability was found after incubation with P4 for 24 h. Incubation with imatinib led to a decrease in cell viability, this decrease being more significant whenever cells were treated with imatinib combined with P4 (P4+Imat) (*p<0.05; #p<0.01). Cell viability is expressed in terms of percentage of control cells (EtOH), and is reported as mean ± SD.

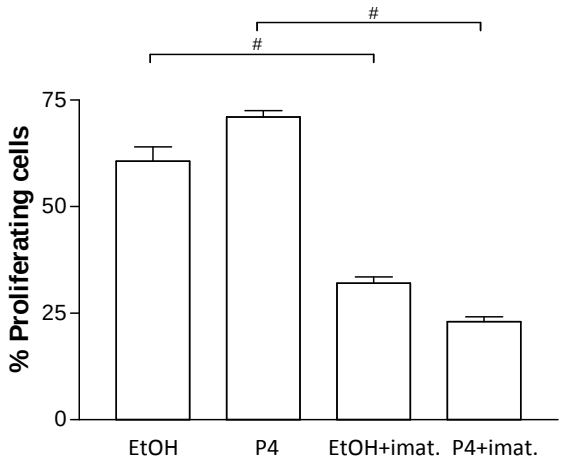

Figure 3. Proliferation levels were assessed in HAoSMC by BrdU assay. Incubation with P4 resulted in a significant increase in the percentage of proliferanting cells. Whenever cells were treated with imatinib a very significant decrease of the proliferation levels could be noticed (#p<0.01). Bars represent the percentage of proliferating cells evaluated by the number of BrdU-stained cells in 1000 hematoxylin-stained cells.

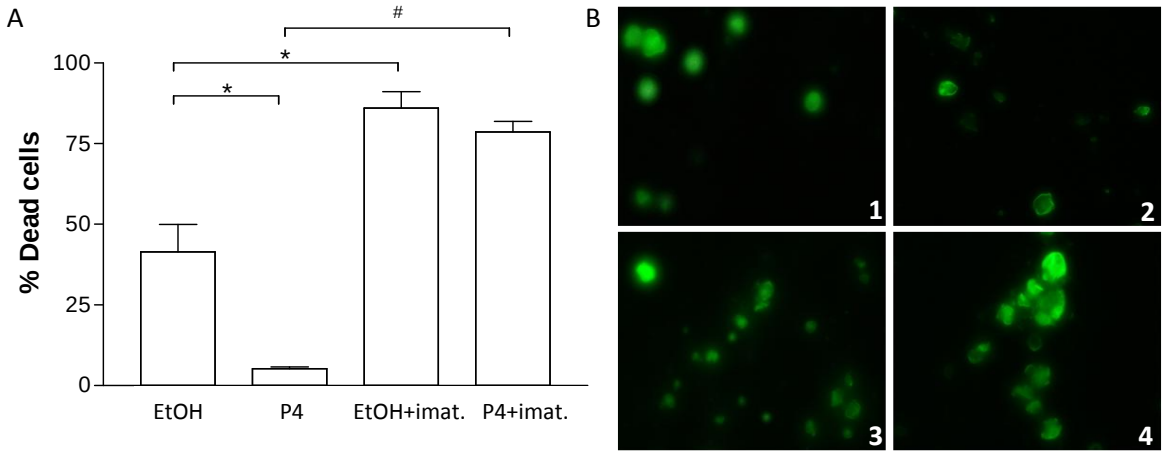

Figure 4. Apoptosis was evaluated in HAoSMC cells by TUNEL assay. **(A)** P4 resulted in a marked reduction in the percentage of apoptotic cells. Treatment with imatinib significantly increased apoptosis (*p<0.05 vs. EtOH; #p<0,01 vs. P4). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results. **(B)** TUNEL assay images. 1, HAoSMC incubated with Ethanol. 2, HAoSMC incubated with Progesterone. 3, HAoSMC incubated with Ethanol and Imatinib. 4, HAoSMC incubated with Progesterone and Imatinib. Green fluorescent spots correspond to apoptotic cells.

Imatinib prevented migration of HAoSMC cells

The migration ability was quantified by double-chamber assay. In comparison to ethanol, P4 led to an obvious increase in migration capacity of HAoSMC. The number of migrating cells found after incubation with imatinib was significantly reduced in the presence or in the absence of P4 (Figure 5).

