Selective RNAi-mediated inhibition of mutated c-kit

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ABSTRACT

The proto-oncogene c-kit plays an important role in the development and survival of mast cells. Gain-of-function mutations in c-kit are one of the most characteristic events in mast cell leukemia (MCL) but as yet there is no clinically approved treatment for the disease. Here we describe growth inhibition of human MCL cell lines by the use of RNAi against c-kit or its mutant form. Retroviral transduction of HMC1.1 and HMC1.2 cell lines with vectors carrying DNA to be transcribed to RNAi against the wild type or mutant c-kit messengers reduced Kit protein levels considerably, decreased cell proliferation, and increased the apoptotic levels five days after retroviral infection. Thus RNAi targeted against Kit or its mutant form could be considered as a new antiproliferative agent against human mast leukemia cell lines, especially HMC1.2 cells which are resistant to the Kit tyrosine kinase inhibitor, imatinib mesylate.

KEYWORDS: RNAi, c-kit, allelic discrimination, mast cell leukemia (MCL), apoptosis

INTRODUCTION

The c-kit proto-oncogene codifies for a tyrosine kinase receptor that is essential for the proliferation and survival of hematopoietic stem cells, melanocytes, interstitial cells of Cajal, germinal cells and mastocytes. Mutations in c-kit have been associated with several diseases, such as gastrointestinal stromal tumours (GISTs) and mast cell leukemia (MCL) (Orfao et al, 2007), a rare form of mastocytosis characterized by the infiltration of large burdens of tissue mast cells into bone marrow and peripherical blood. Allele-specific gain-of-function mutations either at the juxtamembrane or the catalytic domain of c-kit have been found in MCL (Longley et al, 2001). Imatinib mesylate, the clinically approved c-Kit tyrosine kinase inhibitor, is able to block the survival of cells from the human leukemia mast cell line HMC1.1 as well as some cases of both MCL and GISTs, all of them harbouring mutations in the juxtamembrane domain of the protein (Hartmann et al, 2005). The drug nevertheless has no effect on the survival of MCL cells harbouring mutations at the catalytic domain of c-Kit, such as the human leukemia MCL line HMC1.2 (Akin et al, 2003). Additionally, tumours harbouring c-kit mutations that respond to imatinib mesylate, may develop new point mutations that confer them resistance to the drug (Haller et al, 2007; Nakagomi and Hirota, 2007).

RNAi has the potential for treatment of disorders where dominant gene mutations contribute to the progression of the disease (Kim and Rossi, 2007). In this report, we have investigated the effect of using shRNA to silence Kit in both the imatinib-sensitive HMC1.1 and the imatinib-resistant HMC1.2 cell lines. We found that RNAi may be used as an alternative treatment for MCL.

MATERIALS AND METHODS

Cell culture

HMC 1.1 and HMC1.2 cell lines were a generous gift from Drs Luis Escrúbano (Hospital Virgen del Valle, Toledo, Spain) and David Butterfield (Mayo Clinic, Rochester,
USA), respectively. Cells were grown in Iscove’s Modified Dulbecco’s medium (IMDM) supplemented with 10% (v/v) foetal bovine serum and 100 µg/ml penicillin. The Phoenix amphotropic cell line was cultured in DMEM supplemented with 10% (v/v) foetal bovine serum.

**Antibodies and reagents**

Rabbit polyclonal anti-Kit (sc-168) was obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-actin secondary antibody was obtained from Sigma-Aldrich, Inc. STI-571 (Imatinib mesylate) was a gift from Novartis; working stocks were prepared by dissolving it in polybrene (8 µg/ml). Cells were incubated overnight and by single PCR reactions using the appropriate primer pairs human RNA from HMC1.2 cells (which carry c-kit wild type or mutant). The amplified products were isolated and cloned into pGEM-T vector. The PCR cycles were: 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The following primers were used in PCR amplification:  

