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ORIGINAL ARTICLE

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Imatinib mesylate inhibits platelet-derived growth factor activity and increases chemosensitivity in feline vaccine-associated sarcoma

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Abstract Feline vaccine-associated sarcoma (VAS) is a biologically aggressive soft-tissue sarcoma that can develop at sites where inactivated feline vaccines have been administered. We showed that platelet-derived growth factor (PDGF) and its receptor (PDGFR) play a role in the growth of VAS cells. The presence of PDGFR-β was confirmed in each of five VAS cell lines evaluated, one non-vaccine-associated feline fibrosarcoma (FSA) cell line and a feline fibroblast-derived cell line. The PDGF/PDGFR signaling pathway was inhibited in the VAS cell lines and the FSA cell line using the tyrosine kinase inhibitor imatinib mesylate (formerly called STI-571). Imatinib inhibited PDGF-BB-induced autophosphorylation of PDGFR in VAS cells and feline FSA cells in vitro in a dose-dependent manner. Imatinib also significantly inhibited growth of feline VAS tumors in a murine xenograft model. Imatinib reversed the protective effect of PDGF-BB on growth inhibition by doxorubicin and carboplatin. PDGF-BB protected VAS cells from serum starvation and doxorubicin-induced apoptosis but not carboplatin-induced apoptosis, and imatinib eliminated this protection. These observations suggest that imatinib inhibits PDGFR tyrosine kinase activity in feline soft tissue sarcomas in vitro and inhibits tumor growth in a xenograft model.

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Abbreviations C/10: Complete minimal essential medium supplemented with 10% FBS · CPT: Carboplatin · DOX: Doxorubicin · EGF: Epidermal growth factor · FBS: Fetal bovine serum · FSA: Fibrosarcoma · HGF: Hepatocyte growth factor · IGF-1: Insulin-like growth factor-1 · MTS: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium · PDGF: Platelet-derived growth factor · PDGFR: Platelet-derived growth factor receptor · PI: Propidium iodide · VAS: Vaccine-associated sarcoma

Introduction

Feline VAS is a soft-tissue tumor that can develop at sites where inactivated feline vaccines (e.g., feline leukemia virus and rabies) have been administered [20, 24, 27]. Evidence suggests that vaccine adjuvants, such as aluminum hydroxide or aluminum phosphate, enhance the chronic immune response and result in inflammatory granulomas at the site of vaccination, which may promote the neoplastic transformation of resident fibroblasts and myofibroblasts in genetically predisposed cats [21, 23, 34]. In an ultrastructure study, aluminum was identified in the cytoplasm of macrophages in postvaccinal granulomas and in some sarcomas that developed at vaccine sites [35]. It was suggested that aluminum may directly cause tumor development; however, since this crystalline particulate material is not always detected in VAS, the role of the vaccine adjuvants remains uncertain.

Histopathologically, FSA are the most common reported type of VAS; however, various types of mesenchymal tumors can develop (e.g., rhabdomyosarcoma, malignant fibrous histiocytoma, soft-tissue osteosarcomas, chondrosarcomas, and myofibrosarcoma) [14, 15, 22]. While similar to feline FSA that are not associated

with vaccinations, chronic inflammation, infiltration, rapid proliferation, ulceration, incomplete excision, and necrosis are more common features in VAS [39]. These tumors have a high rate of recurrence [11, 24] and poor prognosis after simple surgical excision, with reported median survival times of 12–19 months [11, 26]. Radiation therapy and several antineoplastic drugs, including carboplatin (CPT), doxorubicin (DOX), Doxil, cyclophosphamide, and vincristine, have been used as adjuvant therapy following excision, resulting in prolonged disease-free survival; however, the majority of cats eventually succumb to disease despite these treatments [3, 5, 11, 29, 42]. Although the majority of VAS are locally invasive, approximately 5–25% will metastasize to the lung or other sites [11, 24, 26].

Platelet-derived growth factor (PDGF) is critical for the growth of fibroblasts, vascular smooth muscle cells [19], brain glial cells [25, 40], and kidney mesangial cells [16]. PDGF is also a potent controller of platelet aggregation during wound healing [43, 44], a stimulator of atherosclerosis [19], and plays an important role in the maintenance of interstitial fluid pressure [45]. The PDGF family contains five isoforms of ligands (PDGF-AA, -BB, -AB, -CC, -DD) and their PDGF receptors (PDGFR-$\alpha\alpha$, -$\alpha\beta$, -$\beta\beta$). PDGFR-$\alpha\alpha$ binds to all types of ligands, PDGFR-$\alpha\beta$ binds to PDGF-AB and -BB, and PDGFR-$\beta\beta$ binds to only PDGF-BB [19]. PDGF-CC and -DD have been discovered recently, and bind to PDGFR-$\alpha\alpha$ and -$\beta\beta$, respectively [4, 32, 33]. The B-chain of PDGF is identical to the v-*sis* protooncogene, and overexpression of PDGF-BB modulates endothelial proliferation, malignant transformation, invasion, angiogenesis, metastasis, and inhibits apoptosis of mesenchymal cells [19]. Ligand binding of PDGF to extracellular immunoglobulin-like domains of PDGFR induces receptor dimerization, activates the intracellular tyrosine kinase, and promotes receptor autophosphorylation on its tyrosine residues. This autophosphorylation activates downstream signal transduction, and results in cell proliferation and inhibition of apoptosis [19].