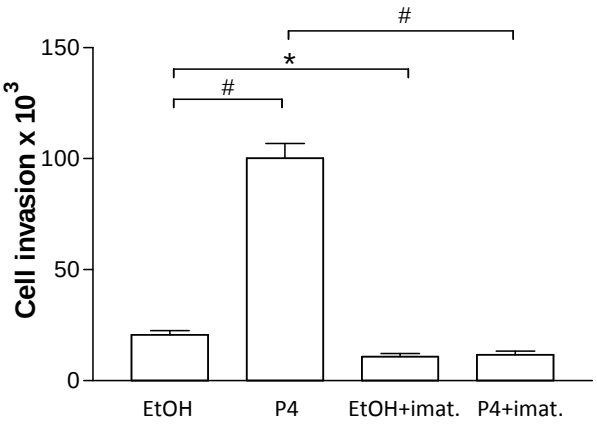

Figure 5. Effects of imatinib in HAoSMC migration were quantified in a double-chamber assay using medium complemented with 10% FBS as a chemoattractant. Incubation with P4 resulted in effective migration of cells (#p<0.01 vs. EtOH). Treatment with imatinib completely abrogated migration capacity (*p<0.05 vs. EtOH and #p<0.01 vs. P4). Bars represent the number of invasive cells. Two independent assays were performed in triplicate.

Imatinib had no significant effects in HUVEC

The effect of imatinib on HUVEC viability, proliferation, apoptosis and migration was addressed using MTT assay, BrdU analysis, TUNEL and Double-chamber assay respectively. P4 significantly enhanced cell viability (Figure 6), proliferation (Figure 7) and migration (Figure 9). Apoptosis was effectively reduced by P4 as well (Figure 8). Incubation with imatinib did not significantly affect any of these cellular processes (Figures 6, 7, 8 and 9), despite the faint expression of phosphorylated PDGFR-α found in these cells.

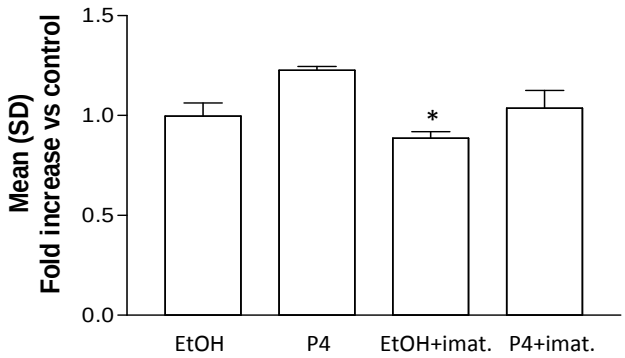

Figure 6. Cell viability by MTT assay in HUVECs. As expected, a significant increase in cell viability was found after incubation with P4 for 24 h. Incubation with imatinib led to no significant change (*p<0.05 vs. EtOH).

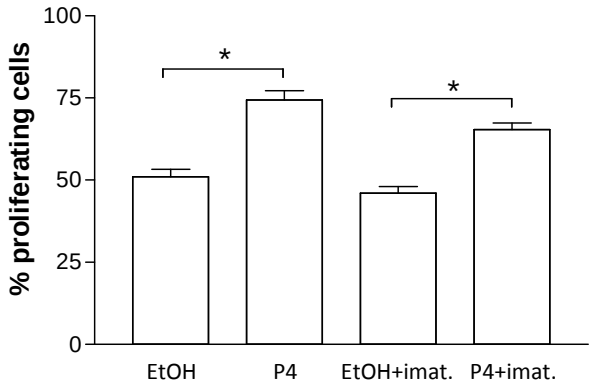

Figure 7. Proliferation levels were evaluated, in HUVECs, by BrdU assay. Incubation with P4 resulted in a significant increase in the percentage of proliferanting cells. Incubation with imatinib had no significant effect in the proliferation values (*p<0.05). Bars represent the percentage of proliferating cells evaluated by the number of BrdU-stained cells in 1000 hematoxylin-stained cells.

