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On the ability of imatinib mesylate to inhibit smooth muscle cell proliferation without delaying endothelialization: An in vitro study

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ABSTRACT

Restenosis, the re-occlusion of a diseased vessel following a surgical intervention, is a major cause of failure of angioplasty, stenting, and bypass grafting with natural and synthetic vessels. In healthy vessels, the endothelium exerts a control over smooth muscle cell (SMC) proliferation and migration. Unfortunately, revascularization procedures damage the endothelium of natural vessels and bypass vessels are completely devoid of endothelial cells. Many strategies have been developed to inhibit SMC proliferation and reduce intimal hyperplasia, yet most of the drugs tested thus far simultaneously inhibit endothelialization and do not selectively target SMCs. The ideal biological agent should have anti-proliferative effects on SMCs while preserving vascular healing and endothelialization so as to prevent late thrombosis. Imatinib mesylate is a specific inhibitor of three tyrosine kinase receptors, two of which, PDGF-R and c-Kit, are implicated in the pathogenesis of intimal hyperplasia. In this study, we investigated in vitro the potential of imatinib mesylate to inhibit SMCs and its effect on ECs. Our findings indicate that low doses of imatinib mesylate successfully inhibit SMC proliferation. Furthermore, at these concentrations, the drug was not only harmless to ECs, but also enhanced their proliferation. In light of these in vitro results, imatinib mesylate shows potential as a good candidate to inhibit intimal hyperplasia without delaying neo-endothelialization.

1. Introduction

Restenosis caused by intimal hyperplasia is a major cause of failure of most revascularization procedures such as angioplasty, stenting, and bypass grafting with natural and synthetic vessels (Bhoday et al., 2006; Vamvakopoulos et al., 2006). The intima is the innermost layer of blood vessels and is normally composed of an endothelium and its underlying basal lamina. In healthy vessels, the endothelial cell (EC) monolayer releases several factors (e.g. nitric oxide, prostacyclin) which force the smooth muscle cells (SMCs) into a lower proliferating and invasive phenotype (McCormick et al., 2007; Popowich et al., 2007). However, following surgical revascularization, the endothelium is either damaged or absent, thus impeding proper function (McCormick et al., 2007; Popowich et al., 2007; Sumpio et al., 2002). Intimal hyperplasia is characterized by a thickening of the intima which is due primarily to SMC proliferation and extracellular matrix production (Kraitzer et al., 2008). Synthetic prostheses, designed to replace beyond-repair damaged large vessels, have demonstrated a fair amount of efficiency (Prager et al., 2001). However, small to medium-size vessels remain harder to replace by these synthetic prostheses (Klinkert et al., 2004; Pereira et al., 2006). In this context, our work in recent years has been to characterize new ways to optimize the surface of such grafts so as to reduce intimal hyperplasia while enabling their endothelialization (Gagne et al., 2006; Gauvreau and Laroche, 2005; Vallieres et al., 2007a,b).

In a previous study, we demonstrated that fibronectin (FN) grafted in active conformation onto PTFE greatly improved EC adhesion onto this polymer (Vallieres et al., 2007b). However, FN is an adhesion protein present in most extracellular matrix and is not recognized exclusively by endothelial cells (Lodish et al., 2003). Because smooth muscle cell proliferation in the anastomotic sites of prostheses leads to intimal hyperplasia, these cells must be selectively inhibited.

The origin of these SMCs is unclear. It was first assumed that they originated from the media and migrated into the intima (Ross, 1993; Clowes et al., 1983; Reidy, 1985), however some recent studies have shown that bone marrow cells are key factors in the development of intimal hyperplasia (Sata et al., 2002; Wang et al., 2006, 2007) and that due to an increase in stem cell factor (SCF) expression, c-Kit$^+$ bone marrow-derived progenitors are recruited at the site of vascular injury and give rise to a significant proportion of SMCs that contribute to intimal hyperplasia (Wang et al., 2006). SCF promotes cell survival, proliferation, mobilization, and adhesion through the interaction with its receptor (c-Kit) (Wang et al., 2007; Miyamoto et al., 1997; Taylor

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and Metcalfe, 2000). Furthermore, c-Kit expression in SMCs, which express both the SCF and c-Kit, was shown to increase considerably following vascular injury (Hollenbeck et al., 2004). This suggests that SCF/c-Kit signalling is involved in the pathogenesis of intimal hyperplasia and stimulates SMCs via an autocrine pathway (Wang et al., 2007; Hollenbeck et al., 2004).

