Induction of apoptosis in myeloid leukaemic cells by ribozymes targeted against AML1/MTG8

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Summary The translocation (8;21)(q22;q22) is a karyotypic abnormality detected in acute myeloid leukaemia (AML) M2 and results in the formation of the chimeric fusion gene AML1/MTG8. We previously reported that two hammerhead ribozymes against AML1/MTG8 cleave this fusion transcript and also inhibit the proliferation of myeloid leukaemia cell line Kasumi-1 which possesses t(8;21)(q22;q22). In this study, we investigated the mechanisms of inhibition of proliferation in myeloid leukaemic cells with t(8;21)(q22;q22) by ribozymes. These ribozymes specifically inhibited the growth of Kasumi-1 cells, but did not affect the leukaemic cells without t(8;21)(q22;q22). We observed the morphological changes including chromatin condensation, fragmentation and the formation of apoptotic bodies in Kasumi-1 cells incubated with ribozymes for 7 days. In addition, DNA ladder formation was also detected after incubation with ribozymes which suggested the induction of apoptosis in Kasumi-1 cells by the AML1/MTG8 ribozymes. However, the ribozymes did not induce the expression of CD11b and CD14 antigens in Kasumi-1 cells. The above data suggest that these ribozymes therefore inhibit the growth of myeloid leukaemic cells with t(8;21)(q22;q22) by the induction of apoptosis, but not differentiation. We conclude therefore that the ribozymes targeted against AML1/MTG8 may have therapeutic potential for patients with AML carrying t(8;21)(q22;q22) while, in addition, the product of the chimeric gene is responsible for the pathogenesis of myeloid leukaemia.

Keywords: apoptosis; ribozyme; AML; AML1/MTG8; t(8;21)

The (t(8;21)(q22;q22) translocation is one of the specific chromosome translocations which exists in 7–17% of the cases with acute myeloid leukaemia (AML) and is usually classified as AML M2 according to the French–American–British (FAB) classification criteria (Koeffler, 1987; Schiffer et al, 1989; Tashiro et al, 1992). This translocation results in the formation of the AML1/MTG8 chimeric fusion gene, which has a 5’ portion of the AML1 gene on chromosome 21 which is fused almost to the entire MTG8 gene, also called ETO gene, on chromosome 8 (Erlickson et al, 1992; Miyoshi et al, 1993).

The AML1 is a member of transcriptional factors which contain a region of homology to the Drosophila pair-rule gene, runt (Miyoshi et al, 1993). The runt homology domain is responsible for heterodimerization with core binding factor β (CBFβ) (Lenny et al, 1995) and specific binding to the sequence TGT/cGGT which is the enhancer core motif (Meyers et al, 1993). Several target genes for AML1 have been identified such as neutrophil elastase, myeloperoxidase, GM-CSF, IL-3, M-CSF receptor and TCRβ enhancer (Cameron et al, 1994; Nuchprayoon et al, 1994; Zhang et al, 1994, 1996; Frank et al, 1995; Meyers et al, 1995; Takahashi et al, 1995). AML1 has been found to transactivate the regulatory segments of each of these genes. Three representative forms of proteins, AML1a, AML1b and AML1c, are produced from the AML1 gene by alternative splicing (Miyoshi et al, 1995). AML1a and AML1b are thought to regulate haematopoietic myeloid cell differentiation and transcriptional activation antagonistically (Tanaka et al, 1995). The function of the AML1 protein is regulated by extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinases through phosphorylation (Tanaka et al, 1996). An in vivo study revealed that mice lacking AML1 are embryonically lethal without myeloid or erythroid progenitors originating from definitive haematopoiesis (Okuda et al, 1996; Wang et al, 1996). These data indicate that AML1 is one of the essential transcriptional factors for the definitive haematopoiesis of all types of cell lineage.

AML1/MTG8 chimeric protein shares structural features with these genes and also demonstrates the runt homology domain (Miyoshi et al, 1993). AML1/MTG8 is thought to interfere with AML1b-dependent transcriptional activation as a dominant negative protein (Meyers et al, 1993, 1995). The antisense oligonucleotide targeted against AML1/MTG8 fusion transcript inhibited the growth of the AML cell lines with t(8;21)(q22;q22) (Sakakura et al, 1994). Therefore, AML1/MTG8 chimeric fusion protein is considered to play an important role in the leukaemogenesis of acute myeloid leukaemia with t(8;21)(q22;q22).