The expression of receptors for PDGF has been demonstrated in human soft-tissue sarcomas [1, 2, 31, 41, 48, 52], and PDGF has been shown to modulate sarcoma cell proliferation and migration in vivo and in vitro [1, 2, 49]. Thus, evaluation of PDGFR inhibitors in a relevant, spontaneous large animal model of human soft-tissue sarcoma is reasonable.

Imatinib mesylate, a signal transduction inhibitor formerly known as STI-571 or Gleevec (Glivec), is a small molecule kinase inhibitor derived from 2-phenylaminopyrimidine [6, 7]. Imatinib selectively inhibits PDGFR (both -α and -β) tyrosine kinase activity by binding to the ATP binding site, thus inhibiting downstream signal transduction including the phosphatidylinositol 3′ kinase antiapoptosis pathway and the mitogen-activated protein kinase cell proliferation pathway [6, 7, 8]. Imatinib has been studied in human solid tumors and inflammatory diseases expressing PDGFR, including glioma [28], dermatofibrosarcoma protuberans [46, 47], osteosarcoma [37], and mesangial proliferative glomerulonephritis [17], and results in cell growth arrest. In humans, imatinib also selectively inhibits the Bcr-Abl tyrosine kinase in chronic myeloid leukemia [7, 12, 13, 36], and c-kit tyrosine kinase in small-cell lung cancer [30] and gastrointestinal stromal tumors [50, 51], and results in either inhibition of cell proliferation or apoptosis.

In the studies reported here, we determined that PDGFR-β and its consequent signal transduction contribute to cell growth in VAS and FSA and protection from apoptosis in VAS. We also showed that the PDGFR tyrosine kinase inhibitor imatinib can successfully disrupt the PDGF/PDGFR signaling pathway, leading to VAS and FSA cell growth inhibition both in vitro and in vivo, and can increase sensitivity to apoptosis. The ability to disrupt the PDGF/PDGFR signaling pathway may offer a potential therapy for cats with VAS and similar soft-tissue sarcomas in humans.

Materials and methods

Cell lines

Feline VAS and non-VAS (FSA) cell lines were established from spontaneously arising tumors in client-owned cats treated at the University of Wisconsin Veterinary Medical Teaching Hospital (UW-VMTH). Tissues collected at surgery were minced and dissociated with DNAse (200 U/ml) and collagenase (270 U/ml) into a single-cell suspension, washed and plated onto tissue culture flasks with complete minimal essential medium (Mediatech-Cellgro, Herndon, Va.) supplemented with 10% FBS (C/10). After 24 h, the nonadherent cells were removed and the adherent cells were maintained. Early passage cells were frozen in liquid nitrogen for future use. Tumors were collected from 23 sites on 21 cats. From these tumors, 20 cell cultures were established, and 6 cell lines were used for these experiments (Table 1). Feline normal fibroblasts (CRL-6102; American Type Culture Collection, Bethesda, Md.) and a canine mammary carcinoma cell line (CMT-12; provided by Dr. L.G. Wolfe, Auburn University) were used as controls. Each cell line was cultured in C/10, and maintained in an atmosphere containing 5% CO_2 at 37°C (standard conditions).

Imatinib mesylate

Imatinib mesylate was provided by Novartis Pharma (Basel, Switzerland). For in vitro studies, 50 m *M* stock solutions were prepared in DMSO, and stored at −20°C. Dilutions of imatinib were made from the stock solutions in serum-free medium. For in vivo studies, imatinib was diluted with saline immediately before use.