**Forward primer for the wild type allele:**  
5' - CATGATAAGTGACGTGGAAGGTCT 

**Forward primer for the Val560Gly mutant allele:**  
5' - ATGTGATAGTACAGTGGAAGGCTG 

**Reverse primer for both wild type and mutant alleles:**  
5' - GGAAATGTTGTTG GGTCTATGTAAC 

**Western blot analysis**

Cells were harvested in TNSEV lysis buffer (50mM Tris-HCl pH7.5; 2 mM EDTA; 2mM sodium orthovanadate, 1%, v/v, NP-40) supplemented with 1 mM PMSF, 1 µg/ml leupeptin and 1 µg/ml aprotinin, and incubated on ice for 15 min. The homogenate was centrifuged for 15 min at 14000 rpm and the supernatant was taken for protein quantification. Protein samples (70 µg) were denatured in Laemmli buffer at 95°C, and separated in 8% (v/v) SDS-PAGE gel at 100 V for 2 hr, and electrophoretically transferred onto nylon membranes. The membranes were incubated for 1 hr with the appropriate primary antibodies dissolved in 5% (w/v) powdered non-fat milk and 1% (v/v) Tween-20 in PBS. After washing, membranes were incubated for 45 min with the correspondent secondary antibodies. The bound antibodies were detected by chemiluminescence using ECL reagents.

**Flow cytometric apoptosis assay**

The percentage of cell death was determined according to the supplier’s instructions (BD, Pharmingen). After retroviral infection, HMC1.1 and HMC1.2 cells were washed in PBS and resuspended in 100 µl of annexin/7-AAD binding buffer and incubated for 15 min at room temperature in dark. Thereafter, 400µl of binding buffer was added to each sample and analyzed by flow cytometry using a FACS Calibur cytometer (BD, Heidelberg, Germany).

**Cell viability assays**

These were based on trypan blue exclusion or 3,4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. For the former, infected cells were washed in PBS, and resuspended in 1 vol of PBS and an equal volume of trypan blue. After 1 min incubation viable cells, represented as white spots, were counted using a Neubauer chamber under a light microscope. For the MTT assay, 3,4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was dissolved in PBS at 1 µg/µl. Viable cells converted the MTT to a blue formazan precipitate. After 2 hr incubation at 37°C in the dark, dark-blue formazan was solubilised with 2ml of DMSO and the absorbance at 540nm was measured.

**Statistical analysis**

Values are expressed as mean ± SD; comparisons were made using Student’s t-test.

**RESULTS**

**Knockdown of wild type and mutant Kit proteins by allele-specific shRNAs**

We designed five different shRNAs targeting different regions of c-kit messengers. As shown in Figure 1A,
HMC1.1 cells had an allele-specific point mutation at position-1680 of c-kit cDNA, which corresponds to a Val560Gly substitution, and HMC1.2 cells had two different allele-specific point mutations, one at position 1680 and the other at position 2448 of the same allele of c-kit, which corresponds to a Val560Gly and Asp816Val substitution as described previously in the literature (Akin C et al, 2003). Two shRNAs targeted c-kit mRNA outside the mutated region inhibiting the expression of both mutant and wild type mRNAs (Total 1 and Total 2). Other two shRNAs were directed against the V560G mutant messenger of c-kit present in both HMC1.1 and HMC1.2 cells (RNAi Mut 1, RNAi Mut 2), and finally there was one non-allele specific RNAi (RNAi Mut 3). Both HMC1.1 and HMC1.2 cell lines were infected with either the empty control retrovirus or the retroviral-based shRNAs. Five days after retroviral infection and puromycin selection of HMC1.1 and HMC1.2 cells, we tested the efficiency of retroviral-based shRNAs on Kit protein levels (Figure 1B). Total 2 RNAi against both wild type and mutant messengers of the gene, gave rise to 86% knockdown in HMC1.1 and 95% protein knockdown in HMC1.2, respectively. The Mut 1 RNAi against the mutant messenger V560G inhibited 50 and 58% of the total protein levels in HMC1.1 and HMC1.2, respectively and the Mut 2 RNAi did so for 52% and 57% of Kit protein. The non-allele specific RNAi (Mut 3 RNAi) inhibited Kit protein levels not only in HMC1.2 cells but also in HMC1.1 cells. The empty retroviral vector control did not show any significant reduction of Kit protein.

A:  

Val560Gly  

Wild type: 5’ GTGGAAGGTGTGGTACAGGAG  

Mutant: 5’ GTGGAAGGTGTGGTACAGGAG  

Asp816Val  

Wild type: 5’ TCTAGGCAGAGCATCAAG  

Mutant: 5’ TCTAGGCAGAGCATCAAG

B:  

Figure 1. Localization of c-kit point mutations. (A) Identification of allele-specific c-kit point mutations in HMC1.1 and HMC1.2 cell lines after amplification by RT-PCR and sequencing. The G/T polymorphism for Val560Gly mutation and the A/T polymorphism for Asp816Val mutation in c-kit are underlined. (B) Western blot analysis showing the amount of Kit protein after transduction of HMC1.1(upper panel) and HMC1.2 (lower panel) cells with retroviruses carrying an empty retrovirus (Control vector) and with retroviruses expressing shRNA against: both messenger RNAs of c-kit (Tot1, Tot2); the mutated messenger RNAs (Mut1, Mut 2), or a non-allele specific shRNA (Mut 3). Actin was used as protein loading control.

