for low-risk MDS to improve hematologic conditions [29,30]. We also gave thalidomide to two patients with MM/MDS with der(1;7), and they became transfusion independent. These observations clearly indicate that monitoring the appearance of the der(1;7) and morphologic assessment with hematologic conditions will be required for early detection of hidden myelodysplastic conditions and to judge the appropriate timing for thalidomide administration.

Acknowledgments

We are indebted to Prof. J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript. This work has been supported in part by a grant-in-aid for "Intractable Hematopoietic Diseases" from the Ministry of Health, Welfare, and Labor, Japan; the High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology (MEXT); and by the University–Industry Joint Research Project from MEXT.

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Oncogene (2006) 25, 5719–5725

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ORIGINAL ARTICLE

Telomerase inhibition with a novel G-quadruplex-interactive agent, telomestatin: *in vitro* and *in vivo* studies in acute leukemia

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The telomerase complex is responsible for telomere maintenance and represents a promising neoplasia therapeutic target. Recently, we have demonstrated that treatment with a G-quadruplex-interactive agent, telomestatin reproducibly inhibited telomerase activity in the BCR-ABL-positive leukemic cell lines. In the present study, we investigated the mechanisms of apoptosis induced by telomerase inhibition in acute leukemia. We have found the activation of caspase-3 and poly-(ADP-ribose) polymerase in telomestatin-treated U937 cells (PD20) and dominant-negative DN-hTERT-expressing U937 cells (PD25). Activation of p38 mitogen-activated protein (MAP) kinase and MKK3/6 was also found in telomestatin-treated U937 cells (PD20) and dominant-negative DN-hTERT-expressing U937 cells (PD25); however, activation of JNK and ASK1 was not detected in these cells. To examine the effect of p38 MAP kinase inhibition on growth properties and apoptosis in telomerase-inhibited cells, we cultured DN-hTERT-expressing U937 cells with or without SB203580. Dominant-negative-hTERT-expressing U937 cells stopped proliferation on PD25; however, a significant increase in growth rate was observed in the presence of SB203580. Treatment of SB203580 also reduced the induction of apoptosis in DN-hTERT-expressing U937 cells (PD25). These results suggest that p38 MAP kinase has a critical role for the induction of apoptosis in telomerase-inhibited leukemia cells. Further, we evaluated the effect of telomestatin on the growth of U937 cells in xenograft mouse model. Systemic intraperitoneal administration of telomestatin in U937 xenografts decreased tumor telomerase levels and reduced tumor volumes. Tumor tissue from telomestatin-treated animals exhibited marked apoptosis. None of the mice treated with telomestatin displayed any signs of toxicity. Taken together, these results lay the foundations for a program of drug development to achieve the dual aims of efficacy and selectivity *in vivo*.

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Received 17 August 2005; revised 14 February 2006; accepted 8 March 2006; published online 1 May 2006

Oncogene (2006) **25**, 5719–5725. doi:10.1038/sj.onc.1209577; published online 1 May 2006

Keywords: telomerase inhibition; p38 MAP kinase; MKK3/6; apoptosis; *in vivo* study

Introduction

Telomerase is a cellular RNA-dependent DNA polymerase that serves to maintain the tandem arrays of telomeric TTAGGG repeats at eukaryotic chromosome ends (Blackburn and Greider, 1995). Telomeres are essential DNA–protein structures that cap and protect the end of eukaryotic chromosome from illegitimate recombination, degradation and detection as DNA damage. The reactivation of telomerase activity in most cancer cells supports the concept that telomerase is a relevant target in oncology, and telomerase inhibitors have been proposed as new potential anticancer agents (Bearss *et al.*, 2000; White *et al.*, 2001; Ohyashiki *et al.*, 2002). One effective strategy for designing telomerase inhibitors is to target telomerase indirectly via the telomeric substrate aiming to block the interaction between the enzyme and the telomere (Bearss *et al.*, 2000). At the extreme 3′-termini of telomeres, there are regions of single-stranded DNA formed owing to the limitations of DNA polymerization known as the end-replication problem (Watson *et al.*, 1972; Olovnikov *et al.*, 1973). Each of these regions have a guanine (G)-rich, single-stranded structure assembled around a core stack of guanines arranged in almost-planar, hydrogen-bonded tetrads. Ionic conditions that favor quadruplex formation have been shown to inhibit telomerase, and small molecules that stabilize or promote formation of quadruplexes also have shown inhibitory activity (Zahler *et al.*, 1991; Sun *et al.*, 1997; Wheelhouse *et al.*, 1998; Izbicka *et al.*, 1999). Therefore, stabilization of G-quadruplexes can be considered an original strategy to achieve antitumor activity.

Several classes of agents are potent inhibitors of telomerase and display strong affinities for G-quadruplex structures (Mergeny *et al.*, 1998). Small molecules that selectively stabilize the telomeric G-quadruplex induce telomere shortening and replicative senescence

(Gowan, *et al.*, 2001, 2002; Shin-ya *et al.*, 2001; Riou *et al.*, 2002; Tauchi *et al.*, 2003). Among those, telomestatin appears very promising owing to its high selectivity towards quadruplexes as compared to other nucleic acid conformations (Shin-ya *et al.*, 2001; Tauchi *et al.*, 2003). Telomestatin induces apoptosis in different tumor cell types and displays a selectivity towards cancer cells as compared to normal progenitor cells (Kim *et al.*, 2002, 2003; Gomez *et al.*, 2003; Tauchi *et al.*, 2003; Shammas *et al.*, 2004). Telomere shortening is also observed in cells treated with telomestatin, but arises earlier than expected for a single mechanism involving telomerase inhibition (Tauchi *et al.*, 2003; Gomez *et al.*, 2004). In the present study, we investigated the mechanisms of apoptosis induced by telomerase inhibition in acute leukemia. We found the activation of caspase-3 and poly-(ADP-ribose) polymerase (PARP) in telomestatin-treated U937 cells (PD20). Activation of p38 mitogen-activated protein (MAP) kinase and MKK3/6 was also found in telomestatin-treated U937 cells. Further, we evaluated the effect of telomestatin on the growth of U937 cells in xenograft mouse model. Systemic intraperitoneal administration of telomestatin in U937 xenografts decreased tumor telomerase levels and reduced tumor volumes. Tumor tissue from telomestatin-treated animals exhibited marked apoptosis. Taken together, these results lay the foundations for a program of drug development to achieve the dual aims of efficacy and selectivity *in vivo*.

Results

Effects of telomestatin and the expression of DN-hTERT on telomerase activity and telomere length in U937 cells

We have previously shown that inhibition of telomerase by DN-hTERT reproducibly results in telomere shortening and induction of DNA damage-associated apoptosis in human leukemia cells (Tauchi *et al.*, 2002, 2003; Nakajima *et al.*, 2003) In order to study the mechanisms of apoptosis induced by telomerase inhibition in leukemia cells, first we show the effects of telomestatin and the expression of DN-hTERT on telomerase activity in human acute myelomonocytic leukemia cell line, U937. U937 cells were cultured with telomestatin for 48 h. DN-hTERT-expressing U937 cells (PD25) were generated as described previously (Tauchi *et al.*, 2003). Telomerase activity from each cell line was analysed by telomere repeat amplification protocol (TRAP) assay (Figure 1a). Treatment of 10 μM of telomestatin and the expression of DN-hTERT showed the significant reduction of telomerase activity in U937 cells (Figure 1a). We next defined the telomere shortening by the presence of 2 μM of telomestatin and the expression of DN-hTERT in U937 cells (Figure 1b). Periodically, total DNA samples were prepared from indicated cell lines, and digested with frequently cutting restriction enzymes and the telomere length was examined by Southern blotting (Figure 1b). In the presence of 2 μM of telomestatin, the terminal restriction fragment (TRF) length of U937 cells shortened progressively from 9.5 to 3.8 kb at population doubling (PD) 20. The TRF length of DN-hTERT-expressing U937 cells showed 3.4 kb at PD25.

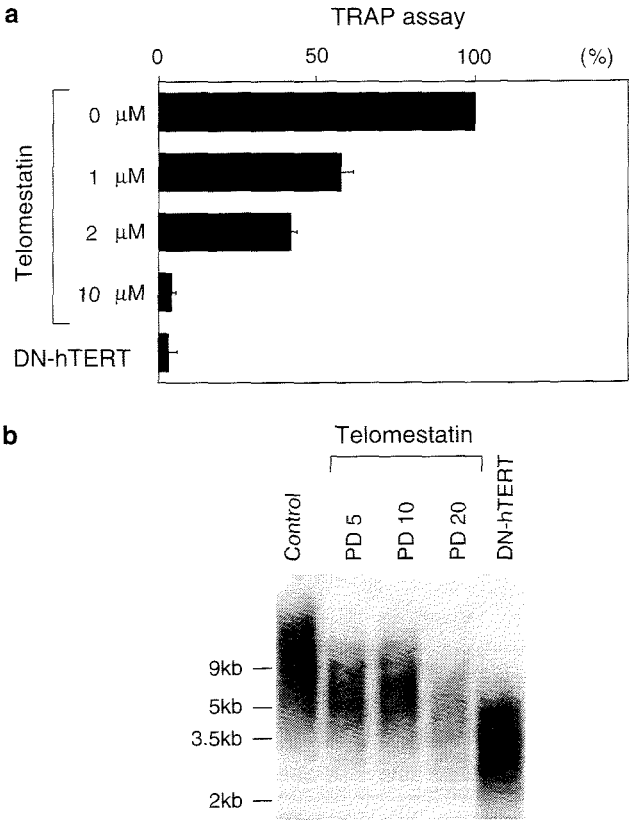

Figure 1 Effects of telomestatin and dominant-negative (DN)-hTERT expression on telomerase activity and telomere length in U937 cells. (**a**) The effect of telomestatin and DN-hTERT on telomerase activity in U937 cells. Telomerase activity was examined by a telomere repeat amplification protocol (TRAP) assay using a $TRAP_{EzE}$ telomerase detection kit (Oncor, Gaithersburg, MD, USA). (**b**) Total genomic DNA from U937 cells was assessed for telomere restriction fragment size by Southern blot analysis with a telomeric probe. PD, population doubling; left margin, molecular size markers (kb).