Table 1 Characteristics of cell lines used

Name	Breed	Age (years)	Gender	Histopathology	Lesion location	Diagnosis
VAS-1	Domestic short hair	13	Spayed female	FSA	Lateral lumbar	VAS
VAS-2[a]	Domestic short hair	14	Spayed female	FSA	Dorsal lumbar	VAS
VAS-3	Domestic short hair	4	Spayed female	FSA	Hind leg	VAS
VAS-4	Domestic short hair	6	Spayed female	FSA	Interscapular	VAS
VAS-5	Domestic short hair	10	Spayed female	FSA	Interscapular	VAS
FSA-1	Domestic short hair	8	Spayed female	FSA	Hind leg	Non-VAS FSA

[a]VAS-2 is a second primary from the same patient as VAS-1

Immunoprecipitation

Confluent cells were incubated in serum-free medium for 24 h. Serum-starved cells were treated for 15 min with different concentrations of imatinib with or without 50 ng/ml of human recombinant PDGF-BB (Sigma, Saint Louis, Mo.). Cells were then lysed with M-PER mammalian protein extraction reagent (Pierce, Rockford, Ill.) containing 100 m *M* sodium orthovanadate, 10 mg/ml phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics, Indianapolis, Ind.). Whole cell lysates were extracted, and equal amounts of total protein, quantified using a BCA protein assay kit (Pierce), were subjected to immunoprecipitation using anti-human PDGFR-β antibody 958 (sc-432; Santa Cruz Biotechnology, Santa Cruz, Calif.). Feline cross-reactive PDGFR-α antibodies were not available.

Western blot analysis

Immunoprecipitates were subjected to 8.0% SDS-PAGE, and transferred to nitrocellulose membranes. Immunoblotting with anti-phosphotyrosine antibody cocktails, PY99 (sc-7020) and PY22 (sc-508) (dilution 1:1000) (Santa Cruz Biotechnology) was performed overnight at 4°C after blocking with TBST (Tris-buffered saline with 0.05% Tween 20) containing 5% nonfat milk. The membranes were washed, incubated with anti-mouse IgG secondary antibody (dilution 1:1200) (Amersham Biosciences, Piscataway, N.J.) for 1 h at room temperature, and developed by the ECL Western blotting system (Amersham). After development, the membranes were stripped using Restore Western blot stripping buffer (Pierce), re-probed using anti-PDGFR-β antibody (dilution 1:400), and then incubated with anti-rabbit IgG secondary antibody (dilution 1:2400) (Amersham).

Cell viability assay

Cells were plated in 96-well microtiter plates at 10,000 cells/well and incubated in C/10 under standard conditions. After 24 h, the plates were washed, serum-free medium containing various concentrations of imatinib with or without 50 ng/ml of human recombinant PDGF-BB was added, and each condition was replicated in five wells. Relative viable cell numbers were measured after 72 h using the CellTiter 96 AQ_{ueous} one solution assay (Promega, Madison, Wis.), a colorimetric system utilizing the tetrazolium salt MTS, according to manufacturer's directions. Absorbance at 490 nm was measured using a Bio-Kinetics EL312e microplate reader (Bio-Tek Instruments, Winooski, Vt.) and KC Junior software (Bio-Tek Instruments). Cell viability data were analyzed using Prism (GraphPad Software, San Diego, Calif.).

In vivo studies with imatinib

Nude mice at 7–8 weeks of age were housed in an AALAC-approved facility and cared for according to the NIH "Guide for the Care and Use of Laboratory Animals". Mice were inoculated subcutaneously with 4.0×10^6 VAS-5 cells in the right hind limb, and 24 h later ten mice in each group were treated with 50 mg/kg of imatinib or saline, intraperitoneally, once daily. This imatinib dose resulted in weight loss and was thus reduced to 25 mg/kg per day on treatment day 16, which resulted in normalization of body weight within 1 week. Mice were treated for 60 days. Tumor volumes were determined using the formula $\pi\times L\times D^2/6$, where L and D are the longest and shortest tumor diameters, respectively.

Chemotherapy studies

Growth inhibition was assessed using the MTS assay as described above. Cells grown in 96-well microtiter plates in C/10 were washed and exposed to serum-free medium containing various concentrations of the antineoplastic drugs DOX or CPT, with or without imatinib (0.5 μM or 1.0 μM) and PDGF-BB (50 ng/ml). After 72 h of incubation, the number of viable cells was measured as above.

For flow cytometric analysis, VAS cells were grown in 100-mm^3 culture dishes and, after 24 h, were exposed to either DOX (0.1 μg/ml) or CPT (20 μg/ml) alone, or combined with PDGF-BB (50 ng/ml) and/or imatinib (2.0 μM) in serum-free medium. After 72 h, 1.0×10^5 cells in each condition were stained with FITC-conjugated

annexin-V and propidium iodide (PI) (PharMingen, San Diego, Calif.), and subjected to flow cytometric analysis to assess the proportion of cells undergoing apoptosis. Early and late apoptosis were defined as annexin-V(+)/PI(−), and annexin-V(+)/PI(+), respectively. Total apoptosis was defined as the sum of the early and late apoptosis. Flow cytometric data were analyzed using ModFit LT V2.0 (Pmac).