Hammerhead ribozymes are one of the useful tools for the specific inhibition of gene expression (Haseloff and Gerlach, 1988). They are oligoribonucleotides with sequence-specific cleavage activity of target RNA and can be designed to cleave any triplet of NUX (N = any nucleotide, X = A, C or U) (Koizumi et al, 1988). One molecule of ribozyme can cleave a plural number of target RNAs by repeating the catalytic cycle. Therefore ribozymes are thought to be more effective than antisense oligonucleotides to suppress the expression of the target genes (Homann et al, 1993).

We have designed two hammerhead ribozymes against...
AML1/MTG8 which specifically cleaved the AML1/MTG8 substrate in a cell-free system and also inhibited the growth of Kasumi-1 cells, an AML cell line with t(8;21)(q22;q22) (Matsushita et al, 1995). The purpose of this report is to disclose the mechanism of growth inhibition of AML cells with t(8;21)(q22;q22) by these ribozymes. We herein demonstrate for the first time that ribozymes can inhibit the proliferation of myeloid leukaemic cells by the induction of apoptosis, but not differentiation.

**MATERIALS AND METHODS**

**Cells and chemicals**

The myeloid leukaemic cell line Kasumi-1 established from a patient with AML M2 carrying t(8;21)(q22;q22) was a generous gift of Dr N Kamada (Hiroshima University, Horoshima, Japan) (Asou et al, 1991). The other human myeloid leukaemic cell lines used in this study were HL-60, KG-1, NB4 (a gift from Dr M Lanotte, Hôpital St. Louis, Paris, France) (Lanotte et al, 1991) and
Ribozyme transfection into various leukaemic cells with lipofection

Ribozymes were transfected into various myeloid leukaemia cell lines with DOTAP (Boehringer Mannheim) for 5 days as described (Matsushita et al, 1995).

Assays for cellular proliferation

The cells were incubated for 5 days with and without ribozymes (Rz1, Rz2 and ScRz) in a 96-well plate (Flow Laboratories, Irvine, CA, USA). Twenty microlitres of MTT (5 μg ml−1) were added to each well. The reaction was stopped after 4 h of incubation by adding 100 μl of 0.04 N HCl in propanol and then the OD570 was measured.

Detection of target RNA cleavage by ribozymes

This quantification of cleavage activity by ribozymes was based on the modified method by Leopold et al (1995). Briefly, UF-1 cells (1 × 10^6) which carry t(15;17) were added to various amounts (0–5 × 10^6) of Kasumi-1 cells carrying t(8;21). Two hundred micrograms of ribozymes with 37.5 μg of DOTAP and 2000 U of rRNasin in 5 ml of Opti-MEM I Reduced Serum Medium (GIBCO-BRL) were added and incubated at 37°C for 12 h, and then 5 ml of RPMI 1640 medium with 20% FBS was added and incubated at 37°C for a further 12 h. The cells were then harvested, and the total RNA was isolated with Isogen (Nippongene, Toyama, Japan), and 1 μg of RNA was then applied to the reverse transcription-polymerase chain reaction (RT-PCR). RT was performed with Superscript II (GIBCO-BRL) and random primers; p(dN)6 (Takara Shuzo, Shiga, Japan) at 37°C for 60 min. PCR was performed with Taq Polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The thermal condition of PCR was as follows: precycle at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The primers for the amplification of AML1/MTG8 were 4S [5′-GAC CAT CAC TGT CCT CAC AA-3′, residue 2022 to 2041 in AML1 (Miyoshi et al, 1993)] and 5R [5′-GTC TTC ACA TCC ACA GGT GA-3′, residue 2143 to 2162 in MTG8 (Miyoshi et al, 1993)] which was used in our previous study (Muto et al, 1996). The β-actin gene was amplified with Human β-Actin Control Amplimer Set (Clontech, Palo Alto, CA, USA) as an internal control. The PCR products were electrophoresed in 3% agarose gel and subsequent Southern blot analysis was performed with the ECL 3′-oligolabelling and detection systems (Amersham, UK). The probe sequence for the AML1/MTG8 junction site was 8P (Muto et al, 1996) [5′-CGA GAA CCT CGA AAT CGT ACT GAG T-3′, residue 2098 in AML1 to 2122 in MTG8 (Miyoshi et al, 1993)]. The density of the signals on the autoradiographs were analysed with Digital Densitotol DM-303 (Advantec Toyo, Tokyo, Japan).