Effects of telomestatin on normal diploid human fibroblasts and alternative lengthening of telomeres-positive cells

We evaluated the effects of telomestatin on normal diploid human fibroblasts (BJ cells and IMR-90 cells) and alternative lengthening of telomere (ALT)-positive GM847 cells. Short-time (3 days) exposure to telomestatin at concentration up to 5 μM did not affect the viability of normal human fibroblasts BJ or IMR-90; however, 5 μM of telomestatin reduced the viability of GM847 cells (data not shown). We used 2 μM of telomestatin as the treatment condition in long-term cultivation experiments for GM847 cells. Treatment of BJ or IMR-90 cells with 2 or 5 μM of telomestatin did not significantly change the proliferation rate or viability to that of control cells (Figure 2a and b). Treatment of 2 μM of telomestatin also did not change the proliferation of GM847 cells (Figure 2c). These results suggest that telomestatin has less effect on normal diploid human fibroblasts and ALT-positive cells.

The telomere shortening associated with DNA-damage-induced apoptosis is associated with caspase-3 activation

One of the molecular events associated with DNA damage-induced apoptosis is the cleavage of caspase-3 proenzyme into a caspase-3 active form. Therefore, we examined the activation of caspase-3 and PARP in telomesatin-treated U937 cells and DN-hTERT-expressing U937 cells. U937 cells were cultured with 2 μM of telomestatin for indicated periods (PD0, PD5, PD10 and PD20). DN-hTERT-expressing U937 cells (PD25) were also used as positive controls of telomerase inhibition. Cell lysates from telomestatin-treated cells (PD0, PD5, PD10 and PD20) or DN-hTERT-expressing cells (PD25) were immunoblotted with anticleaved caspase-3 antibody (Ab) or anticleaved PARP Ab (Figure 3a and b). Caspase-3 and PARP were activated in cell lysates from telomestatin-treated U937 cells (PD20) and DN-hTERT-expressing U937 cells (PD25), but not in lysates from early passaged telomestatin-treated U937 cells (PD0, PD5 and PD10) (Figure 3a and b). These results indicate the involvement of caspase-3 activation in the telomerase inhibition associated apoptosis.

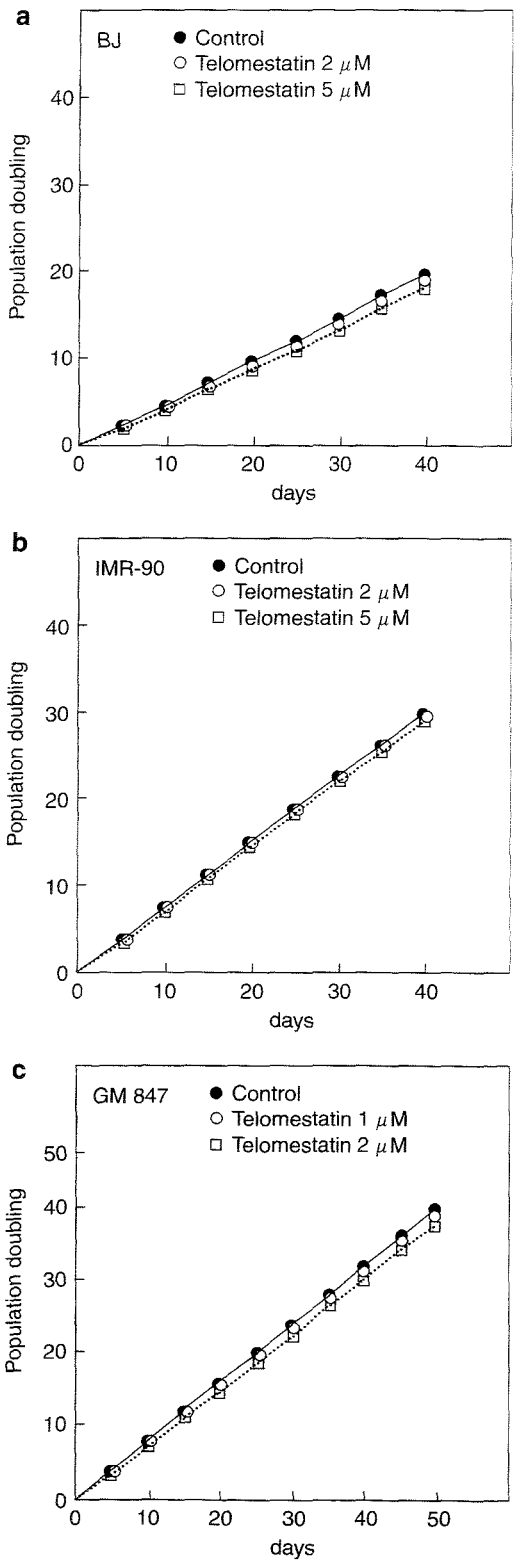

Figure 2 Effects of telomestatin on normal diploid human fibroblasts and alternative lengthening of telomere (ALT)-positive GM847 cells. (**a**) and (**b**) BJ cells and IMR-90 cells were plated in the presence of 2 or 5 μM of telomestatin in 0.1% methanol. Control cells were treated with 0.1% methanol. Cultures were repeated every 3–4 days to maintain log-phase growth and to calculate the growth rate. (**c**) GM847 cells were plated in the presence of 1 or 2 μM of telomestatin in 0.1% methanol. Control cells were treated with 0.1% methanol. Cultures were repeated every 3–4 days to maintain log-phase growth and to calculate the growth rate.

p38 MAP kinase is activated in telomestatin-treated U937 cells and DN-hTERT-expressing U937 cells

As p38 MAP kinase plays causative roles in senescent normal fibroblast cells following telomere shortening (Iwasa *et al.*, 2003), we examined the phosphorylation of p38 MAP kinase, JNK and ASK1, in telomestatin-treated U937 cells and dominant-negative (DN)-hTERT-expressing U937 cells. We found a four- to fivefold increase in the amount of phosphorylated p38

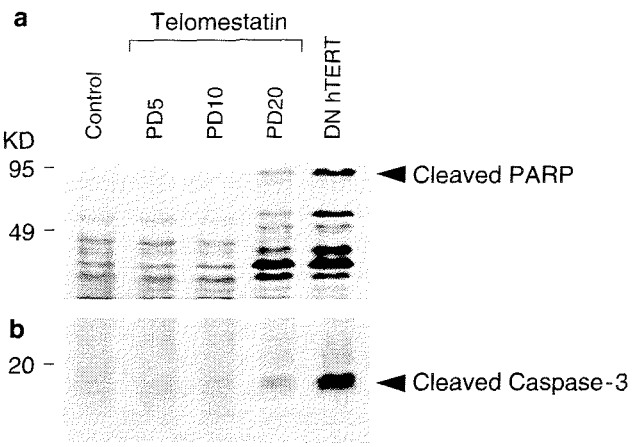

Figure 3 Telomestatin and dominant-negative (DN)-hTERT activate caspase-3 and PARP. Activation of caspase-3 and poly-(ADP-ribose) polymerase (PARP) in whole-cell lysates from the indicated cell lines was examined by immunoblotting with anticleaved caspase-3 antibody (**a**) or anticleaved PARP antibody (**b**).

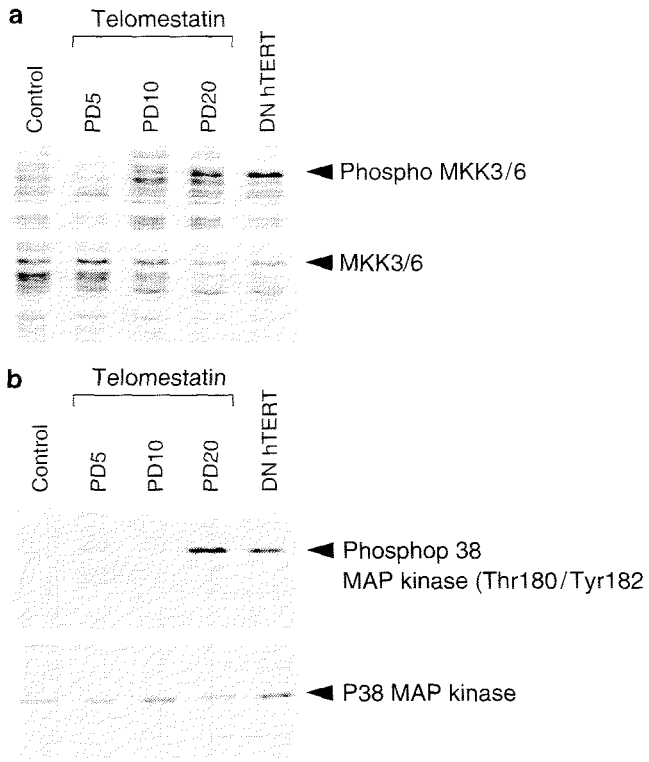

Figure 4 Telomestatin and dominant-negative (DN)-hTERT activate MKK3/6 and p38 mitogen-activated protein (MAP) kinase. Activation of MKK3/6 and p38 MAP kinasein whole cell lysates from the indicated cell lines was examined by immunoblotting with antiphospho-MKK3/6 antibody (**a**) or antiphospho-p38 MAP kinase antibody (**b**).

