Abstract. Evidence suggests that different types of mutation in gastrointestinal stromal tumours (GISTs) correlate with different response rates to imatinib (Glivec, STI571). The purpose of this study was to explore the sensitivity of the PDGFRA\(^ {L839P}\) mutant, a novel gain-of-function mutation isoform related to GISTs, to imatinib \(\textit{in vitro}\). The eukaryotic expression vectors pcDNA3.1-PDGFRA\(^ {\text{Wild}}\), pcDNA3.1-PDGFRA\(^ {D842V}\) and pcDNA3.1-PDGFRA\(^ {L839P}\) were constructed and transfected into Chinese hamster ovary (CHO) cells by liposome methods. The responses of cells with PDGFRA\(^ {\text{Wild}}\), PDGFRA\(^ {L839P}\) and PDGFRA\(^ {D842V}\) mutants to imatinib were determined by methyl thiazolyl tetrazolium (MTT) assay, western blotting and apoptosis assays. Results of the MTT assay revealed that the growth rate of CHO(PDGFRA\(^ {L839P}\)) cells decreased to approximately 60% when exposed to 1 µM imatinib and to approximately 50% with 5 µM imatinib. However, the growth rate of CHO(PDGFRA\(^ {D842V}\)) cells did not significantly change with 5 µM imatinib. Western blot analysis indicated that 1 µM imatinib completely blocked the phosphorylation of PDGFRA\(^ {L839P}\), but did not affect PDGFRA\(^ {D842V}\) phosphorylation. Apoptosis analysis suggested that the percentage of apoptotic CHO(PDGFRA\(^ {L839P}\)) cells increased approximately 4-fold (from 5.90 to 25.2%) with 1 µM imatinib. Although the treatment of CHO(PDGFRA\(^ {D842V}\)) and CHO(PDGFRA\(^ {\text{Wild}}\)) cells with 5 µM imatinib resulted in a slight increase in the number of apoptotic cells, the percentage of apoptotic cells remained approximately 10% of the total population. Our findings showed that the PDGFRA gene mutation isoform L839P is sensitive to inhibition by imatinib. Screening for PDGFRA mutations in GISTs is essential to identify the response to treatment with imatinib.

Introduction

Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract and are believed to originate from the interstitial cells of Cajal (1-3). GISTs are characterised by the expression of the type III receptor tyrosine kinase KIT encoded by the KIT proto-oncogene (4), thus KIT immunohistochemistry has been used as a diagnostic marker of GISTs (4,5). Since Hirota et al (6) first reported the KIT mutation in 1998, a number of studies have reported that the majority of GISTs have oncogenic mutations in KIT (7,8). A small subset of GISTs was also found to possess activating mutations in the platelet-derived growth factor receptor A (PDGFRA) gene (9,10).

Therapeutic targeting with the tyrosine kinase inhibitor imatinib (Glivec, STI571) has been shown to be effective in patients with advanced or unresectable GISTs (11,12). Imatinib is an inhibitor of a number of tyrosine kinases, including the intracellular kinase ABL, the growth factor receptors KIT and PDGFRA-A and -B and their oncogenic activated forms (13). Imatinib competes with adenosine triphosphate (ATP) for the ATP-binding site of the kinases, preventing downstream signalling (14,15). Imatinib is now successfully used in the treatment of advanced GISTs (16,17) and chronic myelogenous leukaemia (18,19). Its clinical activity in other neoplasms has also been reported, including chordoma (20) and dermatofibrosarcoma protuberans (21).