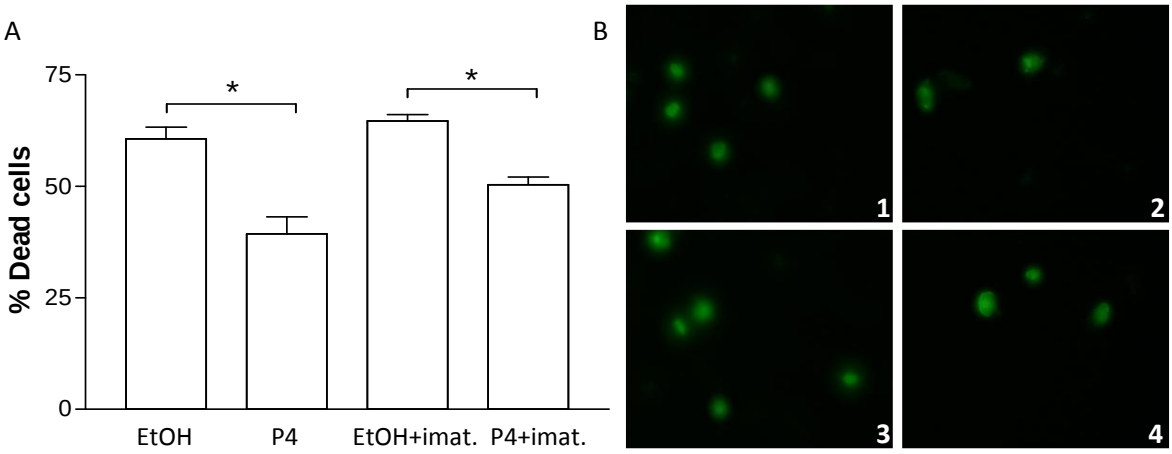

Figure 8. Apoptosis levels were studied in HUVECs by TUNEL assay. **(A)** The percentage of apoptotic cells did significantly increase when cells were incubated with P4. No significant results were found whenever cells were treated with imatinib (*p<0.05). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results. **(B)** TUNEL assay images. 1, HUVEC incubated with Ethanol. 2, HUVEC incubated with Progesterone. 3, HUVEC incubated with Ethanol and Imatinib. 4, HUVEC incubated with Progesterone and Imatinib. Green fluorescent spots correspond to apoptotic cells.

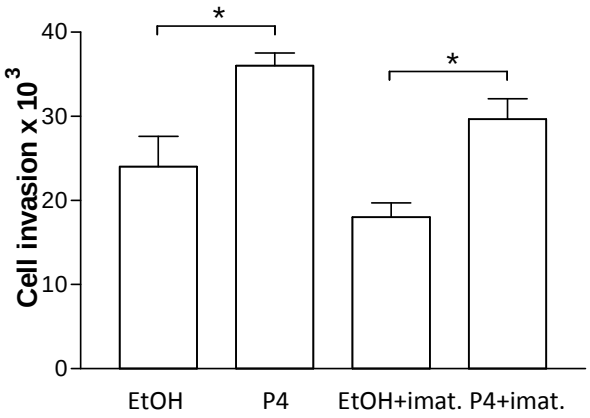

Figure 9. The effect of imatinib in HUVECs migration was quantified in a double-chamber assay using medium complemented with 10% FBS as a chemoattractant. Incubation with P4 resulted in effective migration of cells (*p<0.05). Treatment with imatinib had no significant effect in the migration ability of HUVEC cells. Bars represent the number of invasive cells. Assays were repeated twice and performed in triplicate.

DISCUSSION

Imatinib was designed to target tyrosine kinase receptor activity, including c-kit, Bcr-Abl and PDGFR-α-receptors. In the current study, imatinib was able to prevent SMC growth and migration, as well as to induce cell apoptosis. Accordingly, our study also pointed out that the use of imatinib resulted in down-regulation of PDGFR-α expression and activity, even in the presence of P4, a previously established PDGF signaling promoter (Soares et al. [37]). PDGF signaling transduction pathway plays essential roles in SMC behavior, namely resulting in increased survival and proliferation of these cells (Edelberg et al. [8]; Machens et al. [23]; Tsutsumi et al. [40]). In addition, expression of the two PDGF receptors has been described in these blood vessel support cells (Taylor [39]). Neovessels maturation is accomplished by the release of PDGF, which activates their specific receptors at the SMC membrane, leading to the recruitment of these cells into the angiogenic vessels (Taylor [39]; Edelberg et al. [8]; Machens et al. [23]). As a result, neovessels stabilize, a critical step for the development of functional vasculature. Absence of SMC adhesion leads to vessel leakage. Furthermore, these vessels are also prone to regression (Distler et al. [7]; Armulik et al. [2]).