Statistical analysis

Changes in proliferation, as measured by the growth inhibition assay, for each cell line under various conditions were compared using nonparametric one-way analysis of variance (Kruskal-Wallis). Changes in tumor volumes between groups during the mouse xenograft study were compared statistically using nonparametric repeated measures ANOVA (Friedman). Changes in the proportion of cells undergoing apoptosis, as measured by PI and annexin-V flow cytometric analysis, were compared using two-way Fisher's exact test (95% CI). Statistical analyses were performed using a commercially available desktop computer software program (Prism 4, GraphPad Software, San Diego, Calif.). P values < 0.05 was considered to be statistically significant.

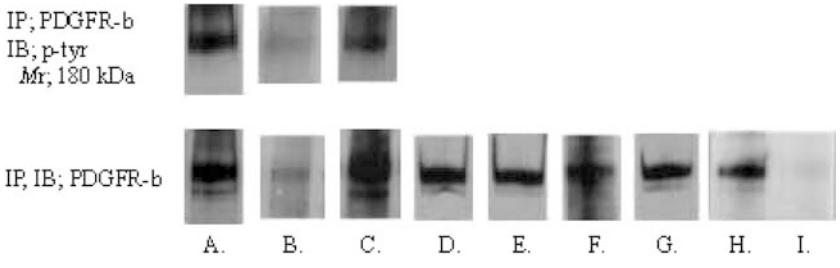
Results

Imatinib inhibits PDGF-induced autophosphorylation of PDGFR-β tyrosine kinase in feline VAS cells

To determine the presence of PDGFR-β on VAS cells, immunoprecipitates were probed with anti-PDGFR-β antibodies. PDGFR-β was confirmed to exist in all five VAS-derived cell lines (VAS-1, -2, -3, -4, and -5), as well as in one non-vaccine-associated FSA-derived cell line, FSA-1, and CRL-6102 (Fig. 1). The feline normal fibroblast-derived cell line (CRL-6102) and canine mammary carcinoma (CMT-12) were used as positive and negative controls, respectively. A canine melanoma cell line, 17CM98, was used to ensure that canine proteins crossreact with anti-human PDGFR-β antibody. Investigations of VAS (VAS-1 and -5) and FSA cell lines showed that PDGF-BB induced PDGFR autophosphorylation (Fig. 1).

VAS-1 cells were used to determine the concentration of imatinib required for inhibition of PDGF-induced autophosphorylation of PDFGR-β. Serum-starved cells were exposed to 10% FBS or increasing concentrations of imatinib with or without PDGF-BB. Equal amounts of total protein (300 μg) were subjected to immunoprecipitation with anti-PDGFR-β, and immunoblotting with anti-phosphotyrosine was performed. Autophosphorylation of PDGFR was not detected in the absence of PDGF-BB (Fig. 2, lanes 1–3). Imatinib blocked tyrosine phosphorylation of PDGFR-β induced by PDGF-BB in a dose-dependent manner (lanes 6–12), with near complete inhibition at a concentration of 2.5 μM (lane 12). After determination of autophosphorylation, the membrane was stripped, and reprobed with anti-PDGFR-β to show equal amounts of receptor for each condition.

Fig. 1 Expression of PDGFR-β in VAS and FSA. Cells were treated with PDGF-BB and lysed. Whole cell lysates were immunoprecipitated with anti-PDGFR-β and analyzed using Western blotting. Immunoprecipitates of VAS-1 (*A*), VAS-5 (*B*) and FSA-1 (*C*) were immunoblotted with anti-phosphotyrosine (*top*), and stripped membranes were reprobed with anti-PDGFR-β (*bottom*). D-I were immunoblotted with anti-PDGFR-β (*D* VAS-2, *E* VAS-3, *F* VAS-4, *G* CRL-6102, *H* 17CM98, *I* CMT-12). CRl-6102 and CMT-12 were used as positive and negative controls, respectively (*IP* immunoprecipitation, *IB* immunoblot, *P-tyr* phosphotyrosine)



Imatinib decreases viability of PDGF-BB-stimulated feline VAS cells in vitro

Diminished cell viability through the blockade of PDGFR-β tyrosine kinase activity using imatinib was evaluated in vitro in four VAS cell lines (VAS-1, -3, -4, and -5) and the FSA-1 cell line. Imatinib decreased viability of PDGF-BB-stimulated VAS and FSA cell lines in a dose-dependent manner (Fig. 3; $P < 0.01$). Concentrations of 0.5 μM were effective in decreasing PDGF-BB-dependent viability in most VAS cell lines. VAS-5 showed the weakest PDGFR-β and autophosphorylation levels, and was consistently the least dependent on PDGF-BB for viability. CRL-6102 and CMT-12 were used as positive and negative controls, respectively. PDGF-BB and imatinib had no effect on growth of CMT-12 cells.