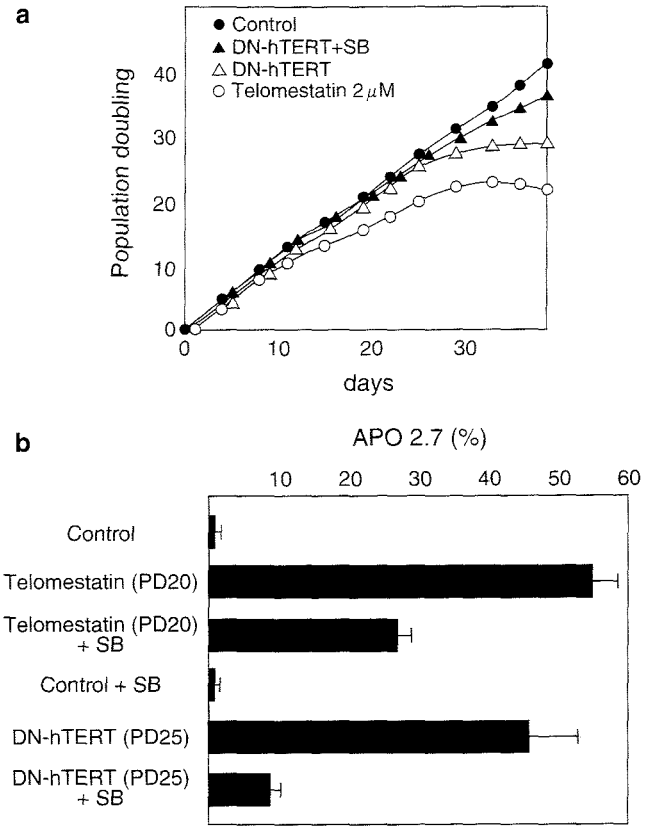

Figure 5 Effects of SB203580 on cell proliferation and induction of apoptosis in telomestatin-treated U937 cells and dominant-negative (DN)-hTERT-expressing U937 cells. (**a**) U937 cells were plated in 24-well plates in the presence of 2 μM of telomestatin in 0.1% methanol. DN-hTERT-expressing U937 cells were also plated in 24-well plates with or without 10 μM of SB203580. Control cells were treated with 0.1% methanol. Cultures were replated every 3–4 days to maintain log-phase growth and to calculate the growth rate. (**b**) Telomestatin-treated U937 cells (PD20) and DN-hTERT-expressing U937 cells (PD25) were incubated with 10 μM of SB203580 for 72 h. Apoptosis was examined by the cell surface expression of APO2.7, as determined by flow cytometry. The percentages of APO2.7-positive cells are shown at the top right of each panel.

MAP kinase in telomestatin-treated U937 cells (PD20) and DN-hTERT-expressing U937 cells (PD25) (Figure 4a). Phosphorylation of JNK and ASK1 in U937 cells was not altered by telomestatin and DN-hTERT (data not shown). MKK3/6 is regulated as the responsible kinase for p38 MAP kinase activation after various stimulations; therefore, we examined the phosphorylation of MKK3/6. Telomestatin and DN-hTERT also enhanced the phosphorylation of MKK3/6 in U937 cells (Figure 4b).

Effects of SB203580 on cell proliferation and inhibition of apoptosis in telomestatin-treated U937 cells and DN-hTERT-expressing U937 cells. To study the roles of p38 MAP kinase in cell proliferation and apoptosis, a p38 MAP kinase inhibitor, SB203580, was applied for the following studies. The growth kinetics of telomestatin-treated U937 cells and DN-hTERT-expressing U937 cells initially did not differ from those of control cells (Figure 5a). However, after 30 days, telomestatin-treated U937 cells and DN-hTERT-expressing U937 cells showed an almost complete inhibition of proliferation (Figure 5a). In the presence of SB203580, a significant increase in growth rate was observed in DN-hTERT-expressing U937 cells (Figure 5a). To determine whether p38 MAK kinase inactivation affect the apoptosis in telomestatin-treated U937 cells and DN-hTERT-expressing U937 cells, we employed flow cytometry analysis with APO2.7 antibody (Figure 5b). Cultivation with SB203580 for 72 h markedly decreased the population of APO2.7-positive cells from 54.5±3.5 to 26.7±1.7% in telomestatin-treated U937 cells (PD20) (Figure 5b). Cultivation with SB203580 also decreased the population of APO2.7-positive cells from 45.7±6.6 to 8.5±1.4% in DN-hTERT-expressing cells (PD25) (Figure 5b). These results indicate that p38 MAP kinase has a critical role for the growth kinetics and the induction of apoptosis in telomerase-inhibited leukemia cells.

Systemic telomestatin treatment decreased tumor telomerase activity and inhibited the growth of U937 xenografts

To further study the activity of telomestatin on tumor growth *in vivo*, we used xenografts of U937 cells in nude mice. When the tumors were established to a size of

100 mm^3, mice were treated with intraperitoneal injections of phosphate-buffer saline (PBS) ($n=5$) or telomestatin (15 mg/kg, $n=5$) two times a week for 4 weeks (Figure 6a). We found that telomestatin treatment significantly reduced tumor growth in U937 xenograft (Figure 6a). U937 xenograft treated with PBS for 21 days had a mean tumor volume of 1395 ± 270 mm^3 compared with telomestatin treated with a mean tumor volume of 291 ± 14 mm^3 (Figure 6a). Mice treated with systemic telomestatin treatment exhibited no adverse effects (body weight loss, clinical signs or survival). Telomerase activity from tumor cells was also examined by a TRAP assay after 48 h treatment (Figure 6b). Systemic administrations of 3 mg/kg or 9 mg/kg or 15 mg/kg of telomestatin decreased tumor telomerase activity by 60.2, 74 and 92.5% compared to control, respectively (Figure 6b). To evaluate whether administration of telomestatin results in induction of apoptosis in U937 cells *in vivo*, we examined the histology of tumors after telomestatin treatment (15 mg/kg, two times a week; day 21). Excised tumor or bone marrow samples were sectioned and stained with hemotoxylin and eosin (H &E) or TdT-mediated dUTP nick-end labeling (TUNEL) (Figure 6c). Tumors of control mice were composed of densely packed U937 cells with no feature of apoptosis (Figure 6c). Tumors from telomestatin-treated mice revealed an increased fraction of dead cells, identified by their amorphous shape and condensed nuclei (Figure 6c). Dead tumor cells and areas of degenerative tissue were observed, appearing as loosely arranged cells with the occurrence of vacuolated structures. Feature of apoptosis were observed in the tumor sections stained with TUNEL (Figure 6c). Administration of telomestatin also reduced U937 cells in bone marrow and recovered the normal hematopoiesis (Figure 6c).

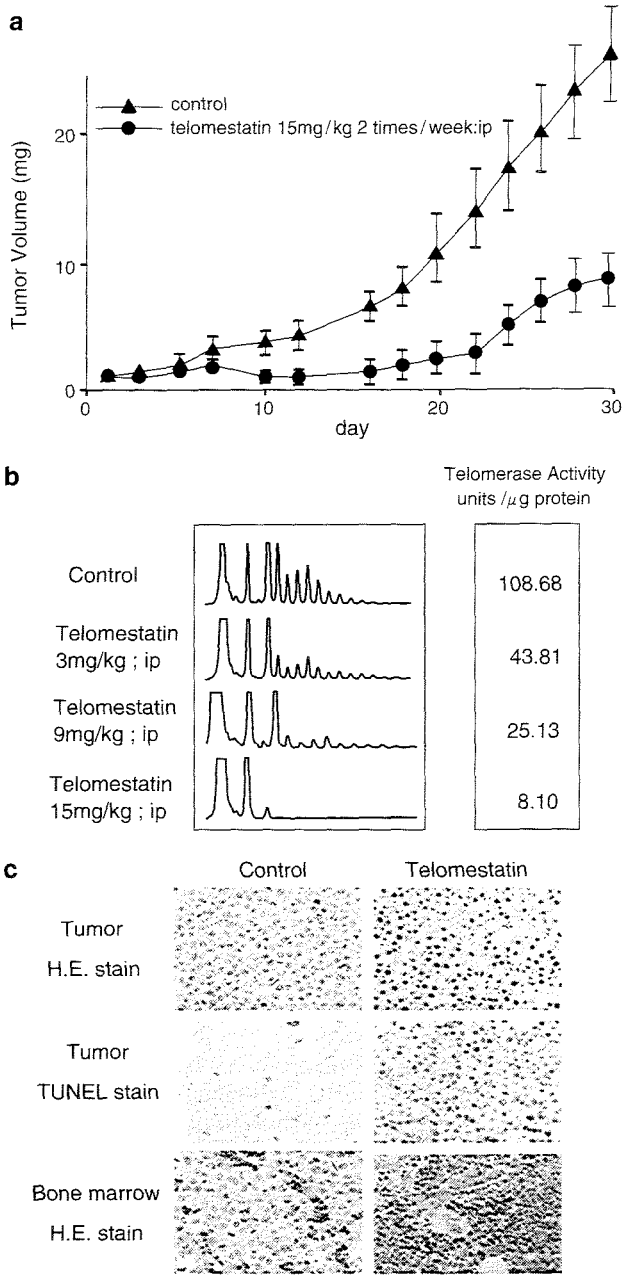

Figure 6 *In vivo* activity of telomestatin in U937 xenografts. (**a**) Systemic administration of telomestatin inhibited the growth of U937 xenografts in mouse models. Nude mice inoculated with 5×10^6 of U937 cells subcutaneously. When the tumors were established to a size of 100 mm^3 (approximately day 28), mice were divided into two groups of 5 mice/group. Mean tumor volumes $\pm$ s.e. (bars) in mice in treated with intraperitoneal telomestatin (15 mg/kg/day/2 times a week) or phosphate-buffered saline (PBS) for 4 weeks are shown. (**b**) Telomestatin decreased tumor telomerase activity *in vivo*. When the tumors were established to a size of 500 mm^3, mice were treated with indicated doses of telomestatin. After 48 h administration, telomerase activity was examined by a telomere repeat amplification protocol (TRAP) assay. (**c**) Representative photographs of biopsy samples from mice treated for 28 days with PBS (control) or telomestatin. H &E: hematoxylin and eosin; TUNEL: TdT-mediated dUTP nick-end labeling. Original magnification $\times 200$.

