Imatinib Mesylate Inhibits Tumorigenicity of Malignant Fibrous Histiocytoma Cells In Vivo

ISTAN IRSAN 1, TOSHIHIRO AKISUE 1, HITOMI HARA 1, TAKUYA FUJIMOTO 1, MASAYA IMABORI 1, MINORU DOITA 1, RYOSUKE KURODA 1, HIROYUKI FUJIOKA 1, TERUYA KAWAMOTO 2, TETSUJI YAMAMOTO 2 and MASAHIRO KUROSAKA 1

1Department of Orthopedic Surgery, Kobe University Graduate School of Medicine, Hyogo; 2Department of Orthopedic Surgery, Kagawa University, Kagawa, Japan

Abstract. Malignant fibrous histiocytoma (MFH) is one of the most diffuse and aggressive tumors among soft tissue sarcomas in adults, but still poorly characterized from the molecular viewpoint. MFH cell proliferation is inhibited selectively by imatinib mesylate, a tyrosine kinase inhibitor. The expressions of platelet-derived growth factor receptors (PDGFRs) and c-Kit have been previously examined in MFH cell lines and the inhibitory effect of imatinib mesylate on the MFH cell proliferation was tested. MFH cell lines showed various patterns of PDGFRs and c-Kit expression. Imatinib mesylate inhibited the proliferation of MFH cells that expressed PDGFRs and/or c-Kit.

Materials and Methods: Four MFH cell lines were used (Nara H, Nara F, GBS-1 and TNMY1). The mRNA expression of PDGFRs and c-Kit was analyzed using RT-PCR; cell proliferation was analyzed using the MTS assay. Immunohistochemistry was used to analyze the inhibitory effect of imatinib mesylate on phosphorylation of PDGFRs and c-Kit in vivo. The Nara H and TNMY1 cell lines were implanted into nude mice and tumor growth was evaluated daily by measuring the two-dimensional diameters of the tumor nodule. Results: PDGFRs and c-Kit were expressed in Nara F, GBS-1 and TNMY1, but not in Nara H cells. Imatinib mesylate inhibited PDGFRs and c-Kit phosphorylation in TNMY1 cells affecting the tumorigenicity, in the control group (139 mm³ SD±1.03) and treatment group (126.2 mm³ SD±1.63) but did not affect the tumorigenicity of Nara H cells. Conclusion: Imatinib mesylate reduced in vivo tumor growth of MFH that express PDGFRs and c-Kit associated with phosphorylation suppression.

Key Words: MFH, c-Kit, PDGFRs, tumorigenicity, nude mice, imatinib mesylate.
causes PDGFR activation, which involves dimerization and autophosphorylation of specific tyrosines in the cytoplasmic domain of PDGFR. The phosphotyrosine serve as targets for cytoplasmic effector proteins involved in signal transduction (6).

With a similar structure and organization to gene encoding with PDGFR, the stem cell factor receptor, c-Kit, is a receptor protein tyrosine kinase (RPTK) that initiates signal transduction in response to stem cell factor binding and results in cell proliferation. The mechanism leading to uncontrolled activation of c-Kit involves both autocrine loops as well as mutational activation (7).

Imatinib mesylate is a derivative of 2-phenylamino-pyrimidine and was originally developed as a competitor for an ATP-binding site of the Abl protein kinase (20). In addition, imatinib mesylate was found to inhibit the kinase activity of PDGFR and c-kit (21). In vitro studies in our laboratory demonstrated the inhibition effect of imatinib mesylate on the cell proliferation of the MFH human cell line that expresses PDGFR and c-kit in a time- and dose-dependent manner (10). Several studies also demonstrated the inhibition effect of imatinib mesylate on bone metastatic tumors that express PDGFR and c-Kit (22, 23). Furthermore, although imatinib mesylate is associated with reduced c-Kit and PDGFR phosphorylation, discrepancies remain between responses to imatinib mesylate in in vitro and in vivo studies (7, 24, 25).

The purpose of this study was to demonstrate the expression of PDGFRs and c-Kit in MFH cell lines and to test the inhibitory effects of imatinib mesylate on MFH tumor growth with various patterns of PDGFRs and c-Kit expression using a xenograft model.