Imatinib inhibits growth of feline VAS in vivo

The ability of imatinib to inhibit growth of VAS cells in vivo was investigated using a nude mouse model.

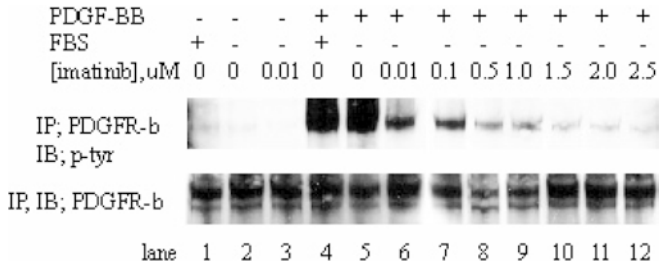


Fig. 2 Dose-dependent inhibition of PDGF-BB-induced autophosphorylation by imatinib in VAS. VAS-1 cells were treated with increasing concentrations of imatinib with or without PDGF-BB or 10% FBS and lysed. Cell lysates (300 μg of total protein each) were immunoprecipitated with anti-PDGFR-β and analyzed using Western blotting. Immunoprecipitates were immunoblotted with anti-phosphotyrosine (*top*), and stripped membranes were reprobed with anti-PDGFR-β (*bottom*) (*IP* immunoprecipitation, *IB* immunoblot, *P-tyr* phosphotyrosine)

Male nude mice at 7–8 week of age were implanted subcutaneously with 4.0×10^6 feline-derived VAS cells (VAS-5), and then treated with 50 mg/kg per day of imatinib or saline as stated previously. The VAS-5 cell line was chosen because it was the most tumorigenic of the VAS lines in nude mice in preliminary studies. Imatinib at 50 mg/kg per day resulted in acute weight loss early in the experiment and was, therefore, reduced to 25 mg/kg per day on treatment day 16. Body weight quickly returned to pretreatment values (data not shown), and no other significant toxicities were observed through the course of the experiment, nor were changes observed at necropsy. Only mice with tumors were evaluated for growth delay since some in both groups did not develop tumors. Imatinib significantly inhibited growth of VAS tumors (Fig. 4). After 60 days of treatment, imatinib-treated mice ($n=5$) had tumors that were 76.25% smaller in volume ($P=0.002$) than those in saline-treated mice ($n=4$).

Imatinib increases chemosensitivity of feline VAS in vitro

We investigated whether PDGF-BB protected VAS cells from the inhibitory effects of antineoplastic agents, and whether imatinib could alter this effect. DOX and CPT were utilized as they have been frequently used for the treatment of VAS in cats. Viability of VAS cells in C/10 was decreased by DOX and CPT in a dose-dependent manner. The 50% inhibitory concentration, IC_{50}, was approximately 0.06–0.4 μg/ml for DOX and 20 μg/ml for CPT (data not shown). The wide range of IC_{50} values