Quantification of c-kit mutant transcripts after RNAi expression in HMC1.1 cells  

Next, we performed a quantitative RT-PCR to confirm the efficacy and specificity of the RNAi directed against the mRNA of the mutant allele of c-kit. We designed specific primers that amplified only the mutant or the wild type c-kit cDNA (Figure 2A). The confirmation of the strict allelic discrimination of these primers was examined in a test experiment primed with either wild type or mutant cDNAs. As shown in Figure 2B wild type but not mutant c-kit cDNA was amplified in the presence of the wild type primers, while conversely mutant but not wild type cDNA was amplified in the presence of the mutant primers. Figure 2B also shows the amplification of both wild type and mutant c-kit cDNAs in retrotranscribed mRNA prepared from HMC1.1 and HMC1.2 cells, with both wild type and mutant primers.

Some but not all of the shRNAs differentially suppressed the wild type and mutant c-kit mRNAs. The results obtained were normalised with respect to those obtained with cells infected with an empty retrovirus. The levels of c-kit mutant mRNA in HMC1.1 cells treated with the Mut 1 and Mut 2 RNAi were reduced relative to wild type mRNA by 93% and 79%, respectively, indicating the specificity of these two shRNAs for the mutant messenger of c-kit (Figure 2C). Nevertheless, when cells were treated with Mut 3 RNAi, the ratio of mutant to wild type c-kit mRNAs was similar to that observed in cells infected with the control empty retrovirus, in agreement with our previous results in which Mut 3 RNAi inhibited mutant and wild type mRNAs in HMC1.1 cells indiscriminately.

Effects of Kit knockdown in HMC1.1 and HMC1.2 cells  

Knowing that Kit is important for the proliferation and survival of mast cells, we tested whether RNAi mediated c-kit knockdown reduced mast cell survival. We used in most of the experiments retrovirus transcribing Mut 1 RNAi, which was the most efficient in the reduction of the mutant mRNAs of c-kit, and in some others Tot 2 RNAi, which reduced both forms of Kit indiscriminately. Twelve days after retroviral infection and puromycin selection, the proliferation of mast cells was markedly reduced by both retroviruses. Both total Kit knockdown (Tot) and mutant Kit knockdown (Mut) effectively reduced HMC1.1 and HMC1.2 cell viability (Figure 3A and B, respectively). To evaluate whether the decrease in cell viability was associated to apoptosis, we measured the percentage of annexin-V positive cells 5 days after infection. The death following infection in the two cell lines represented 27.97% for HMC1.1 and 16.05% for HMC1.2, respectively as compared to cells infected with the control.
empty retrovirus (Figure 3C). We also found that the inhibition of only mutant c-kit mRNA showed even higher levels of apoptosis, being 44.64% and 27.63% in HMC1.1 and HMC1.2 cells, respectively.

Figure 2. Selective knockdown of c-kit mutant messengers in HMC1.1 cells. (A) Sequence details of c-kit wild type and mutant cDNAs. (B) Agarose gel detection of amplified wild type or mutated c-kit cDNAs by standard PCR, using the wild type primers for the upper part of the figure and the mutant primers for the lower part: lane a, DNA amplified from cloned wild type c-kit originally from NCI-H510 lung cancer cells; lane b, DNA amplified from cloned mutant c-kit originally from HMC1.2 cells; lane c, product of RT-PCR amplification of RNA from HMC1.1 cells; d: product of RT-PCR amplification of RNA from HMC1.2 cells. (C) Estimated ratio of mutant to wild type mRNAs by qRT-PCR using sybergreen in HMC1.1 cells after treatment with control empty retrovirus, and retroviruses transcribing Mut 1 RNAi, Mut 2 RNAi and Mut 3 RNAi.