Materials and Methods

Cell culture. Four human MFH cell lines were used in this study: TNMY1, GBS-1, Nara-F and Nara-H. All cell lines were grown in a culture medium consisting of minimum essential Eagle's medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Co.), penicillin G (100 U/ml) and streptomycin (100 Ìg/ml). The cell lines were routinely maintained at 37ÆC in a humidified 5% CO2 atmosphere. For in vivo experiments, tumor cells were harvested by brief exposure to 0.25% trypsin.

Cell proliferation of MFH cell lines. Cell proliferation was assayed using the MTS assay technique. Cells were trypsinized at a density of approximately 5,000 cells/well in 96-well cell culture plate in 100 ìl culture medium with 10% FBS. After 2-h incubation, at 37°C in a humidified atmosphere of 5% CO2, the medium was removed and washed with phosphate-buffered saline and was then refreshed with medium containing MTS reagent (100 ìl medium without FBS plus 20 ìl MTS reagent/well). The optical density was measured at 490 nm using an automatic microplate reader after 2 h of further incubation. The percentage viability of each well was calculated daily for four days.

mRNA expression of the receptor tyrosine kinase. Total RNAs were eluted by selective binding to a silica-gel-based membrane using an Rneasy Mini Kit (QIAGEN Inc., Valencia, CA, USA). Reverse transcription of RNA into cDNA was performed by incubating 1 ìl of RNA in a reaction mixture containing 0.5 mg/ml of oligo (dT) primer, Reverse Transcription 10x buffer, 10 nM dNTP Mix and AMV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). Polymerase Chain Reaction (PCR) was performed using a Perkin-Elmer DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA). Receptor tyrosine kinases were examined by reverse transcription (RT)-PCR. The samples were preheated at 94°C for 5 min, and then cycled 40 times at 94°C for 60 sec, 58°C for 60 sec, 72°C for 60 sec and finally 72°C for 7 min. The sequences of the primers for human PDGFRα were forward primer 5’-ATC AAT CAG CCC AGA TGG AC-3’, reverse primer 5’-TTC ACG GGC AGA AAG GTA CT-3’ expected size 891 bp; human PDGFRβ forward primer 5’-AAT GTC TCC AGC ACC TTC GT-3’, reverse primer 5’-AGC GGA TGT GGT AAG GCA TA-3’ expected size 688 bp; human c-Kit forward primer 5’-CGT TGA CTA TCA GGA TCT CAG G-3’, reverse primer 5’-CTA GGA ATG TGT AAG TGC CTC C-3’ expected size 356 bp. After PCR amplification, 8 ìl aliquots of PCR products were electrophoresed in a 2.5% agarose gel, followed by ethidium bromide dye.

Animal xenograft model. Male athymic nude mice (Charles River Laboratories, Inc., Tokyo, Japan) 6-8 weeks of age were used. Animal maintenance was in accordance with institutional principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals at our Institution.

Implantation of tumor cells. To establish tumors, 12 million cells from Nara H, Nara F, TNMY1 and GBS-1 of MFH human cell line were injected s.c. into the backs of the mice. Tumor growth was monitored daily after implantation. Tumor dimensions were measured using digital calipers and tumor volume was calculated according to the formula V = \( \pi/6 \times a^2 \times b \), where a represents the shorter diameter and b the longer dimension of the tumor.

Treatment of tumor-bearing mice. Twenty-four mice were randomly assigned into two groups, Nara H group (n=12) and TNMY1 group (n=12) and mice from each of these groups were randomly assigned...
into the imatinib mesylate group (treatment groups) and PBS group (control groups). After tumors had reached ~100 mm³, the treatment was started. Imatinib mesylate was provided by Novartis Pharma (Basel, Switzerland). For each treatment, a dose of imatinib mesylate at 100 mg/kg every 24 h for the treatment group and PBS for the control group by oral gavage. After ten days of treatment, the treatment was stopped for three mice in each group and tumor growth was observed for one more week. The remaining three mice in each group were sacrificed and tumor tissue was processed and embedded in paraffin according to conventional techniques for immunohistochemistry.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections by the indirect immunoperoxidase method, in which they were deparaffinized with xylene and routinely dehydrated through a series of graded alcohol administrations. Antigen retrieval was carried out using Proteinase K for 10 min. Following elimination of endogenous peroxidase activity using 3% H₂O₂, the sections were incubated for 1 hour at room temperature with primary antibody for c-Kit (Santa Cruz Biotechnology, Inc. dilution, 1:200), p-c-Kit (Santa Cruz Biotechnology, Inc. dilution, 1:200), PDGFRα (Santa Cruz Biotechnology, Inc. dilution, 1:500), p-PDGFRα (Santa Cruz Biotechnology, Inc. dilution, 1:100), PDGFRβ (Santa Cruz Biotechnology, Inc. dilution, 1:500) and p-PDGFRβ (Santa Cruz Biotechnology, Inc. dilution, 1:100). After washing with Tris-buffered saline, the sections were incubated at room temperature for 30 min with secondary antibody for PDGFRα and c-Kit. 3,3’-Diaminobenzidine tetrahydrochloride was used for color development, and the sections were counterstained with hematoxylin. Negative controls included incubation with irrelevant species matched immunoglobulins and incubations in which the primary antibody was omitted.