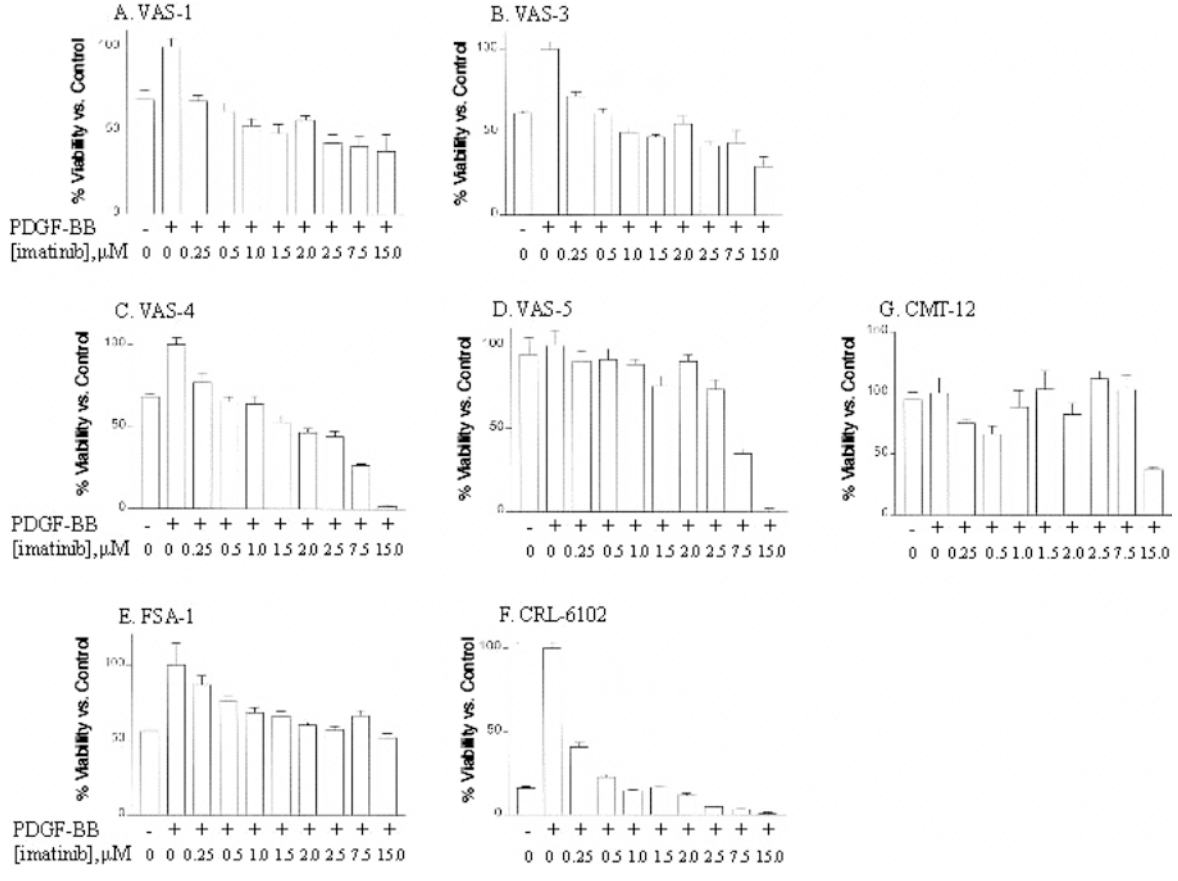


Fig. 3A–F Imatinib decreases viability of PDGF-BB-stimulated VAS and FSA. Cells were plated in C/10 overnight, and then treated for 72 h in serum-free medium. PDGF-BB at 50 ng/ml was added in each treated condition. CRL-6102 (**F**) and CMT-12 (**G**) were used as positive and negative controls, respectively. The effect on cell viability was assessed using MTS. All values are percentages (means ± SD, $n=5$) using the number of cells in PDGF-BB/0% FBS as the reference value. The data shown are representative of two or more independent experiments. $^*P<0.001$

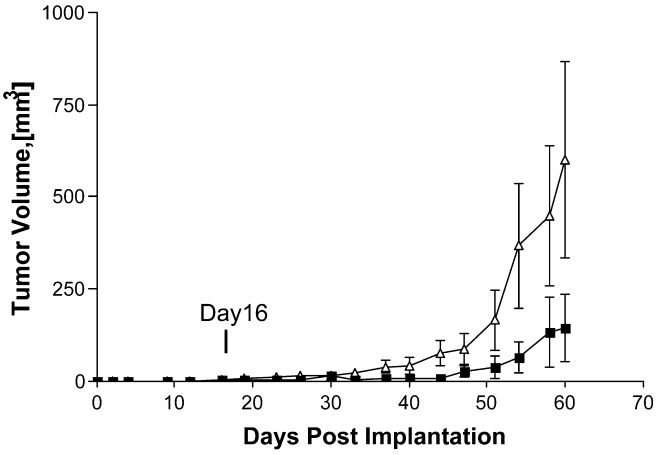


Fig. 4 Inhibition of the in vivo growth of VAS following treatment with imatinib. VAS-5 cells were inoculated s.c. into nude mice. After 24 h, mice were treated with imatinib or saline i.p. once daily for 60 days. Body weight and tumor size were monitored every 2–3 days. Imatinib at a dose of 50 mg/kg per day resulted in weight loss, and thus the dose was reduced to 25 mg/kg per day on day 16 (*arrow*). Only mice with tumors were evaluated (■ imatinib-treated, $n = 5/10$; ▲ control, $n = 4/10$). The values are means ± SD. $P = 0.002$