Discussion

Inhibition of telomerase activity by pharmacological or genetic interventions has been demonstrated to result in continuous telomere erosion, which ultimately induces replicative senescence or apoptosis. Telomerase inhibition does not immediately affect tumor cell growth, but will lead to a delay effect that is dependent on sufficient telomere decapping upon proliferation (de Lange *et al.*, 2002). We have previously demonstrated that telomestatin induced telomere shortening in human leukemia cells earlier than expected for a simple mechanism involving telomerase inhibition (Tauchi *et al.*, 2003). Recent observation demonstrated that treatment of telomesatin resulted in a marked decrease in the 3′-overhang signal, which correlated with the onset of the growth arrest (Gomez *et al.*, 2004).

In the present study, we examined the mechanisms of apotosis induced by telomestatin in acute leukemia. We demonstrated that caspase-3 activation, a key executioner of apoptosis is associated with telomestatin and DN-hTERT-induced apoptosis (Figure 3a). We have also observed the PARP activation in telomestatin-

treated U937 cells and DN-hTERT-expressing U937 cells (Figure 3b). Poly-(ADP-ribose) polymerase is activated during the DNA damage response, and it is involved in the base-excision repair, being cleaved by caspases into two fragments of 115 and 85 kDa, during apoptosis (Ame *et al.*, 1999; Schreiber *et al.*, 2002). Poly-(ADP-ribose) polymerase is the only characterized nuclear protein whose catalytic activity is stimulated by DNA strand break (Ame *et al.*, 1999; Schreiber *et al.*, 2002). Our results support the idea of relationship between telomerase inhibition-induced DNA damage and PARP activation.

The p38 MAP kinase pathway mediates responses to environmental stresses, including DNA-damaging agents such as UV- and γ-irradiation (Nebreda *et al.*, 2000; Wang *et al.*, 2000). A recent study reported that the activation of p38 MAP kinase contributes to the onset of cellular senescence induced by telomere shortening in human fibroblasts (Iwasa *et al.*, 2003). We have shown that telomestatin-treatment (PD20) and DN-hTERT-expression (PD25) enhanced the phosphorylation of p38 MAP kinase and MKK3/6 in U937 cells (Figure 4a and b). We have also shown that inhibition of p38 MAP kinase by SB203580 restored the proliferation in DN-hTERT-expressing U937 cells after PD25 (Figure 5a). The inhibition of p38 MAP kinase by SB203580 decreased the population of apoptosis in telomestatin-treated U937 cells and DN-hTERT-expressing U937 cells (Figure 5b). These results indicate that p38 MAP kinase has a critical role for the growth kinetics and the induction of apoptosis in telomerase-inhibited leukemia cells. Several lines of evidence suggest the association between telomestatin-induced telomere dysfunction and p38 MAP kinase activation. First of all, the activation of MKK3/6 and p38 MAP kinase occurred after PD20 in telomestatin-treated U937 cells (Figure 4a and b). This suggests that the treatment of 2 μM of telomestatin does not directly activate MKK3/6 and p38 MAP kinase. DN-hTERT-expression also activates MKK3/6 and p38 MAP kinase at PD25 (Figure 4a and b). In addition, the telomere loss induced by telomestatin led to telomere dysfunction (Tauchi *et al.*, 2003). Replicative senescence is induced not by the complete loss of telomeric repeats, but by the loss of telomeric function to protect chromosomes from end-to-end fusions (Karlseder *et al.*, 2002). Therefore, accumulation of genetic dysfunctions caused by telomestatin is responsible for p38 MAP kinase activation.

We have previously reported that telomestatin also had less effect on burst-forming unit-erythroid and colony-forming unit-grammulocyte/macrophase colony formation of normal bone marrow CD34-positive cells (Tauchi *et al.*, 2003). There are some difficulties inherent in using *in vitro* normal hematopoietic cell systems to evaluate the long-term cytotoxicities. In order to evaluate the long-term effects of telomestatin on normal cells, we treated normal diploid human fibroblasts with telomestatin over 40 days (Figure 2a and b). We observed that telomestatin had less effect on normal BJ or IMR-90 fibroblasts, suggesting a high anticancer specificity (Figure 2a and b). We also observed that telomestatin had minimum effects on ALT-positive GM847 cells (Figure 2c). In contrast to telomestatin, TMPyP4, a compound that preferentially facilitates the formation of intermolecular G-quadruplex structures, suppresses the proliferation of ALT-positive cells. Kim *et al.* (2003) reported that this difference is caused by the selectivity of compounds for either the intramolecular (telomestatin) or the intermolecular (TMPyP4) G-quadruplex structures.

We evaluated the *in vivo* activity of telomestatin in U937 xenografts (Figure 5). We demonstrated that treatment with telomestatin (15 mg/kg/day, intraperitoneally) was capable of achieving significant downregulation of telomerase activity in U937 cells *in vivo*, and that this was associated with antitumor activity (Figure 6a and b). Histological examination of these tumors by TUNEL staining revealed significant induction of apoptosis, suggesting that the changes in tumor volume may reflect a large population of apoptotic cells (Figure 6c). Intraperitoneal treatment of telomestatin (up to 15 mg/kg) exhibited no significant adverse effects compared with PBS-treated mice. Specifically, no differences in weight loss, or clinical symptoms were observed between telomestatin- and control-treated mice. Although we are unable to resolve the precise mechanism regarding with telomere shortening more than expected, it is clearly a feature that has significant implications for therapeutic applications and warrants further investigation.

In summary, we demonstrate that the telomestatin can cause effective reductions in telomerase activity that lead to induction of antitumor activity. These results, which should not be extrapolated to other types of tumors, highlight the complexity of the cell death process and the myriad of intracellular changes that may be required to achieve it. This, in turn, may shed some additional light on the cellular requirements for the development of effective and specific therapeutic strategies.

Materials and methods

Antibodies and reagents

Anticleaved caspase-3 (ASP175) Ab, anticleaved PARP (ASP214) Ab, anti-p38 MAP kinase Ab, antiphospho-p38 MAP kinase Ab (Thr180/Tyr182), anti-MKK3 Ab, antiphospho-MKK3/6 Ab, anti-JNK Ab, antiphospho JNK Ab, anti-ASK1 Ab and antiphospho ASK1(Ser83) Ab were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). SB203580 was obtained from Calbiochem, LaJolla, CA, USA. Telomestatin was purified as described previously (Shin-ya *et al.*, 2001).

Cells and cell culture

U937 cells, BJ cells and IMR-90 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). GM847 cells were obtained from Coriell Institute for Medical Research (Camden, NJ, USA). U937 cells were cultured in RPMI1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA). BJ cells, IMR-90 cells and GM847 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal calf serum.

Generation of stable clones expressing DN-hTERT mutants
pBABE-DN-hTERT was a gift from Dr Robert Weinberg (Massachusetts Institute of Technology). U937 cells were transfected with the expression vector pBABE-puro-DN-hTERT by electroporation. Beginning 48 h after electroporation, cells were selected with 2 μg/ml of puromycin and cloned by limiting dilution. PD0 was defined as the time at which cultures reached confluence in 10-cm culture dishes.

Telomerase assay and measurement of TRF
Telomerase activity was examined by a TRAP assay using a $TRAP_{EzE}$ telomerase detection kit (Oncor, Gaithersburg, MD, USA). The polymerase chain reaction (PCR) products were subjected to 12% acrylamide denaturing electrophoresis in an automated laser fluorescence DNA sequencer II (Pharmacia LKB Biotechnology, AB, Canada) and analysed by the Fragment Manager program (Pharmacia LKB Biotechnology, AB, Canada). Activity in the extract-based PCR TRAP assay was detected as a periodic 6 bp peak of telomerase products and, in each sample, relative telomerase activity was calculated semiquantitatively in comparison with a 36-bp internal standard. To measure TRF, genomic DNA was digested with the restriction enzymes *Hin*fI and *Rsa*I, fractionated on 0.7% agarose gels and transferred onto nylon membranes. Hybridization was performed by using the Telo TTAGGG telomere length assay kit (Roche Molecular Biochemicals, Mannheim, Germany).

Apoptosis assay
The incidence of apoptosis was determined by flow cytometric analysis with the fluorescein isothiocynate-conjugated APO2.7 monoclonal antibody (clone 2.7), which was raised against the 38 kDa mitochondrial membrane protein (7A6 antigen) and is expressed by cells undergoing apoptosis (Nakajima *et al.*, 2001).

Immunoblotting and immunoprecipitation
Immunoblotting and immunoprecipitation were performed as described previously (Tauchi *et al.*, 1994).

Antitumor effect in vivo
We studied the antitumor effect of telomestatin in mice bearing U937 cells. Tumors were observed 4 weeks after cells were injected into the back of 6-week-old female nude mice. In this study, we started to treat these animals 4 weeks after tumor inoculation, once transplantability was confirmed (tumor weight was 100 mm^3). These mice were treated with telomestatin (15 mg/kg, intraperitoneally two times a week) or PBS for 28 days. Mice were observed daily, and body weight as well as signs of stress (e.g., lethargy, ruffled coat or ataxia) were used to detect possible toxicities. Average tumor weight per mouse was calculated and was used to analyse the group mean tumor weight $\pm$ s.e. ($n = 5$ mice).

Tumor and tissue processing
Tumors were collected at selected times and fixed in paraformaldehyde. Paraffin-embedded tissues were sectioned and processed for gross histopathology by H.&E. staining and TUNEL staining.

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Cancer Genetics and Cytogenetics 165 (2006) 161–166

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Additional cytogenetic changes and previous genotoxic exposure predict unfavorable prognosis in myelodysplastic syndromes and acute myeloid leukemia with der(1;7)(q10;p10)

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Received 23 August 2005; received in revised form 10 September 2005; accepted 12 September 2005

Abstract We analyzed 23 patients with myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) showing a der(1;7)(q10;p10) [hereafter der(1;7)] to identify the exact predictive factor of this cytogenetic change. Eight (34.8%) patients, including six with MDS and two with AML patients, had a previous history of genotoxic exposure, especially radiation and/or antimetabolites. Patients with der(1;7) consisted of three groups: one third of patients had a previous history of genotoxic agents, one third had additional cytogenetic changes at the time of MDS/AML diagnosis without previous exposure history, and the remaining one third had neither a previous exposure history nor additional cytogenetic changes. The current study demonstrated that the poor outcome of MDS/AML with der(1;7) is caused by the high frequency of associated risk factors (i.e., previous history of genotoxic exposure, the presence of additional cytogenetic changes, or both). Identification of prognostic disadvantage might be required for applying the appropriate strategy in managing MDS/AML patients with rare der(1;7) abnormality.