Statistical analysis. The data obtained were analyzed using the Student’s t-test. A value of p<0.05 was regarded as statistically significant.

Results

mRNA expression of the receptor tyrosine kinases. TNMY1, Nara F and GBS-1 MFH cell line all expressed mRNA of PDGFRα, PDGFRβ and c-Kit by RT-PCR. Neither PDGFRα nor c-Kit mRNAs were expressed in Nara H (Figure 1). A low level of PDGFRα was seen in Nara F cell line.

Table I. Tumor volume of TNMY1 cells in a xenograft model. A significant difference in tumor volume between the treatment and control group can be seen beginning on the seventh day of medication (p<0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor incidence</th>
<th>Mean tumor volume±SD (mm³)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>6/6</td>
<td>109.3±1.03</td>
</tr>
<tr>
<td>Imatinib</td>
<td>6/6</td>
<td>107.5±1.63</td>
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Cell proliferation and tumor growth of MFH cell lines. Nara H (PDGFRα– and c-Kit–) cell line had a greater rate of proliferation than the other cell lines (PDGFRα + and c-Kit +). A similar result was observed for tumor growth in vivo. (Figure 2A, B). After eight days of observation, Nara H tumor growth was 50% higher than in the other cell lines.

Inhibition effect of imatinib mesylate on tumor growth in vivo. The TNMY1 (PDGFRα and c-Kit-positive) and Nara H (PDGFRα and c-Kit-negative) cell lines showed the selectivity
of the imatinib mesylate inhibition effect for PDGFRs and c-Kit in the MFH cell line. For the TNMY1 xenograft, tumor volume began to decrease on the seventh day after medication and the differences were significant for the treatment group (126.2 mm³ SD ± 1.63) and control group (139 mm³ SD ± 1.03) (Table I). Imatinib mesylate had no effect on Nara H (PDGFRs and c-Kit negative) tumor growth (Figure 3).

**Inhibition of PDGFRs and c-Kit Phosphorylation of human MFH cell lines in vivo.** After the tenth day of medication, three mice from each control and treatment group of TNMY1 and Nara H were sacrificed for immunohistochemistry evaluation of PDGFRs and c-Kit phosphorylation. All PDGFR and c-Kit were expressed in the TNMY1 MFH xenograft at the same level for both the treatment and control group. Nevertheless, there was a significant reduction in expression of phosphorylation of PDGFRs and c-Kit in the treatment group compared to the control group. All results showed an immunoreactivity of more than 50% positive tumor cells for TNMY1 control group (Figure 4). The Nara H xenograft did not express PDGFRs or c-Kit.

**Discussion**

MFH is one of the most diffuse and aggressive tumors among soft tissue sarcoma in adults but remains poorly characterized from a molecular viewpoint (1). Some studies demonstrated the expression of growth factors in human epithelial and mesenchymal malignancies cell line suggesting that they provide an important regulating system for the proliferation of tumor cells by playing a paracrine and/or autocrine role. Our present study showed that three out of the four MFH human cell lines (TNMY1, GBS-1 and Nara F) expressed PDGFRs and c-Kit at the mRNA and protein level, suggesting that PDGFR and c-Kit play an important role in MFH tumorigenesis.