Detection of DNA ladder formation in Kasumi-1 cells by ribozymes

After incubation with ribozymes for 7 days, Kasumi-1 cells were washed with PBS twice and then DNA was extracted by incubating with 100 μl of buffer which includes 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA (pH 8.0) and 0.5% Triton X-100 at 4°C for 10 min. After centrifugation, the supernatant was transferred into a new tube and incubated at 37°C for 1 h with 40 μg of RNase A (Sigma), and for a further hour with 40 μg of Proteinase K (Sigma). Ethanol precipitation was performed and resuspended
with TE buffer. Purified DNA was labelled with $^{32}$P-dCTP and labelled DNA was then electrophoresed in 1.8% agarose gel (Rösl, 1992). The gel was then dried on 3MM Whatman paper and thereafter autoradiography was performed.

Assays for cellular differentiation by ribozymes

After incubation for 5 days with ribozymes, the cells were incubated for 30 min with human AB serum, and then were stained with PE-conjugated mouse anti-human CD11b antibodies and FITC-conjugated mouse anti-human CD14 antibodies (Becton Dickinson, San Jose, CA). Control studies were performed with a nonbinding control mouse IgG isotype antibody (Becton Dickinson). A flow cytometric analysis was performed with Ortho Cytoron Absolute (Ortho Diagnostic systems, Tokyo, Japan).

RESULTS

Cell-specific inhibition by ribozymes against AML1/MTG8

To confirm the cell-specific inhibition by ribozymes against AML1/MTG8, we transfected these ribozymes into various myeloid leukaemia cell lines, including Kasumi-1, HL-60, KG-1, U937, NB4 and UF-1 cells. Incubation with myeloid leukaemic ribozymes for 5 days and then cellular proliferation was evaluated by an MTT assay. Both ribozymes only inhibited the proliferation of Kasumi-1 cells, but not the other myeloid leukaemia cell lines. Rz2 was slightly more potent than Rz1 in decreasing the absorbance of the MTT assay (Figure 2). These results suggest that Rz1 and Rz2 specifically inhibit the growth of myeloid leukaemia cells carrying t(8;21)(q22;q22).

Cleavage activity of ribozymes in cells

To address the specific cleavage activity of ribozymes against AML1/MTG8 in vitro, we next transfected Rz2 into Kasumi-1 cells diluted with UF-1 cells. UF-1 cells were used for dilution because they do not have t(8;21)(q22;q22), and were not inhibited by Rz1 and Rz2 while they also demonstrated a long doubling time (72 h) (Leopold et al, 1995) (Figure 2 and data not shown). Rz2 decreased the detectable level of $AML1/MTG8$ mRNA at all corresponding lanes (Figure 3). The signal intensity was decreased to 41.1% by Rz2 in the presence of $5 \times 10^3$ Kasumi-1 cells (Figure 3). These results suggest that the growth inhibition of myeloid leukaemia cells with t(8;21)(q22;q22) by ribozymes are thus thought to be due to the reduction of $AML1/MTG8$ mRNA.

Detection of apoptosis in Kasumi-1 cells by ribozymes

After 7 days of culture in Rz1-, Rz2- and ScRz-transduced Kasumi-1, HL-60, NB4 and U937 cells by lipofection, morphological
changes of apoptosis in only Kasumi-1 cells occurred with chromatin condensation, fragmentation, and the formation of apoptotic bodies (Figure 4). However, neither Rz1 or Rz2 induced apoptosis of myeloid leukaemic cells without t(8;21). In addition, ScRz, an unrelated ribozyme, did not respond to Kasumi-1 cells (Figure 4).

The time course of the appearance of apoptotic cells during Kasumi-1 culture for 7 days was determined (Figure 5). In Rz1- and Rz2-treated Kasumi-1 cells, the percentage of apoptotic cells began to increase after day 3, and became maximal at day 5 (35% with Rz1 and 32% with Rz2). In contrast, apoptosis was not induced by the treatment of ScRz and DOTAP alone (control) in Kasumi-1 cells over a 7-day period (Figure 5). Apoptosis was also confirmed by DNA electrophoresis which showed a pattern of DNA fragments that results from the activation of endogenous endonuclease (Figure 6). Taken together, these results indicate that the ribozymes against AML1/MTG8 chimeric transcript specifically inhibited the proliferation of Kasumi-1 cells through the apoptotic pathway.

**Investigation of differentiation in Kasumi-1 cells by ribozymes**

Induction of differentiation in Kasumi-1 cells into mature granulocytes or monocytes by Rz1 and Rz2 was assumed by the expression of CD11b and CD14 antigens. NB4 and Kasumi-1 cells were treated with all-trans RA (10^{-7} M) for 4 days. Kasumi-1 cells were also incubated with liposome alone and ribozymes against AML1/MTG8 (Rz1 and Rz2) for 5 days. The cells were incubated for 30 min with human AB serum to block Fc receptors and then were stained with direct immunofluorescence using FITC-conjugated mouse antihuman CD14 and PE-conjugated mouse antihuman CD11b antibodies. Control studies were performed with non-binding control mouse IgG_{1} and IgG_{2a} isotype antibodies.
both antigens in Kasumi-1 cells by a flow cytometric analysis. In addition, no morphological changes accompanied with differentiation were observed in the Rz1-treated Kasumi-1 cells (Figure 4).