While many strategies have been developed to inhibit SMC proliferation, there remains no long-term effective therapy to prevent intimal hyperplasia in humans. The best results thus far have been obtained with the immunosuppressant drug sirolimus (rapamycin) and the microtubular inhibitor paclitaxel. These drugs have been approved for clinical use as stent coatings and have shown good short-term results (Duda et al., 2003; Moses et al., 2003; Stone et al., 2004; Suzuki et al., 2001), despite the fact that their long-term safety has been questioned (Camenzind et al., 2007; Pfisterer et al., 2006) because of the continual increase in late stent thrombosis (Roy et al., 2008). Most of the tested drugs do not specifically target SMCs and they inhibit endothelialization at the same time. The ideal biological agent should have an anti-proliferative effect on SMCs while preserving vascular healing and endothelialization (Kraitzer et al., 2008).

Imatinib mesylate is a specific inhibitor of three tyrosine kinase receptors: PDGF-R, c-Kit, and Bcr/Abl. This inhibitor has been used to treat chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST), which are characterized by the presence of mutated Bcr/Abl and c-Kit, respectively (Radford and Imatinib, 2002). Many studies have also demonstrated an anti-angiogenic effect of imatinib mesylate mediated via PDGF-R inhibition (Kim et al., 2005; Kvasnicka et al., 2004). Because muscle cells express both c-Kit and PDGF-R, two important mediators of intimal hyperplasia pathogenesis (Sata et al., 2002; Wang et al., 2006, 2007; Hollenbeck et al., 2004; Banai et al., 2005; Simper et al., 2002), this drug may potentially be highly effective in inhibiting SMC growth. Furthermore, mature ECs do not express PDGF-R and Bcr/Abl and their expression of c-Kit is uncertain (Miyamoto et al., 1997; Miettinen et al., 2000). In this context, the possibility that imatinib mesylate does not interfere with EC growth should not be ruled out.

In this study, we evaluated in vitro the potential of imatinib mesylate to inhibit human and bovine SMC proliferation with no harm to human and bovine ECs. Proliferation assays were also performed on co-cultures of muscle and endothelial cells to evaluate the drug's activity in a scenario which more closely mimicked the in vivo competition between the two cell types.

2. Materials and methods

2.1. Chemicals

A 100 mg caplet of imatinib mesylate (Novartis Pharmaceuticals Canada Inc., Dorval, QC, Canada) was obtained from the St-François d'Assise Hospital pharmacy. The caplet was crushed in a mortar and solubilised in 2 mL DMSO (Sigma-Aldrich, Oakville, ON, Canada), and 3 mL of PBS was added to obtain a final concentration of 40% DMSO. Paclitaxel (Sigma-Aldrich) was used to induce endothelial cell apoptosis. Human fibronectin (FN) was supplied by Roche Applied Sciences (Laval, QC, Canada). Resazurin and the secondary antibodies conjugated with AlexaFluor 488 (anti-rabbit and anti-mouse) and AlexaFluor 568 (anti-mouse) were obtained from Invitrogen (Burlington, ON, Canada). The polyclonal Von Willebrand factor antibody was supplied by Dako (Mississauga, ON, Canada), while the monoclonal alpha actin antibody and the Hoechst 33342 were purchased from Sigma-Aldrich. Antibodies to the cleaved caspase-3 and PARP were supplied by Cell Signalling Technology (Danvers, MA, USA). HRP-conjugated anti-rabbit and ECL detection reagent were obtained from Amersham/GE Healthcare (Piscataway, NJ, USA) and PCNA antibody was supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell cultures