Evidence suggests that different types of mutation in GISTs correlate with different response rates to imatinib (22). Previous studies have demonstrated that the majority of GISTs with a KIT mutation were sensitive to imatinib, but GISTs with a PDGFRA mutation were mostly resistant to imatinib. Most of the PDGFRA mutations found in GISTs have been identified in exon 18 and are the imatinib-resistant substitution D842V. Only approximately one-third of PDGFRA mutations...
in GISTs have been found in exons 12 and 14 and these mutations have different responses to imatinib (23). We previously studied the PDGFRA mutation types of exon 12 and 18 by PCR amplification and DNA sequencing in a series of Chinese GISTs. Compared with the reports in the published data, a point mutation at codon 839, L839P, lies outside the hot spot area (23). Further studies confirmed that PDGFRA\textsuperscript{L839P} is a gain-of-function mutation (24). To explore the response of PDGFRA\textsuperscript{L839P} to imatinib, we transfected different isoforms of the human PDGFRA gene into Chinese hamster ovary (CHO) cells and compared the inhibitory effects of imatinib on PDGFRA\textsuperscript{L839P} with the effects on PDGFRA\textsuperscript{D842V} in vitro concerning cell growth, apoptosis and receptor phosphorylation level.

**Materials and methods**

**PDGFRA expression constructs.** The wild-type human PDGFRA cDNA (HD Biosciences Co, Shanghai, China) was cloned into the pcDNA3.1hygro+ vector to create pcDNA3.1-PDGFRA\textsubscript{Wild}. cDNA encoding the human PDGFRA mutant isoforms was generated using a MutanBEST site-specific mutagenesis kit (Takara Bio, Inc., Shiga, Japan) using the primers: 5’-CGTGTGACTTTGGCCCGGCCAGAGACATCATG-3’ and 5’-CATGATGTCTCTGGCCGGGCCAAAGTCACAG-3’ for the PDGFRA\textsuperscript{L839P} cDNA, and 5’-GGCCCTGGCCAGAGTAGCATCATGAGTATTGCC-3’ and 5’-CGAATCATGCTATGATGACCTGGCCAGGC-3’ for the PDGFRA\textsuperscript{D842V} cDNA. A ~3.3-kb product was obtained by denaturation for 1 min at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C for 30 cycles. These fragments were digested with XhoI and NheI and directionally cloned into the pcDNA3.1hygro+ vector. All vectors were confirmed by restriction endonuclease digestion and bidirectional sequencing.

**Transfection of CHO cell lines.** To transfect CHO cells with plasmids encoding human PDGFRA\textsubscript{Wild}, PDGFRA\textsubscript{D842V} and PDGFRA\textsubscript{L839P}, Lipofectamine\textsuperscript{TM} 2000 (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer’s instructions. Infectants were selected with 1 mg/ml Hygromycin B (Merck, Darmstadt, Germany) until all the uninfected control cells were killed. Following selection, the CHO cells were cultured in medium containing 0.5 mg/ml Hygromycin B.

**Flow cytometric analysis of apoptosis.** The CHO cell lines were cultured in the presence or absence of imatinib for 24 h. Subsequently, apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) staining kit (Roche, Mannheim, Germany). The cells were harvested by trypsinisation and labelled with annexin-V-FITC for 15 min at 4°C and analysed by FACSCalibur flow cytometry.

**Methyl thiazolyl tetrazolium (MTT) assay.** Cells were added to 96-well plates at a density of 2x10\textsuperscript{4} cells/well. After the cells were maintained for 24 h at 37°C in a 5% CO\textsubscript{2} atmosphere, a 200 \(\mu\)l solution containing imatinib (0, 0.001, 0.01, 0.1, 1 or 5 \(\mu\)M) was added. After 72 h, 20 \(\mu\)l of a 5 mg/ml solution in PBS of the MTT (Sigma, St. Louis, MO, USA) tetrazolium substrate was added and the cells were incubated for 4 h at 37°C. The resulting violet formazan precipitate was solubilised by the addition of 150 \(\mu\)l DMSO (Amresco, Solon, OH, USA) and incubated for 10 min at room temperature. Sample absorbances were then measured on a plate reader at 540 nm.

**Western blotting.** Whole-cell lysates were prepared by resuspending the cells in cold SDS buffer [1% SDS, 0.04 mol/l Tris-HCl (pH 6.8), 5% glycerol]. The protein concentrations were determined using a commercial BCA protein assay kit (Merck). Protein extracts were added at a 4:1 ratio to 5X SDS sample buffer and boiled. The protein (50 \(\mu\)g) was resolved on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with anti-PDGFRA rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-GAPDH goat polyclonal antibody (GenScript, Piscataway, NJ, USA) or anti-phosphotyrosine monoclonal antibody (Long Island Biotech Inc., New York, USA). The signals were detected using the western blotting luminal reagent (Santa Cruz Biotechnology, Inc.).