**DISCUSSION**

AML1/MTG8 is thought to play a key role in leukaemogenesis because the growth of leukaemic cells with t(8;21)(q22;q22) was inhibited by antisense oligonucleotides or ribozymes against AML1/MTG8 fusion transcript (Sakakura et al, 1994; Matsushita et al, 1995; Kozu et al, 1996). The molecular mechanism of leukaemogenesis by AML1/MTG8 has been studied, and AML1/MTG8 has been shown to block AML1b transcription as the dominant negative protein which is mediated by the runt homology domain (Meyers et al, 1993, 1995). More recently, it has been reported that AML1 is essential for definitive haematopoiesis of all lineages by using AML1 knockout mice (Okuda et al, 1995; Wang et al, 1996) and AML1/MTG8 knock-in analyses (Yergeau et al, 1997; Okuda et al, 1998). On the other hand, few studies about the biological effect of AML1/MTG8 have been reported which suggest that Kasumi-1 cells were induced to differentiate to monocytic lineage by antisense oligonucleotide complementary to the AML1/MTG8 transcript, however, only 12% of the cells treated with 10 mm antisense oligonucleotide were positive for NSE staining (Sakakura et al, 1994).

We constructed two hammerhead ribozymes targeted against AML1/MTG8, and proved that these ribozymes cleaved the target substrate specifically in a cell-free system and also inhibited the growth of leukaemic cells with t(8;21)(q22;q22) (Matsushita et al, 1995). Another group has also reported similar results (Kozu et al, 1996), however, the mechanisms of the ribozymes against AML1/MTG8 on the growth inhibition of myeloid leukaemic cells have yet to be elucidated. The ribozyme-induced apoptotic cells were identified based on morphology. The percentage of apoptotic cells was increased in a time-dependent manner by ribozymes targeted against AML1/MTG8. In contrast, apoptosis was not induced by the treatment of unrelated ribozyme. Therefore, the ribozyme-induced growth inhibition of Kasumi-1 cells was associated with the induction of apoptosis. We could also detect DNA ladder formation in ribozyme-treated Kasumi-1 cells. These findings suggest that the decrease in the expression of AML1/MTG8 by ribozymes may thus induce leukaemic cells to apoptosis, and this is the first report which suggests that blocking the expression of AML1/MTG8 by ribozymes induces apoptosis of leukaemic cells. A previous report showed that AML1/MTG8 fusion protein activates the transcription of Bcl-2 (Klamper et al, 1996). We therefore examined the expression of Bcl-2, Bcl-Xs/L and Bax in ribozyme-treated Kasumi-1 cells by a Western blot analysis, however, the expression of these proteins was equal to those in the nontreated cells (data not shown). Further studies are thus needed to clarify the molecular mechanisms of apoptosis in ribozyme-treated leukaemic cells.

A previous study reported that the antisense oligonucleotide complementary to AML1/MTG8 inhibited the growth and induced differentiation of the cell lines derived from AML containing t(8;21) (Sakakura et al, 1994). However, we could not observe any apparent morphological differentiation changes in the ribozyme-treated Kasumi-1 cells, or any apparent increase in the granulocytic lineage marker CD11b and monocytic lineage marker CD14 in these cells by a flow cytometric analysis. These data suggest that the induction of differentiation did not occur in the ribozyme-mediated growth inhibition of Kasumi-1 cells.

The transduction efficiency was evaluated by methods based on those of Leopold et al (1995) and the ribozyme decreased the level of target RNA with liposome by one-log in their study. The reduction of target RNA in our case was thus the same level as that reported in their study (Figure 3). It is possible that a more effective induction of apoptosis might also occur by improving the transduction method.

In summary, we observed both specific growth inhibition and the induction of apoptosis by inhibiting the expression of chimeric RNA by ribozymes in Kasumi-1 cells. Therefore, chimeric RNA may be useful as therapeutic targets in patients with AML. Ribozymes are thus expected to be potentially useful as specific gene modifiers for various malignancies including leukaemia (Lange et al, 1993; Shore et al, 1993; Snyder et al, 1993; Pace et al, 1994; Pachuk et al, 1994). We thus conclude that ribozymes may be useful as a new therapy based on the molecular pathogenesis in the treatment of AML with t(8;21).

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