1. Introduction

Chromosome 7 abnormalities (–7/7q–) are the most common chromosome changes in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), especially in secondary MDS and AML [1–4]. Nonetheless, the unbalanced translocation of chromosomes 1 and 7, der(1;7)(q10;p10) [hereafter der(1;7)], which results in trisomy 1q and monosomy 7p, is relatively rare in MDS and/or AML with cytogenetic abnormalities [5–7]. This abnormality was first reported by Geraedts et al. [8], and other investigators also confirmed this as a nonrandom abnormality in myeloid disorders, sometimes found to be therapy related [9–11]. The abnormality appears to be associated with dysplastic features in the marrow and poor prognosis [11–15]: the International Prognostic Scoring System (IPSS) for MDS adopted an abnormality of chromosome 7, including der(1;7), as one of the poor cytogenetic indicators [16]. Although some MDS cases with der(1;7) were actually therapy related and had unfavorable prognoses, the exact clinico-hematologic features are not fully understood because the number of patients with der(1;7) is still small. In this report, we analyzed 23 MDS/AML patients with der(1;7) from a single institution to attempt to evaluate the prognostic impact of this abnormality.

2. Materials and methods

2.1. Patients

From 1988 to 2004, a total of 27 patients with der(1;7) were detected in chromosome examinations in our hospital. Among them, four patients with chronic myeloproliferative disorders with der(1;7) were excluded from this study and will be discussed elsewhere [17]. A total of 23 cases, including 19 MDS patients (3.7% = 19/515 total MDS patients) and 4 AML patients (1.6% = 4/244 total AML patients), were eligible and all gave their informed consent to enroll in the study. Clinical data, past history, treatment protocol, response status, and follow-up duration were

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0165-4608/06/$ – see front matter
doi:10.1016/j.cancergencyto.2005.09.003

analyzed retrospectively by reviewing the medical records. Leukopenia was defined as total white cell count less than 3×10^9/L or neutrophils less than 1.8×10^9/L, anemia was defined as hemoglobin level below 100 g/L, and thrombocytopenia was a platelet count below 100×10^9/L. The IPSS on MDS patients was used as described in a previous report [16]. Marrow films from some refractory anemia (RA) patients were re-assessed and all of them were re-diagnosed with refractory cytopenia with multilineage dysplasia by World Health Organization (WHO) classification [18].

2.2. Cytogenetic study

Cytogenetic analysis was performed on unstimulated bone marrow specimens after Q-banding. Chromosome abnormalities were classified according to the International System for Human Cytogenetic Nomenclature (ISCN).

2.3. Statistics

Student's *t*-test was used to evaluate the differences of variables between patients with a single chromosome abnormality and those with additional abnormalities, and also between those with a history of exposure and those without history. The chi-square test was performed to evaluate the association between chromosome groups and exposure groups. The Kaplan-Meier method was used to calculate survival in relation to different variables, and long-rank analysis was used to assess significance. The Cox regression model was used for multivariate analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Cytogenetics results

Ten of 23 patients (43.5%) had a sole der(1;7) abnormality, including 8 RA, 1 RA with excess blasts (RAEB), and 1 AML patients (Table 1): 2 patients (unique patient numbers 110 and 113) showed an unrelated clone with a missing Y chromosome, thus they were included in the group of sole der(1;7) rather than in the additional changes group. The other 13 cases, including 8 RA, 2 RAEB, and 3 AML patients, had other abnormalities in addition to der(1;7): 9 cases showed a single additional abnormality, while 4 had complex abnormalities. The most frequent additional abnormalities were −20/20q− (seven cases) and trisomy 8 (four cases). The metaphase of the analyzed cells showed high frequency of der(1;7) with a mean percentage of 71.3% (ranging from 10 to 100%).

There was no significant difference between the sole abnormality group and the additional abnormality group with regard to age, blast percentage, white cell count, hemoglobin concentration, platelet count, or percentage of der(1;7). Even the among the IPSS scores of MDS patients, these two groups showed no significant difference (data not shown).

3.2. Genotoxic exposure

Among 23 cases, 8 patients (34.8%), including 6 RA and 2 AML, had previous histories of genotoxic exposure (Table 2). Seven cases received chemotherapy and/or radiation therapy to treat various solid tumors. One female AML patient was exposed to radiation from a nuclear weapon when she was 7 years old. Although five cases had radiation therapy, it is noteworthy that four patients received antimetabolites [i.e., fluorouracil (UPN 110, 111, 113, and 119)] and two (UPN 110 and 122) received cisplatinum. The duration from exposure to the detection of der(1;7) also varied from a half year to 10 years, except for one patient, UPN 121 (mean, 4.2 years; median, 4 years). Patients with a previous history of genotoxic exposure had no significant high incidence of additional cytogenetic abnormalities when compared to those without exposure [6/8 (75%) vs. 7/15 (46.7%); $P = 0.192$].

3.3. Outcome

All four AML patients received intensive induction chemotherapy, including anthracycline, but two did not respond to the therapy and died within a year (Table 1). Among the three cases of RAEB, only one young patient received intensive chemotherapy without obtaining remission. In 16 RA patients, all patients received supportive care and/or oral medication. Among 15 RA and RAEB patients with long-term follow-up for more than 100 days, 6 cases (40%) had transformation with intervals of 86 to 1,541 days (median, 357 days; Table 1).

The median survival of all patients was 403 days after detection of the der(1;7). Kaplan-Meier univariate analysis showed a significant survival advantage only in patients with blast percentage less than 5% ($P = 0.0128$) and no leukopenia (white cell count under 3×10^9/L; $P = 0.0305$). A significant survival difference was also noted between RA and RAEB ($P = 0.0438$; Table 3 and Fig. 1). Patients with additional changes tended to have short survival ($P = 0.0528$), while a previous history of genotoxic exposure alone was not a significant survival factor ($P = 0.2299$). We tentatively categorized patients with der(1;7) into three groups: (1) patients without previous genotoxic exposure and no additional changes (group A, $n = 8$ in Table 1); (2) patients with previous genotoxic exposure (group B, $n = 8$ in Tables 1 and 2); and (3) patients without previous genotoxic exposure but additional cytogenetic changes (group C, $n = 7$ in Table 1). Kaplan-Meier analysis demonstrated that group A had a significant survival advantage compared to group B ($P = 0.0381$) and group C ($P = 0.0025$; Fig. 1B). Of the patients with sole der(1;7) and no exposure history (group A), 2/6 RA patients developed AML and died on 602 days and 708 days, respectively.

Table 1
Characteristics of MDS/AML patients with der(1;7)

No	Sex/age	Diagnosis (FAB)	WBC ($\times 10^9$/L)	Hb (g/L)	Plt ($\times 10^9$/L)	Blast (%)	Follow up (day)	Trans-formation	Cytogenetics (no. of cells observed)
Group A: Patients without prior history of genotoxic exposure and no additional cytogenetic changes									
101	M/75	RA	4.8	78	75	4.5	708[a]	M4	46,XY,+1,der(1;7)(q10;p10)[18]
102	M/56	RA[b]	7.1	97	226	2.8	2709		46,XY,+1,der(1;7)(q10;p10)[2]/46,XY[18]
103	M/59	RA	4.1	127	42	0.4	1365		46,XY,+1,der(1;7)(q10;p10)[3]/46,XY[18]
104	M/56	RA	2.1	106	12	0.4	886	RAEBT	46,XY,+1,der(1;7)(q10;p10)[18]/46,XY[4]
105	M/53	RAEB	1.7	47	425	8.8	602[a]	M2	46,XY,+1,der(1;7)(q10;p10)[15]/46,XY[7]
106	M/81	RA	4.1	98	58	1.4	663		46,XY,+1,der(1;7)(q10;p10)[13]/46,XY[1]
107	M/68	RA	2	108	66	2.2	56		46,XY,+1,der(1;7)(q10;p10)[9]/46,XY[6]
108	M57	AML-M4	10.9	22	92	45	185		46,XY,+1,der(1;7)(q10;p10)[15]/46,XY[5]
Group B: Patients with a prior history of genotoxic exposure									
110	M/70	RA	2.4	78	37	1.8	602[a]	M4	45,X,−Y[7]/46,XY,+1,der(1;7)(q10;p10)[6]/46,XY[7]
111	M/69	RA	4.3	79	116	0.2	1705	RAEBT	45,XY,+1,der(1;7)(q10;p10),−20[11]
113	M/86	RA	2.4	102	53	2.0	127[a]		45,X,−Y[2]/46,XY,+1,der(1;7)(q10;p10)[11]/46,XY[8]
115	M/72	RA	3	11	47	0.4	657[a]		46,XY,inv(9)[5]/46,XY,+1,der(1;7)(q10;p10),inv(9)[9]/46, XY,+1,der(1;7),del(20)(q11)[6]
119	M/75	RA	3.1	107	94	4.0	54		46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[12]/45,XY,+1, der(1;7),−20[4]
120	M/68	RA	2.9	118	43	2.8	98		45,XY,+1,der(1;7)(q10;p10),−20[2]/45,XY,+1, der(1;7),del(20)(q11)[12]/45,XY,−20[1]/46,XY[7]
121	F/61	AML-M1	2.8	62	182	86	338[a]		48~53,XX,del(5)(q33),+del(5)(q33),add(7)(p21),+1, der(1;7)(q10;p10),−8,+11,−13, add(15)(p?)[19]
122	M/68	AML-M2	1.9	100	26	32	619[a]		46,XY,+1,der(1;7)(q10;p10)[7]/47,XY, idem+8[12]/48,XY,idem,+8,+18[1]/46,XY[1]
Group C: Patients without prior history of genotoxic exposure but who had additional cytogenetic changes									
119	M/46	RA	3.7	125	87	3.8	416[a]		46,XY,+1,der(1;7)(q10;p10),add(10)(q?)[20]/
112	M/59	RA	5.6	97	193	4.8	189		46,XY,+1,der(1;7)(q10;p10)[3]/47,XY,+1, der(1;7)(q10;p10),+8[16]46,XY[8]/
114	M/68	RAEB	1.5	59	13	5.2	403[a]		46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[2]46,XY[10]/
116	M/84	RA	2.4	82	208	1.8	20		47,XY,+1,der(1;7)(q10;p10),del(20)(q11), add(20)(q11)[9]
117	M/65	RA	3.3	75	472	4.0	346[a]	RAEBT	46,XY,+1,der(1;7)(q10;p10)[4]/46,XY,+1,der(1;7), del(20)(q11)[17]46,XY[2]/
118	M/70	RAEB[c]	2.4	70	11	5.6	208[a]		43~52,XY,+X,1,+3,+7,der(1;7),+8,+9, +10,+11,+12,+14,+21,+22[20]
123	M/59	AML-M6	0.9	67	16	31	401[a]		46,XY,+1,der(1;7)(q10;p10)[1]/46,XY,idem, del(3)(q21)[2]/47,XY,idem,del(3)(q21),+8[21]