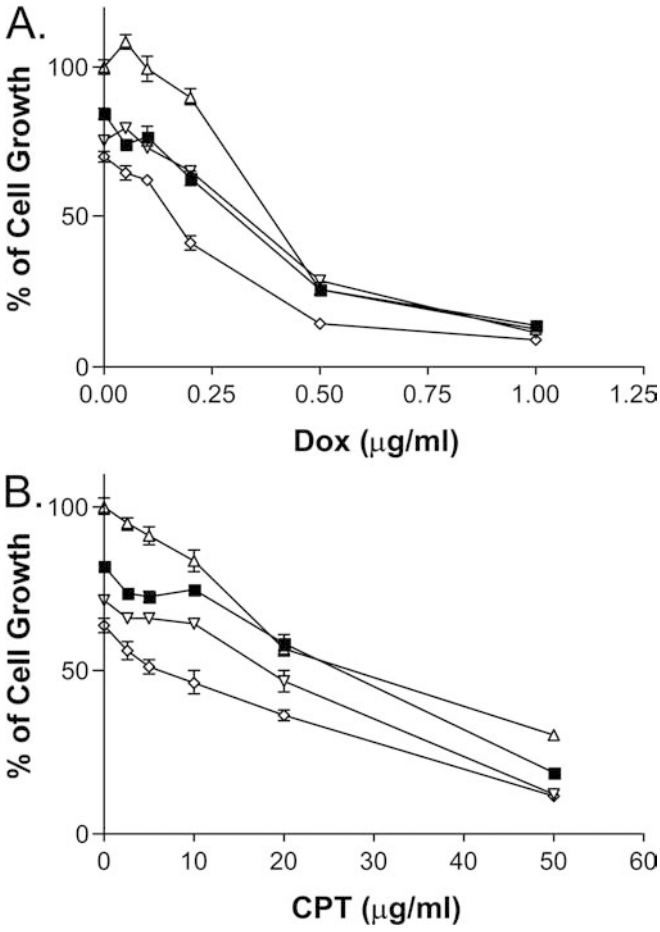


Fig. 5A, B Decreased viability of VAS following treatment with a combination of imatinib and antineoplastic drugs. VAS-1 cells were plated in C/10 overnight, and then treated with various concentrations of DOX (**A**) or CPT (**B**) for 72 h in serum-free medium. The effect on cell proliferation was assessed using MTS (■ untreated, △ PDGF-BB, ▽ PDGF + 0.5 μ*M* of imatinib, ◇ PDGF + 1.0 μ*M* of imatinib. The data shown are representative of two or more independent experiments. The values are means ± SD ($n = 5$)

observed for DOX was dependent on the cell line evaluated. PDGF-BB protected cells from the inhibitory effects of DOX and CPT in VAS-1, and imatinib eliminated this PDGF-BB-mediated protection in a dose-dependent manner when combined with either drug (Fig. 5). These results were repeatable in VAS-4, but DOX- and CPT-mediated growth inhibition were not affected by PDGF-BB or imatinib in CMT-12 cells (data not shown).

Imatinib eliminates PDGF-BB protection from apoptosis in feline VAS cells

Flow cytometric analysis using annexin V-FITC and PI double staining was performed to assess the proportion of cells undergoing apoptosis under various conditions (Fig. 6). PDGF-BB inhibited serum starvation-induced apoptosis in VAS-1 and imatinib reversed this effect. Additionally, VAS-1 cells treated with DOX showed an increasing proportion of cells undergoing apoptosis. PDGF-BB diminished DOX-induced apoptosis and, once again, imatinib was shown to eliminate this protective effect. Conversely, while CPT was shown to induce a small degree of apoptosis in VAS-1 cells, PDGF-BB and imatinib had no significant effect. This result was repeatable in VAS-4 (data not shown).

Discussion

In this study, we demonstrated the role of PDGFR-β in enhancing the viability and survival of feline VAS and FSA cells both alone and in the presence of DOX and CPT chemotherapy. Using RT-PCR and Northern blotting, we showed in preliminary studies that VAS and FSA cell lines express mRNA for c-met (the HGF receptor), IGF-1R, PDGFR-β, and EGFR [9]. Also, in preliminary unpublished data from our laboratory, we found that VAS and feline FSA cells proliferate more vigorously upon stimulation with PDGF-BB than with either HGF or IGF-1. We concluded that PDGF-BB and PDGFR-β play an important role in the growth of VAS and FSA.

We have also demonstrated that PDGFR-β exists on feline VAS and FSA cells and that it is autophosphorylated in the presence of PDGF-BB. In addition, we established that imatinib can inhibit the PDGF-stimulated growth of VAS cells in vitro and in vivo. Imatinib efficiently eliminates PDGF-BB/PDGFR-β induced autophosphorylation in a dose-dependent manner. Furthermore, imatinib inhibits PDGF-BB-dependent cell growth in several VAS cell lines in a dose-dependent manner. The in vitro growth-inhibitory effects of imatinib observed in VAS cells was also observed in vivo