**Results**

**Identification of recombinant plasmids.** Following digestion by XhoI and NheI, bands at 3.3 kDa were detected for the positive clones, suggesting that PDGFRA\textsubscript{Wild}, PDGFRA\textsubscript{L839P} and PDGFRA\textsubscript{D842V} fragments were inserted into the pcDNA3.1 vector, designated as recombinant plasmids pcDNA3.1-PDGFRA\textsubscript{Wild}, pcDNA3.1-PDGFRA\textsubscript{L839P} and pcDNA3.1-PDGFRA\textsubscript{D842V}, respectively.

pcDNA3.1-PDGFRA\textsubscript{Wild}, pcDNA3.1-PDGFRA\textsubscript{L839P} and pcDNA3.1-PDGFRA\textsubscript{D842V} DNA was prepared for sequencing. The sequence obtained was the same as the reported sequence of PDGFRA cDNA and mutant PDGFRA cDNA, indicating that the wild-type and mutant PDGFRA genes were successfully cloned into the eukaryotic expression vector pcDNA3.1.

**Liposome transfer of PDGFRA\textsubscript{Wild}, PDGFRA\textsubscript{D842V} and PDGFRA\textsubscript{L839P} into CHO cell lines.** CHO cells were transfected with Lipofectamine\textsuperscript{TM} 2000 encoding human PDGFRA\textsubscript{Wild}, PDGFRA\textsubscript{D842V} and PDGFRA\textsubscript{L839P}. Following selection in medium containing 1 mg/ml Hygromycin B, the CHO cells were raised in medium containing 0.5 mg/ml Hygromycin B. The expression of human PDGFRA was

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**Figure 1. Expression of human PDGFRA protein in each group of CHO cells.** The results of the western blot analysis showed that human PDGFRA protein was expressed in CHO(Wild), CHO(D842V) and CHO(L839P) cells, but not CHO(vector) cells. The level of expression of human PDGFRA in CHO(D842V) and CHO(L839P) cells was higher than that in CHO(Wild) cells. CHO, Chinese hamster ovary.
examined using western blot analysis (Fig. 1). Results indicated that human PDGFRA was expressed in CHO(Wild), CHO(D842V) and CHO(L839P) cells, but not CHO(vector) cells. The level of expression of human PDGFRA protein in the CHO(D842V) and CHO(L839P) cells was higher than that in the CHO(Wild) cells.

**Mutation isoforms of the PDGFRA gene affect the sensitivity to imatinib.** The effect of imatinib on the growth of CHO(vector), CHO(Wild), CHO(D842V) and CHO(L839P) cells was evaluated using an MTT assay that measures the number of live cells at the end of a 72-h culture period. The proliferation of CHO(L839P) cells was inhibited by imatinib at concentrations of 1 µM (Fig. 2A and D). By contrast, the remaining cell lines, including CHO(D842V), were not greatly affected by the presence of imatinib at concentrations of ≥5 µM (Fig. 2A-C). These results suggest that the CHO(L839P) cell line is more sensitive to inhibition by imatinib than CHO(D842V) and CHO(Wild) cells, indicating that the L839P mutation of the PDGFRA gene may be sensitive to imatinib, but the D842V mutation is resistant to imatinib.

**Imatinib induces apoptosis in CHO cells.** To determine whether growth inhibition was caused by the induction of apoptosis, staining with Annexin-V and propidium iodide was used. Following the incubation of CHO(vector) cells in 5 µM imatinib, the percentage of apoptotic cells remained ~10% of the total population. By contrast, incubation of CHO(L839P) in 1 µM imatinib resulted in a ~4-fold increase in the number of apoptotic cells, the percentage of apoptotic cells remained ~10% of the total population. Although the incubation of CHO(D842V) and CHO(Wild) cells in 5 µM imatinib resulted in a slight increase in the number of apoptotic cells, the percentage of apoptotic cells remained ~10% of the total population. Similar results were obtained when the experiment was repeated.