Interestingly, these findings were restricted to SMC. A very tenuous expression of PDGFR-α was found in HUVEC cultures. Additionally, these expression levels were not changed by incubating HUVEC with P4 or imatinib. Immunostaining for phosphorylated-PDGFR-α also showed that this receptor activity was practically inexistent in these cells, indicating the lack of responsiveness of these cells to PDGFR-α. In agreement with this, imatinib did not exert any effect in HUVEC, even in the presence of P4. Remarkably, P4 was able to promote cell growth and migration, and strongly prevented apoptosis in the current study. However, since PDGF signaling pathway does not play a significant role, we anticipate that other angiogenic pathways are probably activated by P4 in these cells. A few studies reported the role of P4 as an angiogenic promoter (Walter et al. [41]; Mirkin et al. [23]; Wu et al. [43]; Liang et al. [20]; Hyder et al. [13]). VEGF, a well-known EC mitogen, is a good candidate for the role of P4 in endothelial cell growth and survival, observed in our study, since P4 has been claimed to induce VEGF signaling activation (Hyder et al. [14]; Liang et al. [20]; Hyder et al. [13]).

The effect of imatinib as an anti-angiogenic agent has been previously reported (Kim et al. [20]; Kvasnicka et al. [21]). However, given the action of imatinib in targeting tyrosine kinases involved in tumor progression, this agent has been used in tumor settings that specifically depend on the activity of these tyrosine kinase receptors, such as Bcr-Abl in CML and c-kit and PDGFR in GIST. Recent findings, though, showed that imatinib-treated tumors often develop resistance (Ault [3]). Therefore, a more careful use of imatinib has lately been provided to patients carrying CML and GIST. The current work provides evidence that imatinib is able to prevent SMC proliferation, survival and migration. In contrast to tumor cells, SMC fail to develop drug resistance mechanisms, rendering these cells good candidates for the action of imatinib. SMC proliferation and migration are essential for the fulfillment of angiogenesis. In addition, this process is associated with several distinct pathological situations. Restenosis, for instances, is characterized by increased smooth muscle cells growth (Chan [6]).

Unbalanced SMC behavior is also associated with atheroma plaque formation, a highly incident disease in the western world (Qin and Liu [32]).

Our findings that imatinib abrogates SMC proliferation, viability and migration leads to the assumption that it might be useful in a wide range of disorders that exhibit imbalanced SMC behaviour.

OVERALL CONCLUSION

The current study focused on the effect of imatinib on breast cancer that responds to progesterone and on angiogenesis. Altogether, our findings showed that imatinib prevented cell viability, proliferation and migration, and increased apoptosis in MCF7 cells. Although no relevant effect has been observed in EC, imatinib also seems to effectively abrogate SMC proliferation and migration, decreasing viability and increasing apoptosis, relevant processes for vessel stabilization. Furthermore, these effects, in MCF7 and in SMC, were enhanced by the concomitant incubation of cells with P4, implying that this hormone is rendering cells prone to the action of imatinib.

Given the relevance of progesterone in hormone-dependent tissues, this study provides novel data regarding the use of imatinib as a putative anti-cancer and anti-angiogenic agent, particularly in progesterone receptor-expressing organs.

FUTURE PERSPECTIVES

- To investigate the effect of imatinib on hormone-dependent breast cancer angiogenesis *in vivo,* by inoculating human MCF7 breast cancer cells into nude mice and administrating imatinib.

- To test other progesterone-dependent cancer cell lines in order to examine whether the effect of imatinib is still found.

- To investigate the role of progesterone in vascular wall cells (ECs and SMC), blocking the PDGF signaling pathway, using PDGF neutralizing antibodies or siRNA assays.

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