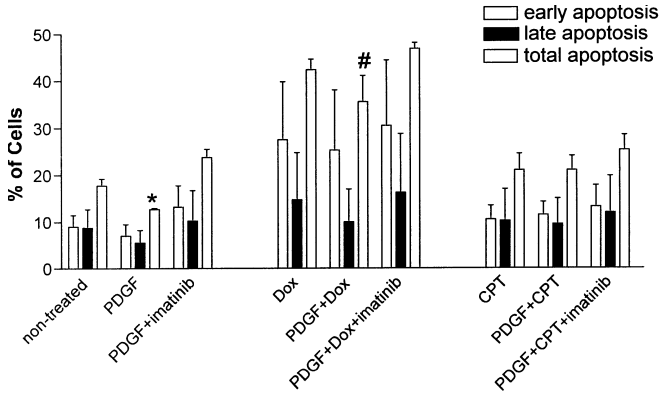


Fig. 6 Apoptotic analysis of imatinib and/or antineoplastic drugs in VAS. VAS-1 cells were plated in C/10 and incubated overnight. Cells were exposed to combinations of PDGF-BB, imatinib and/or antineoplastic drugs in serum-free medium (0% FBS). After 72 h, cells were stained with FITC-conjugated annexin-V and PI, and analyzed for the proportion of cells undergoing apoptosis. *Early apoptosis* annexin-V(+), PI(−); *late apoptosis* annexin-V(+), PI(+); *total apoptosis* early apoptosis + late apoptosis. The data are presented as means ± SD ($n = 2$). $^{*}P < 0.01$ vs non-treated; $^{\#}P < 0.01$ vs DOX alone

at dose levels that were well tolerated in nude mice. These observations, taken together, suggest that interruption of the PDGFR signaling pathway by imatinib inhibits the proliferation of VAS.

DOX and, to a lesser extent CPT, inhibited the growth of VAS cells in a dose-dependent manner in vitro. The antiproliferative effects of DOX and CPT were blocked by PDGF-BB leading to chemoresistance, and imatinib reversed this resistance.

Although the degree to which imatinib induces apoptosis depends on the cell lines or tumor tested [28, 30, 37, 47], imatinib itself is effective in inducing apoptosis of PDGF-BB dependent VAS cells as well as inhibiting PDGF-BB dependent cell proliferation.

In addition to eliminating the protective effects of PDGF-BB on DOX and CPT sensitivity, the addition of 1.0 μM imatinib resulted in greater antiproliferative activity than either DOX or CPT alone (Fig. 5). It appears, therefore, that imatinib may have additional antiproliferative effects unrelated to the PDGF/PDGFR signaling pathway. These additional effects could be due to imatinib's known activity against other tyrosine kinases such as c-kit and/or PDGFR-alpha and should be the subject of further study. This also would explain the apparent increase in apoptotic frequency observed over nontreated controls when imatinib was added to reverse PDGF protection (Fig. 6).

Interestingly, we demonstrated that PDGF-BB protects VAS cells from apoptosis induced by either serum-starvation or DOX, and imatinib reverses this protection. However, PDGF-BB and imatinib had no effect on CPT-induced apoptosis. Krystal et al. have also reported that imatinib does not synergize with the effects of CPT in Kit-dependent small-cell lung cancer cells [30]. This difference with CPT may be due to its chemical structure. The platinum component of CPT may block the biological effect of imatinib by weakening the affinity of imatinib for the ATP binding site of tyrosine kinase, or it may stimulate the IP3/(AKT) antiapoptosis pathway whose signal transduction should be blocked by imatinib. Both antineoplastic agents have been used for the treatment of cats with spontaneously arising VAS; however, their use is sometimes restricted by dose-limiting side effects including myelosuppression, anorexia and, in the case of DOX, nephrotoxicity [10]. The effective sensitization of combination imatinib and antineoplastic therapy observed in this study may allow the use of lower effective doses of antineoplastic drugs and avoidance of antineoplastic therapy-related morbidity. Phase I trials evaluating the safety of imatinib and imatinib-antineoplastic therapy combinations in cats will be necessary before clinical trials can be undertaken to investigate efficacy for VAS in cats.

Higher doses of imatinib were required for growth inhibition in vitro when VAS cells were incubated in C/10, when compared with incubation in serum-free medium supplemented with PDGF (data not shown). This observation suggests that other growth factors present in serum likely also contribute to the growth of VAS cells or perhaps that albumin binding may have decreased available imatinib for interaction. This could result in at least some degree of ineffectiveness in clinical trials using imatinib alone. This also would suggest a more important role for imatinib use in combination with other modalities such as chemotherapy.

In this study, we used VAS cell lines that were histopathologically diagnosed as VAS of the FSA histotype. Therefore, we cannot determine to what extent PDGF-BB/PDGFR-β contributes to the growth of VAS of other mesenchymal types. However, PDGFR is prominent on a variety of cells, including many mesenchymal cells [18, 38], so we may expect similar observations with non-fibroblast-derived VAS.

In conclusion, we demonstrated that imatinib interrupts the PDGF-BB induced autophosphorylation of PDGFR-β and results in the inhibition of PDGF-BB-stimulated VAS cell proliferation in vitro, and tumor growth in vivo. This suggests a possible role for imatinib as a novel treatment for cats with VAS, either alone or in combination with other antineoplastic therapy.

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