Figure 3. Cell survival of human mast leukemia cells after Kit knockdown. (A) Survival assay of HMC1.1 cells after gene silencing of all c-kit mRNAs (Tot) or only the mutant mRNA (Mut) at different times after retroviral infection. (B) Survival assay of HMC1.2 cells as in A. (C) Percentage of HMC1.1 (grey) and HMC1.2 (black) apoptotic cell death 5 days after Kit knockdown (annexin-V positive cells).
Imatinib mesylate resistant HMC1.1 cell line is sensitive to shRNA-mediated c-kit gene silencing

Imatinib mesylate is a potent tyrosine kinase inhibitor of Kit, PDGFR, and the fusion protein ABL-BCR (Kovalenko et al, 1994; Druker et al, 1996; Heinrich et al, 2000). We treated both HMC1.1 and HMC1.2 MCL cell lines with imatinib mesylate and compared the results with the ones obtained by shRNA-mediated c-kit gene silencing (Figure 4), showing as expected, that the drug drastically reduced cell viability in HMC1.1, but not in HMC1.2 cells as previously described, (Akin et al, 2003). On the other hand, Tot and Mut sh-RNAs silencing c-kit reduced considerably the viability of both HMC1.1 and HMC1.2 cells.

DISCUSSION

Kit is up-regulated in several malignant diseases such as myeloproliferative disorders (Beghini et al, 2002), gastrointestinal stromal tumours (Hornick et al, 2007) and neuroblastomas (Vitali et al, 2003) although its role in the progression of the different types of cancer is not clearly established (Sattler and Salgia, 2004).

We have knockdown Kit protein in both HMC1.1 and HMC1.2 cell lines with RNAi. The ability of the RNAi to discriminate between the mutant and wild type messengers of c-kit has also been shown. Similar discriminatory inhibition has been demonstrated for other genes such as p53, k-ras and SOD-1, whose mutations are related to the progression of Li-Fraumeni syndrome, pancreatic cancer and amyotrophic lateral sclerosis respectively (Martinez et al, 2002; Duursma and Agami, 2003; Raoul et al, 2005).

The expression of RNAi specific for the mutant form of c-kit resulted in a reduction of the corresponding mRNA in HMC1.1 cells of up to 93%, and also to a greater decrease in cell viability than when both mutant and wild type forms of the mRNA were inhibited. These results confirm the possibility of using allele discrimination to alleviate illness caused by negative dominant mutations.

Both cell lines HMC1.1 and HMC1.2 were responsive to the anti-proliferative effects of either total c-kit RNAi or mutant c-kit RNAi in a time-dependent manner. The observed apoptotic cell death is probably a result of the down-regulation of Bel-2 and consequent reduction in the activation of downstream effectors such as Akt, Stat3 and Erk1/2, which are necessary for the normal development of mast cells as previously described (Ning et al, 2001; Nishida et al, 2002; Wandzioch et al, 2004). Mast leukemia HMC1.1 and HMC1.2 cell lines are good models to study the susceptibility of mutant c-kit variants to tyrosine kinase inhibitors such as imatinib mesylate. The HMC1.2 cell line, as shown here and previously (Akin et al, 2003), is resistant to imatinib mesylate as a consequence of a point mutation at D816V, the catalytic domain of the protein, while HMC1.1 harbouring exclusively the V560G point mutation is sensitive to the drug. The highly specific effect of RNAi in reducing c-kit mRNA could be used for the treatment of other cancers resistant to imatinib mesylate, such as GISTs which, although they respond to this drug at first, often develop new mutations that confer them resistance. We have demonstrated here that the exclusive inhibition of the mutant allele of c-kit is more efficient than the inhibition of both mutant and wild type alleles and this could be a good strategy for decreasing cancer cell survival. The system could also be used in combination with other therapies for the treatment of c-kit related malignancies.

CONCLUSIONS

• RNAi-mediate inhibition is efficient in discriminating between mutant and wild type alleles from c-kit oncogene, thus have therapeutic potential; such as in the case of mast cell leukaemia, caused by dominant, gain-of-function mutations.

• RNAi-mediated degradation of mutant c-kit triggers a decrease in cell proliferation and induces apoptosis.

• Cell death is induced in both sensitive to imatinib mesylate (HMC1.1) or resistant (HMC1.2) cell lines.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

MCL: Mast cell leukemia
GIST: Gastrointestinal stromal tumours
pac: puromycin-resistance gene
V560G: Val560Gly mutation
D816V: Asp816Val mutation
Tot: Total
Mut: Mutant
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