Abbreviations: FAB: the French–American–British classification system; WBC: white blood cell count; Hb: hemoglobin; Plt: platelets; RAEBT: refractory anemia with excess blasts in transformation.
[a] Patients expired.
[b] This patient had concurrent Hodgkin's disease.
[c] This patient had a 34-year history of aplastic anemia.

Table 2
Exposure history of patients with MDS/AML and der(1;7)

No.	Diagnosis	Past history	Duration (y)	Treatment before presence of der(1;7)
110	RA	Oral cancer	10	Operation, radiotherapy, and chemotherapy (cisplatinum, fluorouracil, UFT (fluorouracil+tegafur))
111	RA	Lung cancer	1	Operation and chemotherapy [UFT (fluorouracil+tegafur)]
113	RA	Lung cancer	0.5	Operation, radiotherapy and chemotherapy [UFT (fluorouracil+tegafur)]
115	RA	Esophagus cancer	4	Operation and radiotherapy
119	RA	Rectal cancer	1	Operation, radiotherapy and chemotherapy (fluorouracil)
120	RA	Gastric cancer	4	Operation and radiotherapy
121	M1	Radiation exposure[a]	54	
122	M2	Malignant sarcoma	9	Operation and chemotherapy (cisplatinum, vincristine, anthracycline)

[a] Patient was exposed to radiation from a nuclear weapon at age 7.

4. Discussion

The unbalanced translocation between chromosomes 1 and 7, der(1;7)(q10;p10), is a nonrandom translocation in hematologic diseases. Since the initial report by Geraedts et al. [8], more than 100 cases have been reported [19].

Table 3
Statistical analysis of prognostic factors of patients with MDS/AML and der(1;7)

Factors	P Univariate	P Multivariate
Age (<60 years old)	0.2004	0.200
Blast (<5%)	0.0128*	0.701
RA vs. RAEB vs. AML	0.0438*	
RA vs. RAEB	0.0439*	
RA vs. AML	0.0599	
RAEB vs AML	0.6939	
Exposure vs. no exposure	0.2299	0.303
Sole abnormality vs. additional abnormality	0.0528	0.345
Group A vs. group B vs. group C	0.0217*	
Group A vs. group B	0.0381*	
Group A vs. group C	0.0025*	
Group A vs. group B + C	0.0074*	
White cell count ($< 3 \times 10^9$/L)	0.0305*	0.071
Hemoglobin (<100 g/L)	0.6728	0.822
Platelet count ($<100 \times 10^9$/L)	0.7305	0.727
Percentage of der(1;7) (<90%)	0.3744	0.143

Group A, sole der(1;7) abnormality and no exposure history to genotoxic agents.

Group B, exposure history to genotoxic agents with or without additional cytogenetic changes.

Group C, additional cytogenetic changes at the MDS/AML diagnosis without exposure history.

* $P < 0.05$

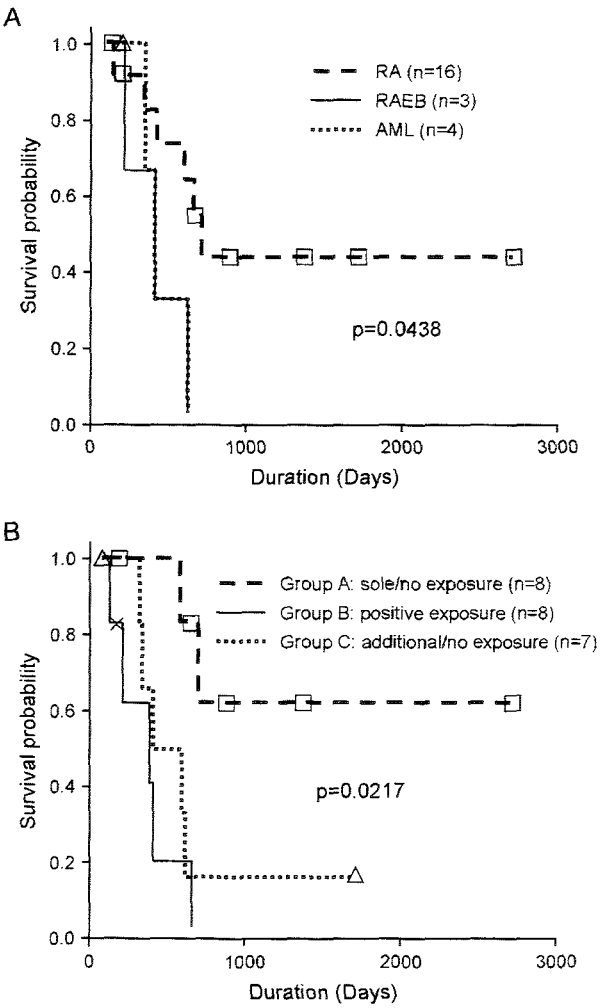

Fig. 1. Survival curves of patients. (A) There was a significant difference in survival among the RA, RAEB, and AML groups. The RA patients showed better survival than the RAEB patients ($P = 0.0439$), while no significant difference was noted between RAEB and AML ($P = 0.6939$). (B) There was a significant difference in survival between patients with sole der(1;7) abnormality without genotoxic exposure (group A in the text) and those with an exposure history (group B; $P = 0.0381$) or those with additional abnormalities without any genotoxic exposure (group C; $P = 0.0025$).

Though the exact mechanism of the formation of this translocation is still unclear, this abnormality is frequently found in myeloid disorders and is associated with genotoxic exposure and poor prognosis [11,15]. In the series, we found that 34.8% of patients (8/23) with der(1;7) had a previous history of genotoxic exposure, in accordance with the review by Pedersen [i.e., 49.3% (36/73) of patients with der(1;7) had a previous history of occupational or therapeutic exposure to genotoxic agents] [15]. Of note in the current study is that administration of antimetabolites, e.g., fluorouracil, was frequently observed as the abnormality-causing agent, apart from radiation, in contrast to previous reports implicating alkylating agents as the most frequently involved in generating chromosome abnormalities [5,11,15]. Our data also showed no significant association between exposure to

cytotoxic agents and additional chromosome changes at the time of diagnosis ($P = 0.192$).

We found a distinct male predominance (22 of 23 patients), which might indicate that Japanese men are highly susceptible to the genotoxic agents linked to this aberration. This retrospective study from a single institution, however, requires confirmation.

Unfavorable outcomes, including poor response to chemotherapy, high incidence of transformation, and short survival, were the characteristics of the patients in our study and other reports [12,15]. The loss of certain genes in the unbalanced translocation and chromosomal instability in the dicentric pattern of the der(1;7) were considered to contribute to the pathomechanisms of these conditions [20–23]. Based on the IPSS, abnormalities of chromosome 7, including der(1;7), are categorized as poor cytogenetic indicators [16], and MDS patients with der(1;7) are believed to have a poor prognosis. This study clearly showed that MDS/AML patients consist of three groups; one third of patients with previous history of genotoxic exposure, one third with additional changes and no previous exposure history, and the remaining one third with no previous history nor additional cytogenetic changes. Survival advantage is notable in patients without previous genotoxic exposure and no additional cytogenetic changes at the time of MDS/AML diagnosis. Therefore, the poor outcome of patients with der(1;7) is actually based on two major disadvantages, i.e., previous genotoxic exposure and additional cytogenetic changes in der(1;7)-positive myelopoietic malignancy.

We found that the aberration was characterized by male predominance and specific clinical features. Approximately one-third of our patients had no previous history of genotoxic exposure and no additional cytogenetic changes, and had a survival advantage. Although cytogenetically categorized MDS/AML patients with der(1;7) had poor prognoses, patients with der(1;7) consisted of heterogeneous prognostic groups, and detailed cytogenetic determination and review of previous genotoxic exposure history are required to identify subsets of patients to further clarify therapeutic strategies for patients with this rare cytogenetic anomaly. Moreover, most patients with der(1;7) showed multilineage dysplasia, thus requiring morphologic re-assessment of this issue using the new WHO classification. The number of patients in this study is still small to provide final conclusions, thus further detailed study of additional patients with der(1;7) is required.

We could not find any additional factors for AML development in MDS patients with der(1;7), so further molecular studies are also required to identify disease progression of this cytogenetic category, even in cases with favorable prognostic factors.

Acknowledgments

The authors are indebted to Prof. J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript. This work has been supported in part by a Grant-in-Aid for Intractable Hematopoietic Diseases from the Ministry of Health, Welfare, and Labor, Japan, the High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and by the University-Industry Joint Research Project from MEXT.