**Effect of imatinib on receptor phosphorylation.** To determine the effect of imatinib on the autophosphorylation of PDGFRA, a western blot for PDGFRA and phosphotyrosine was...
performed (Fig. 4). The phosphotyrosine levels revealed that PDGFRA phosphorylation in CHO(Wild) cells was low. By contrast, the receptor phosphorylation in CHO(D842V) was unaffected by 5 µM imatinib. There was no phosphorylation of PDGFRA in CHO(L839P) cells treated with 1 and 5 µM imatinib. These results correlate well with those of the MTT assays, indicating that CHO(L839P) cells are sensitive to 1 µM imatinib, whereas CHO(D842V) cells are resistant to imatinib.

Discussion

GISTs are the most common mesenchymal neoplasms of the gastrointestinal tract (1-3). Despite clinicopathological differences, most GISTs share a similar genetic profile, including KIT or PDGFRA gene gain-of-function mutations (25,26), which are targets for the kinase inhibitor imatinib (13,27). Previous clinical studies have shown that imatinib is effective in patients with advanced or resectable GISTs (11,12). Further studies have demonstrated that different responses of GIST patients to imatinib correlate with different mutation types (22,28). Results of the study by Corless et al suggest that approximately 62.6% of PDGFRA-mutant tumours are resistant to imatinib (23). In other words, only just over a third of GISTs with PDGFRA mutations may respond to imatinib. Therefore, mutation screening may aid in the management of GIST patients.

In our previous studies, a novel point mutation in exon 18 of the PDGFRA gene (L839P) was found in two GIST cases (24) and lies outside of the hot spot. To compare the inhibitory effects of imatinib on PDGFRA<sup>L839P</sup> with the effects on PDGFRA<sup>D842V</sup> in vitro with the mutations expressed individually in the same cellular background, we used liposome transduction to transfect these forms of PDGFRA into CHO cells. The D842V mutation is the most common mutation of the PDGFRA gene in GISTs. Preliminary data suggested that D842V is resistant to imatinib in vitro and in vivo (22). In the present study, the MTT assay results indicated that the PDGFRA mutant isoform D842V shows significant resistance to imatinib at 1 µM, which is equivalent to the highest serum levels generally achieved in patients, according to other studies (29). Compared with D842V, the L839P mutation of the PDGFRA gene was sensitive to 1 µM imatinib in vitro, suggesting that GISTs carrying the PDGFRA<sup>L839P</sup> mutation may have a better response to imatinib. To determine whether growth inhibition was caused by the induction of apoptosis, staining with Annexin-V was used. The results suggest that imatinib notably induces cell apoptosis in CHO(L839P) cells and enhances the response to imatinib.

To determine the effect of imatinib on autophosphorylation of PDGFRA, western blots for PDGFRA and phosphotyrosine were performed. The results indicate that the differences in the sensitivity of PDGFRA mutants to imatinib are a direct consequence of the ability or inability of imatinib to inhibit PDGFRA phosphorylation.

In conclusion, to compare the inhibitory effects of imatinib on PDGFRA<sup>D842V</sup> and PDGFRA<sup>L839P</sup> with the effects on PDGFRA<sup>Wild</sup>, with the mutations expressed individually in the same cellular background, we used liposome transduction to transfect these forms of PDGFRA into CHO cells. Our data concerning cell growth, apoptosis and receptor phosphorylation indicate that the D842V mutant was resistant to imatinib, which is consistent with the results of previous studies (22,23). Our data also demonstrate that a new mutant, PDGFRA<sup>L839P</sup>, was more sensitive to imatinib than PDGFRA<sup>Wild</sup> and PDGFRA<sup>D842V</sup>. This study suggests that screening patients for PDGFRA mutations is essential to identify malignancies that are likely to be sensitive or resistant to treatment with imatinib.

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References

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