Human vascular smooth muscle cells (HVSMCs) were kindly provided by Dr. François Marceau from the Centre de Recherche du Centre Hospitalier Universitaire de Québec. Human umbilical vein endothelial cells (HUVECs) were isolated by limited digestion using bacterial chemotrypsin. Bovine aortic smooth muscle cells (BAOSMCs) were isolated using the explant technique by tissue sections from cow aortas obtained from a local slaughterhouse. The explants were first kept in a minimum volume of DMEM containing 10% FBS and 4% penicillin/streptomycin until the cells were seen migrating out of the tissue. The bovine aortic endothelial cells (BAECs) were purchased from Cambrex Bio Science (Walkersville, MD, USA). The human cells were cultured in medium M199 (Sigma-Aldrich, Milwaukee, WI, USA) containing 20% inactivated FBS and 1% penicillin/streptomycin (Invitrogen). For the human endothelial cells, 4 μL/mL of endothelial cell growth supplement (ECGS) was added to the culture medium. The bovine cells were cultured in high-glucose DMEM (HyClone, Logan, UT, USA) containing 10% inactivated FBS and 1% penicillin/streptomycin. All of the cells were used between passages 3 and 6.

2.3. Resazurin proliferation assay

To verify the potential of imatinib mesylate to inhibit smooth muscle cell growth without harming endothelial cells, 72-h proliferation assays were performed using increasing concentrations of the drug in the culture medium. These assays were performed with human vascular smooth muscle cells (HVSMCs), human umbilical vein endothelial cells (HUVECs), bovine aortic smooth muscle cells (BAOSMCs), and bovine aortic endothelial cells (BAECs). A preliminary assay was performed to estimate the concentration of imatinib that was associated with 50% inhibition (IC50) of BAOSMCs growth. Since IC50 was found to be around 10 μM, a 1/3 serial dilution ranging from 100 to 1.2 μM was used for further experiments. The 100 μM concentration was abandoned later because it was lethal to all cells. The cells were seeded in 48-well plates in their respective medium with serum. Imatinib was added directly to the medium at time zero and the plates were incubated at 37 °C for 72 h. At the end of the proliferation period, the cells were washed with PBS and incubated with 50 μg/mL of resazurin in culture medium without serum for 2 h at 37 °C. Fluorescence measurements were recorded using excitation and emission wavelengths of 485 and 590 nm, respectively, in a BioTek FL600 reader (Winooski, VT, USA). The relative percentage of cells was calculated with respect to a control without imatinib and was expressed as a mean ± standard error of at least 12 determinations.

2.4. Immunofluorescence

Glass coverslips were placed in 24-well plates and sterilized in alcohol. Following two PBS washes, the coverslips were gelatinized overnight at 4 °C. The wells were then washed with PBS and the cells were seeded in DMEM with serum and imatinib mesylate at 0, 1.2, 3.7, 11.1, and 33.3 μM. The plates were incubated for 72 h at 37 °C. All of the wells were washed with PBS, fixed for 15 min in formaldehyde 3.7%, and washed again in PBS. The cells were then blocked and permeabilized for 1 h with PBS containing 3% bovine serum albumin (BSA) and 0.1% saponin prior to being incubated for 2 h with the primary antibodies (anti-Von Willebrand factor and anti-α actin) diluted in PBS, 3% BSA, and 0.1% saponin. After washing with PBS containing 0.05% Tween 20, the secondary antibodies (Alexa Fluor 488 conjugated anti-rabbit and Alexa Fluor 568 anti-mouse) and Hoechst 33342 were added for 1 h. The wells were washed 5 times and the coverslips were mounted onto glass slides to be viewed under a fluorescence microscope (Olympus BX51, Markham, ON, Canada). Images were captured with a Retiga Exifast digital camera (QImaging, Surrey, BC, Canada) driven by Image Pro software (Media Cybernetics Inc., Bethesda, MD, USA).