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Cancer Genetics and Cytogenetics 164 (2006) 90–91

Cancer Genetics and Cytogenetics

Letter to the editor

The translocation (4;12)(q31;q21) in myelofibrosis associated with myelodysplastic syndrome: impact of the 12q21 breakpoint

Myelofibrosis is sometimes associated with clonal hematopoietic stem cell disorders, including myelodysplastic syndromes (MDS), agnogenic myeloid metaplasia, and polycythemia vera. Nonrandom cytogenetic abnormalities have been found in myelofibrosis with myeloid metaplasia (MMM), but a single consistent abnormality such as Ph translocation in chronic myeloid leukemia has not been identified even though the new World Health Organization classification has proposed that abnormal activation of tyrosine kinase–dependent signal translocation pathways is essential in chronic myeloproliferative disorders. The frequent chromosomal changes in MMM are del(13q), del(20q), del(20p), and partial trisomy 1q [1]. Here we report on a case of MDS that finally evolved into myelofibrosis associated with t(4;12)(q31;q21).

A 70-year-old man complained of malaise in October 1994. Initial hematologic examination showed normocytic anemia: hemoglobin level of 87 g/L, a white blood cell count of 5.3 × 10^9/L, and a platelet count of 26.4 × 10^9/L. Upon physical examination, no organomegaly was observed. Bone marrow aspiration revealed slightly hypocellular marrow with trilineage dysplasia and 2% myeloblasts. Cytogenetic analysis of bone marrow cells revealed a normal male karyotype 46,XY (17 cells). He was diagnosed as having MDS refractory anemia (RA) and was treated conservatively. In May 2003, his hemoglobin decreased gradually and his liver and spleen enlarged progressively. Peripheral blood cells revealed dyserythropoetic changes such as anisocytosis, tear drop cells, and polychromasia with four erythroblasts in the blood cells. The bone marrow aspirate was a dry tap. Trephine biopsy revealed myelofibrosis with prominent multilineage dysplasia. Chromosomal analysis on peripheral blood cells revealed 46,XY,t(4;12)(q31;q21) (22 cells) (Fig. 1). Danazole therapy was initiated in association with transfusion as palliative therapy, but the latter did not appear to be effective. Thalidomide (200 mg) and prednisolone (20 mg/day, PO) were then administered on the basis of a diagnosis of MDS with myelofibrosis.

Balanced translocations in myeloproliferative disorders such as MMM are rare events. To our knowledge, balanced translocations have been reported in only 14 patients with myelofibrosis (Table 1). The translocation (4;12)(q31;q21) has not been reported in association with any myeloproliferative disorder other than MMM. Abnormalities at 12q21 and 12q24 alone or in combination with other abnormalities have been reported in several cases of myelofibrosis. Andrieux et al. [2] reported abnormalities at 12q21 or 12q24 in seven cases of MMM. They suggested that two different "hot spots" on 12q – 12q21 and 12q24 – might be involved in the malignant process of myelofibrosis. Breakpoints at 12q13~q21 or 12q24 subsequently have been identified in translocation-type aberrations in myelofibrosis. Our case demonstrates a clinical profile that may fit such a concept. The translocation (4;12)(q31;q21) was not detected during the MDS phase but appeared subsequently during evolution to myelofibrosis. Considering the long history of anemia and the recent marked splenomegaly in our case, we believe that 12q21 might be a secondary event rather than an initial aberration.

Recent studies have shown that the single point mutation of the cytoplasmic tyrosine kinase, JAK2, has a potential role in myeloproliferative disorders [3,4]. Baxter et al. investigated that a single point mutation (Val617Phe) in JAK2 was identified in 97% of patients with polycythemia

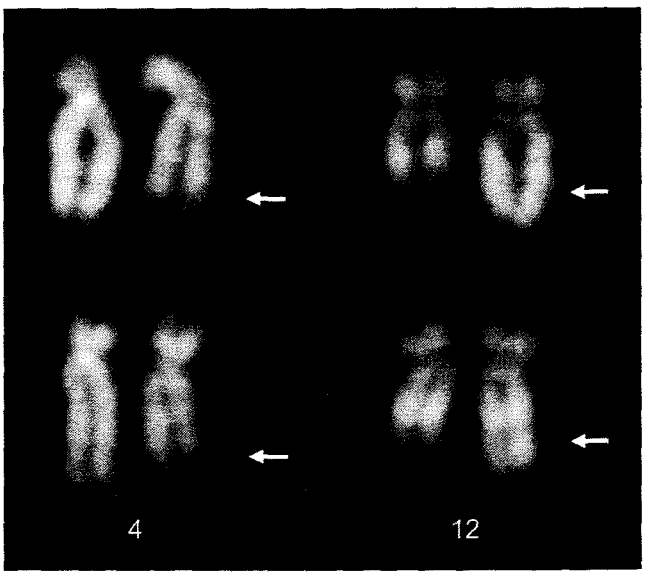

Fig. 1. Partial Q-banded karyotypes of unstimulated peripheral blood cells showing t(4;12)(q31;q21). Arrowheads indicate possible breakpoints of chromosomes 4 and 12.

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doi:10.1016/j.cancergencyto.2005.06.014

Table 1
Clinical features of well-documented cases showing balanced translocations in myelofibrosis

No.	Sex/age	Sample	Disease stage	Karyotypes of the abnormal clone	Report (reference)
1	M/43	BM	MMM at diagnosis	46,XY, t(4;12)(q33;q21)	Andrieux et al. [2]
2	M/75	BM	MMM at diagnosis	46,XY, t(5;12)(p14;q21)	Andrieux et al. [2]
3	F/67	BM	MMM at diagnosis	46,XX, t(1;12)(q22;q24)	Andrieux et al. [2]
4	M/58	BM	MMM at diagnosis	46,XY, t(7;12)(p11;q24)	Andrieux et al. [2]
5	F/44	BM	MMM at diagnosis	46,XX, t(1;12)(p21;q12)	Andrieux et al. [2]
6	M/ND	PB	MF at diagnosis	46,XY, t(6;12)(q13;q23)	Miller et al. [5]
7	M/59	PB	AMF on therapy	46,XY, t(1;4)(q32;q35)	Shah et al. [6]
8	M/76	ND	IMF at diagnosis	46,XY, t(1;12)(p31;q21)	Reilly et al. [1]
9	M/65	PB	IMF at blast crisis	46,XY, t(4;12)(q26;q15), t(5;12)(q13;q24)	Przepiorka et al. [7]
10	F/ND	PB	MF with AMM at diagnosis	46,XX, t(1;7)(p31;p22), t(10;20)(q26;q11)	Jean et al. [8]
11	M/64	BM	AMM at diagnosis	46,XY, t(8;12)(p23;q21)	Borrego et al. [9]
12	M/68	BM	CMPD with MF	46,XY, t(2;3)(p21;p26)	Herens et al. [10]
13	M/52	PB	MDS with MF	46,XY, t(5;12)(q33;p12-3)	Lerza et al. [11]
14	M/69	PB	MDS with MF	46,XY,t(4;12)(q31;q21)	Present case

Abbreviations: MF, myelofibrosis; BM, bone marrow; PB, peripheral blood; MMM, myelofibrosis with myeloid metaplasia; AMF, acute myelofibrosis; IMF, idiopathic myelofibrosis; AMM, agnogenic myeloid metaplasia; CMPD, chronic myeloproliferative disorder; ND, no description.

vera and in 50% of patients with idiopathic myelofibrosis. They suggested that these results have important implications for the classification, diagnosis, and treatment of these diseases and provide insight into their pathogenesis. However, the pathogenesis of half the patients with myelofibrosis has not been established yet. Other tyrosine kinases may be involved in chronic myeloproliferative disorders, including myelofibrosis. Of note is that translocation-type abnormalities in MF showed clustering breakpoints on chromosome 12 [i.e., 12q21 in 6/14 cases, 12q24 in 3/14 cases, and 12q12~q13 in 2/14 cases of myelofibrosis showing translocation-type abnormalities (Table 1)].

Our case confirms an association between myelofibrosis and abnormalities of chromosome 12, especially 12q21, regardless of the etiology of myelofibrosis. The role of the other chromosomal aberrations seen in myelofibrosis remains unclear, however, though we have not had a chance to investigate the JAK2 mutation. The present well-documented report of a case of myelofibrosis in MDS with balanced translocation (4;12)(q31;q21), in association with other well-characterized cases of myelofibrosis, should help in future analyses to identify the elusive genetic basis of this disease.

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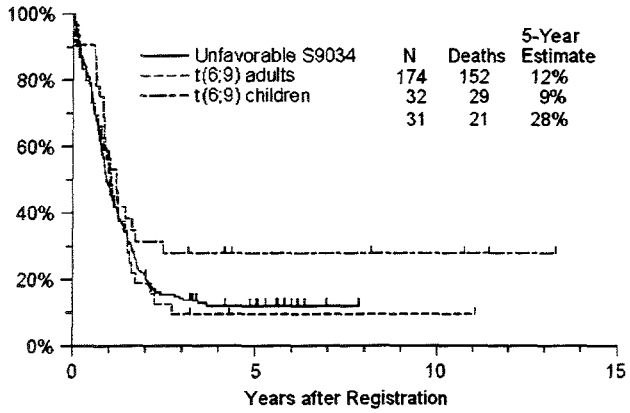

Figure 1 Estimated probabilities of overall survival of t(6;9) adult and pediatric cases compared with SWOG/ECOG S9034/E3489 younger adult AML study[8] unfavorable risk cytogenetics subgroup. Table 1 describes the t(6;9) patients included in this analysis.

perhaps molecular monitoring among the various centers or cooperative groups involved.