Dysregulation of PDGFRs and/or c-Kit has been seen in many types of malignancies including MFH. We previously established a TNMY1 human MFH cell line and reported that the expression of c-Kit and its ligand stem cell factor had a paracrine activity for cell growth (26). Taniuchi et al. (27) immunohistochemically demonstrated that PDGFRβ expression might play an important role in the cell proliferation of fibrohistiocytic tumor cells. Abdiu et al. (11) also reported the possibility of PDGF-related auto/paracrine regulation in MFH cell lines. The cellular effect of dysregulation of PDGFRs and/or c-Kit enables the subsequent engagement of several proteins with a Src homology domain, including phosphatidylinositol 3'-kinase (PI3K), phospholipase γ C, GTP-ase activating protein and Grb, which conveys a mitogenic signal to the nucleus (6-8). In this study, we demonstrated that the cell proliferation rate of the Nara H cell line without expression of PDGFR and c-Kit was 50% higher compared to other cell lines positive for PDGFRs and c-Kit (Nara F, GBS-1 and TNMY1). A similar effect was shown for tumor growth in nude mice; tumor growth for Nara H was faster than for Nara F, GBS-1 or TNMY1.

Imatinib mesylate (Glivec®, formerly STI571) was developed as a specific inhibitor of the Ber-Abl protein tyrosine kinase (28). In addition to various forms of Ber-Abl tyrosine kinase, imatinib mesylate selectively inhibits the ABL-related gene (ARG) protein, the platelet-derived growth factor (PDGF) receptor and c-Kit, but does not inhibit other receptor or cytoplasmic tyrosine kinases. We previously reported that imatinib mesylate inhibited the cell proliferation of human MFH cell line in vitro (10). The inhibition effect of imatinib mesylate on MFH cell lines were at concentrations of 5 μM or higher, a similar concentration required to inhibit small cell lung cancer (SCLC) cells and higher than IC50 of 1 μM for chronic myeloid leukemia (29). Buchdunger et al. (20) reported the cellular activity of imatinib on PDGFRs and c-Kit, with the
IC$_{50}$ values of 0.1 and 0.15 μM, respectively. Therefore, before the current in vivo study, we expected that imatinib mesylate may not inhibit tumor growth in the MFH xenograft model doses similar those used previous studies for CML or small cell lung cancer were administered. In the present study, imatinib was also effective in inhibiting the tumor growth of TNMY1 cells in which PDGFRs and c-Kit were positive in the xenograft model. Our results indicate that the inhibition effect of imatinib mesylate began at the seventh day of treatment. The tumors decreased significantly compared to the control group until the tenth day of treatment. One week after the treatment had been stopped, the tumor volume still showed a tendency to decrease. In a clinical trial in a CML patient, Peng et al. (30) reported that imatinib mesylate was absorbed with oral administration with t$_{max}$ at 2 to 4 h and was detectable in plasma 30 min after oral administration. The pharmacokinetic profile of imatinib mesylate is compatible with once-daily dosing. The steady state was reached in about one week following a continuous once or twice daily dose. Wolff et al. (29) reported that imatinib mesylate rapidly reached therapeutic intra tumor concentration, in BALB/c mice carrying small cell lung cancer (SCLC) 2 h after an oral dose of 100 mg/kg at a steady state and after 8 h plasma imatinib mesylate concentration remained in the therapeutic range. Imatinib mesylate does not inhibit the growth of Nara H cells in which PDGFRs and c-Kit are negative. Our results also show that imatinib mesylate required more time to inhibit tumor growth than in previous study for dermatofibrosarcoma protuberans (13). One explanation might be that the redundancy of the receptor type could influence imatinib mesylate. Nevertheless, the real mechanism is still unclear. Further study is needed to evaluate other explanations for the mutant form of the receptors in MFH cells.

Figure 4. Phosphorylation inhibition of PDGFRs and c-Kit in the specimen retrieved from MFH xenograft model showing PDGFRs and c-Kit expression by imatinib mesylate of MFH cell lines (TNMY1) by immunohistochemistry (400x).
We found, via immunohistochemical studies, that PDGFRs and c-Kit phosphorylation were inhibited in mice bearing TNMY1 cells treated with imatinib mesylate. However, the expressions of PDGFRs and c-Kit were not different in the treatment and control groups. These findings suggest that imatinib mesylate may work at the kinase domain of the receptor to inhibit phosphorylation, but may not function at the receptor level in the MFH cell line. We believe that the concentration of imatinib mesylate used in our study was appropriate and the PDGFRs were functional.

In conclusion, our data demonstrate that imatinib mesylate reduces tumor growth in MFH cells that express PDGFRs and c-Kit, associated with phosphorylation suppression. In addition, PDGFRs and c-Kit might play a specific role in controlling the cell proliferation and tumor growth of MFH cells positive for PDGFRs and c-Kit.

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References