In closing, we report the largest t(6;9) patient cohort to date confirming and refining consistent t(6;9) pathological features including increased basophilia, single or multilineage dysplasia in adult patients, variable FAB morphology, a CD13+, CD33+, CD38+, CD45+, and HLA-DR+ immunophenotype, and a high incidence (71%) of FLT3 internal tandem duplications. Furthermore, the low incidence of secondary aberrations may be important for targeted therapeutic options in the future. Like all retrospective studies, data collection procedures were discordant among the Groups, complicating the analyses; nevertheless, our study objective is to raise awareness of this very poor risk AML subtype that tends to be more common in younger patients. Accordingly, t(6;9) AML may warrant a prospective multi-center investigation of aggressive and/or novel therapeutic strategies, including allogeneic SCT for patients with an HLA-matched donor, and perhaps because of the presence of CD33 and FLT mutations, the addition of anti-CD33-based therapies plus a FLT3 inhibitor for patients without suitable donors. Finally, to facilitate scientific collaboration to accomplish such an initiative, we support the proposal of adding AML with t(6;9)(p23;q34), DEK/NUP214, as a separate disease entity to the World Health Organization (WHO) Classification of Hematological Malignancies.[3,9]

Acknowledgements

We thank all clinicians and scientists who contributed the material for this study. This study was supported in part by the following PHS Cooperative Agreement grant numbers awarded by the National Cancer Institute, DHHS: CA32102, CA21115, CA31946, CA33601, CA41287, CA77658, CA101140, CA13539, CA98543, CA30969, K23 CA92405, and The Leukemia Clinical Research Foundation.

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Reply to Kremer M *et al.*, The JAK2 V617F mutation occurs frequently in myelodysplastic/myeloproliferative diseases, but is absent in true myelodysplastic syndromes with fibrosis

Leukemia (2006) **20**, 1297–1298. doi:10.1038/sj.leu.2404232; published online 13 April 2006

The current approach to detect JAK2 V617F mutation in patients with chronic myeloproliferative disorders (CMPD) revealed the molecular category of this disease entity and related diseases.[1,2] Kremer *et al.*[3] found JAK2 V617F mutation in three of four patients with myelodysplastic (MDS)/myeloproliferative (MPD) diseases, whereas none of 25 patients with MDS with myelofibrosis showed the mutation. They further stated that

MDS/MPD patients with the JAK2 V617F mutation showed clinical features somewhat resembling MPD, including splenomegaly, leukocytosis, and/or thrombocytosis, and morphologic features of CMPD in marrow biopsy.[3] They suggested that detection of the JAK2 V617F mutation could be a help to identify the borderline between typical MDS and atypical MPD.Myelodysplastic syndrome with myelofibrosis is believed to be a heterogeneous entity; some patients show myelofibrosis at the time of MDS diagnosis and are closely associated with acute myeloid leukemia with megakaryoblastic nature, or 'acute myelodysplasia with myelofibrosis'.[4] On the other hand, some MDS patients show myelofibrosis at a later stage of the disease.[5] Moreover, some patients with MPD have clinico-hematologic features resembling MDS at the first manifestation: some of them progress into MPD several years later. The WHO criteria may not cover all of these heterogeneous entities, therefore, we should probably diagnose such patients tentatively at the present juncture. For example, late appearing myelofibrosis in MDS patients should not be categorized as MDS/MPD at the first manifestation.

In our current study published in *Leukemia*,[6] we did not have the chance to identify the JAK2 V617F mutation during the MDS phase lacking myelofibrosis. We detected the mutation in the myelofibrosis phase; therefore, we considered that the difference of the JAK2 V617F mutation,[6] unlike the contention of Kremer *et al.*,[3] might mainly be owing to different diagnostic categorization. Owing to the lack of a concrete diagnostic definition of MDS with myelofibrosis, we cannot completely eliminate the possibility of overestimating 'true' MDS with myelofibrosis, but we do not know the biological or definite clinical significance of the disease, and such an overestimation may cause some confusion concerning the data on the JAK2 V617F mutation.

Eventually, as Kremer *et al.*[3] also suggested, MDS/MPD might be one domain of MDS, but probably only a part of even those may show the JAK2 V617F mutation, as about 90% of polycythemia vera and 50% of essential thrombocythemia or primary myelofibrosis carry this mutation. Therefore, we can only say that the JAK2 V617F mutation might be one diagnostic tool for the identification of MPD. An accumulation of conditions overlapping MDS to ascertain degree, including a compilation of the MPD-like clinico-hematologic features, in combination with the detection of the JAK2 V617F mutation, might be required to resolve the problematic topology of MDS with myelofibrosis.

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Chromosomal aberrations in leukaemia cells may delete tumour target antigens of stem cell-based immunotherapy

Leukemia (2006) **20**, 1298–1300. doi:10.1038/sj.leu.2404237; published online 20 April 2006

A novel approach to eliminate residual disease after human leucocyte antigen (HLA)-matched stem cell transplantation (SCT) for leukaemia is tumour-specific adoptive immunotherapy. Excellent immunotherapeutic targets for adoptive immunotherapy are the haematopoietic system-specific minor-histocompatibility antigens (mHags) HA-1 and HA-2, which are expressed on all normal and malignant haematopoietic cells.[1,2] HA-1 and HA-2 epitopes are presented on the cell surface in the binding groove of HLA-A2 molecules. Immunotherapy with HA-1 or HA-2-specific cytotoxic T lymphocytes (CTLs) is restricted to leukaemia patients positive for the immunogenic mHag HA-1 or HA-2 alleles, that is HA-1H or HA-2V, respectively. The mHag status of patients is routinely determined by allele-specific genomic polymerase chain reaction (PCR) on peripheral blood mononuclear cells (PBMCs).[2] Generally, leukaemic cells have the same mHag allelic patterns as the PBMCs. Most leukaemias, however, have karyotypic abnormalities,[3] some of which affect mHag encoding genomic regions (e.g. 19p13.3 harbouring the HA-1 gene[4] or 7p12-13 harbouring the HA-2 gene[5]).

Here, we describe an isochromosome 7 causing loss of HA-2 CTL recognition of leukaemic cells in a patient with pre-B acute lymphoblastic leukaemia. The patient was typed on PBMCs to be heterozygous for both HA-1 (H/R) and HA-2 (V/M) (Figure 1e and f). The leukaemia karyotype was 46,XX,i(7)(q10),der(19)t(1;19)(q23;p13). Hereby, the HA-1 encoding region on one chromosome 19 and the HA-2 encoding region on one chromosome 7 were deleted (Figure 1a). The bone marrow of the patient contained 90% leukaemic blasts. We separated these CD10 positive leukaemia cells from nonleukaemic cells with magnetic beads and used them as targets in a chromium release

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Blood Cells, Molecules, and Diseases 36 (2006) 308–314
BLOOD CELLS, MOLECULES, & DISEASES
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Stromal-cell-derived factor-1/CXCL12-induced chemotaxis of a T cell line involves intracellular signaling through Cbl and Cbl-b and their regulation by Src kinases and CD45

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Submitted 21 November 2005; revised 21 December 2005

(Communicated by M. Lichtman, M.D., 22 December 2005)

Abstract

Stromal-cell-derived factor-1α (SDF-1α/CXCL12) is a potent chemoattractant for T cells. We report that Cbl family members, Cbl and Cbl-b, are tyrosine-phosphorylated after SDF-1α/CXCL12 stimulation of Jurkat T cells. Enhanced phosphorylation of Cbl and Cbl-b was regulated by src family kinases, and perhaps Fyn. Activated Cbl and Cbl-b interacted with Crk-L, Zap-70, Nck, PLC-γ and Fyb after SDF-1α/CXCL12 stimulation, implicating association of these proteins in SDF-1α/CXCL12 actions. SDF-1α/CXCL12 did not induce tyrosine phosphorylation of Cbl or Cbl-b in Lck-deficient T cell line J.CaM1.6 or CD45-deficient T cell line J45.01. Thus, Lck Src kinase and tyrosine phosphatase CD45 are likely involved in regulating activation of Cbl family members. A functional role for Cbl and Cbl-b in migration was demonstrated by the decrease in SDF-1/CXCL12-induced migration in a T cell line in which transfected small interfering RNA for Cbl and Cbl-b decreased expression of Cbl and Cbl-b, but not MAPK activity. SDF-1α/CXCL12-induced chemotaxis was greatly reduced in the CD45-deficient T cell line. Our results implicate CD45, Cbl, Cbl-b, src kinases and potentially other associated proteins as mediators of SDF-1α/CXCL12-induced cell migration of Jurkat T cells.

Keywords: T lymphocytes; Jurkat cells; Chemotaxis; SDF-1/CXCL12; Signal transduction; Cbl; Src kinases; Lck; CD45

Introduction

Stromal-cell-derived factor-1 (SDF-1/CXCL12) is a CXC chemokine produced by bone marrow stromal cell types [1]. CXCR4, a receptor for SDF-1/CXCL12, is widely expressed by a variety of hematopoietic cell types, including T cells [2]. SDF-1 is chemotactic for human lymphoid, myeloid and CD34 positive progenitor cells and has been implicated in cell migration of human and murine hematopoietic cells (reviewed in [3,4]). Lymphopoiesis and myelopoiesis are markedly reduced in CXCR4 and SDF-1/CXCL12-deficient mice [5].

Towards a better understanding of events mediating SDF-1/CXCL12 effects, a number of investigators have begun to elucidate the intracellular signals triggered by SDF-1/CXCL12 and the role these proteins may play in SDF-1/CXCL12-induced cellular activities. The full range of intracellular signals regulating SDF-1/CXCL12 actions remains to be elucidated. Among a number of intracellular proteins implicated in SDF-1/CXCL12 actions are Cbl [6] and CD45 [7]. Cbl is tyrosine-phosphorylated and associates with PI3 kinase, Crk-L and 14-3-3 when cells are stimulated with SDF-1/CXCL12 [6]. The protein tyrosine phosphatase CD45 was shown to differentially regulate CXCR4-mediated chemotaxis as well as MAP kinase activation by regulating activities of focal adhesion components and other effectors downstream of the T cell receptor [7]. Cbl is a 120 kDa phosphoprotein expressed in the cytoplasm of many cell types. Cbl is the cellular homologue of the v-Cbl oncogene and was first cloned from a recombinant murine retrovirus that

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doi:10.1016/j.bcmd.2005.12.035