Bull Yamaguchi Med Sch 50(1-4):1-5, 2003

The novel ribozyme, maxyzime, that inhibits leukemia progression in disease mouse

Tsuyoshi Tanabe

Age Dimension Research Center, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6-13th site, 1-1-1, Higashi, Tsukuba, Ibaraki, 305-8566, Japan

Key words : ribozyme; maxizyme; bcr-abl; leukemia

Abstract We have constructed an allosterically controllable novel enzyme (designated maxizyme) that can be transcribed in vivo under the control of a human tRNAVal promoter. The maxizyme has sensor arms that can recognize target sequences, and in the presence of such a target sequence only, it can form a cavity that can capture catalytically indispensable Mg2+ ions. As a target for a demonstration of the potential utility of the maxizyme, we chose *BCR-ABL* mRNA, the translated products of which cause chronic myelogenous leukemia. Only the maxizyme (but not conventional ribozymes) had extremely high specificity and high-level activity, not only in vitro but also in cultured cells including BV173 cells derived from a patient with a Philadelphia chromosome. The maxizyme induced apoptosis only in leukemic cells with this chromosome. We have also found that this ribozyme completely inhibits tumor-cell infiltration in mice disease model.

Introduction

The chimeric *BCR-ABL* gene generates two different *BCR-ABL* mRNAs, known as K28 b3a2 and L6 b2a2, both of which yield p210 *BCR-ABL* on translation, an oncoprotein[1] with tyrosine kinase activity[2] that causes malignant transformation of white blood cells in CML.

Although ribozyme-based gene therapy is arousing great interest, its application to disrupt chimeric mRNA has been restricted by the lack of a specific cleavable site at the junction of the chimeric gene. To disrupt *BCR-ABL* mRNA, a ribozyme must exclusively target the junction sequence, otherwise normal *ABL* mRNA that shares part of the chimeric sequence will also be cleaved by the ribozyme, with resultant damage to host cells3.

We have generated a novel ribozyme ('maxizyme') that is dimeric and specifically cleaves *BCR-ABL* mRNA, inducing apoptosis in CML cells3. The maxizyme has sensor arms that recognize two target sequences that induce it to form a cavity for capturing Mg2+ ions essential for catalysis. The activity of the heterodimeric ribozyme can be allosterically controlled through one of the two substrate-binding regions. The maxizyme remains in an inactive conformation in the presence of normal *ABL* mRNA or in the absence of the junction sequence.

We find that this ribozyme completely inhibits tumor-cell infiltration in mice disease model. To our knowledge, this is the first application of an artificial, allosterically controllable enzyme in animals, opening up the possibility of using ribozyme technology in the treatment of CML.

Results and discussion

Specific Design of a Novel Maxizyme under the Control of a Human tRNA Val Promoter

For potential application of a maxizyme to gene therapy for the treatment of CML, it is important that the maxizyme be expressed constitutively and under the control of a strong promoter in vivo. We embedded each monomeric unit downstream of the sequence of a human tRNAVal promoter[3] that is recognized by RNA polymerase III[4], to generate MzL (maxizyme l eft) and MzR (maxizyme right; Figure 1B). High-level expression under the control of the pol III promoter would clearly be advantageous if maxizymes are to be used as therapeutic agents, and such expression would also increase the likelihood of dimerization. The specific design of the tRNAVal constructs was based on our previous success in attaching a ribozyme sequence to the 3Åå-modified side of the tRNAVal portion of the human gene (S. Koseki and K. T., unpublished data). This strategy yielded ribozymes that were very active in cultured cells[3].

In order to achieve high substrate specificity, our maxizyme should adopt an active conformation only in the presence of the abnormal *BCR-ABL* junction (Figure 1 B, top panel), while the conformation should remain inactive in the presence of the normal *ABL* mRNA and in the absence of the abnormal *BCR-ABL* junction (Figure 1B, bottom panel). The specifically designed sequences, which are shown in Figure 1B (note that the lengths and sequences of sensor arms and those of common stem II are the variables), should permit such conformational changes depending on the presence or absence of the abnormal b2a2 mRNA. This phenomenon would resemble the changes in conformation of allosteric proteinaceous enzymes in response to their effector molecules. In order to compare the activity and specificity of our maxizyme with that of a conventional wild-type ribozyme (wtRz) targeted to the same cleavage site, and with those conventional antisense-type ribozymes (asRz52 and asRz81[5]), we embedded the latter two types of ribozyme similarly in the 3Aa portion of the gene for tRNAVal.

In vivo Efficacy of Novel Ribozyme.

To test this technology *in vivo*, we embedded genes encoding the maxizyme downstream of genes for the human transfer RNA tRNAVal[3)6)7]. We used a retroviral system for the expression of the maxizyme in leukaemic cells[8], which involved subcloning two tRNAVal-driven expression cassettes, corresponding to each component of the heterodimer, in tandem into the retroviral vector. A line of CML cells (BV173) was transduced either with a control vector in which the maxizyme sequence had been deleted, or with the maxizyme-encoding vector. We then injected 2106 transduced BV173 cells into the tail veins of individual NOD-SCID mice. (These mice, produced by crossing SCID and non-obesity diabetic mice, are a powerful tool for growing human hematopoietic cells *in vivo*.) Survival of animals was monitored daily for 100 days after inoculation. Eight weeks after inoculation, eight animals from each group were killed by cervical dislocation and their tissues examined.

The pathological changes found at necropsy were remarkably consistent in mice that had received control-transduced BV173 cells (Fig. 2A). The spleen was red, firm and enlarged, weighing two to three times more than the spleen from mice injected with maxizyme-transduced BV173 cells. Histological examination revealed leukaemic nodules, and the liver and kidney were enlarged and firm, with evidence of extensive metastasis of BV173 cells, which was particularly marked in the brain. By contrast, the spleens, livers, kidneys and brains of mice treated with maxizyme-transduced BV173 cells appeared normal (Fig. 2A).

The differences between the two groups of mice were reflected in their mortality rates (Fig. 2B). All of the mice injected with control BV173 cells died of diffuse leukaemia, confirmed at necropsy, 6-13 weeks afterwards (median survival time, 9 weeks), whereas mice injected with maxizyme-transduced BV173 cells remained healthy (Fig. 2B).

Our results indicate that each subunit of the maxizyme introduced by the retroviral

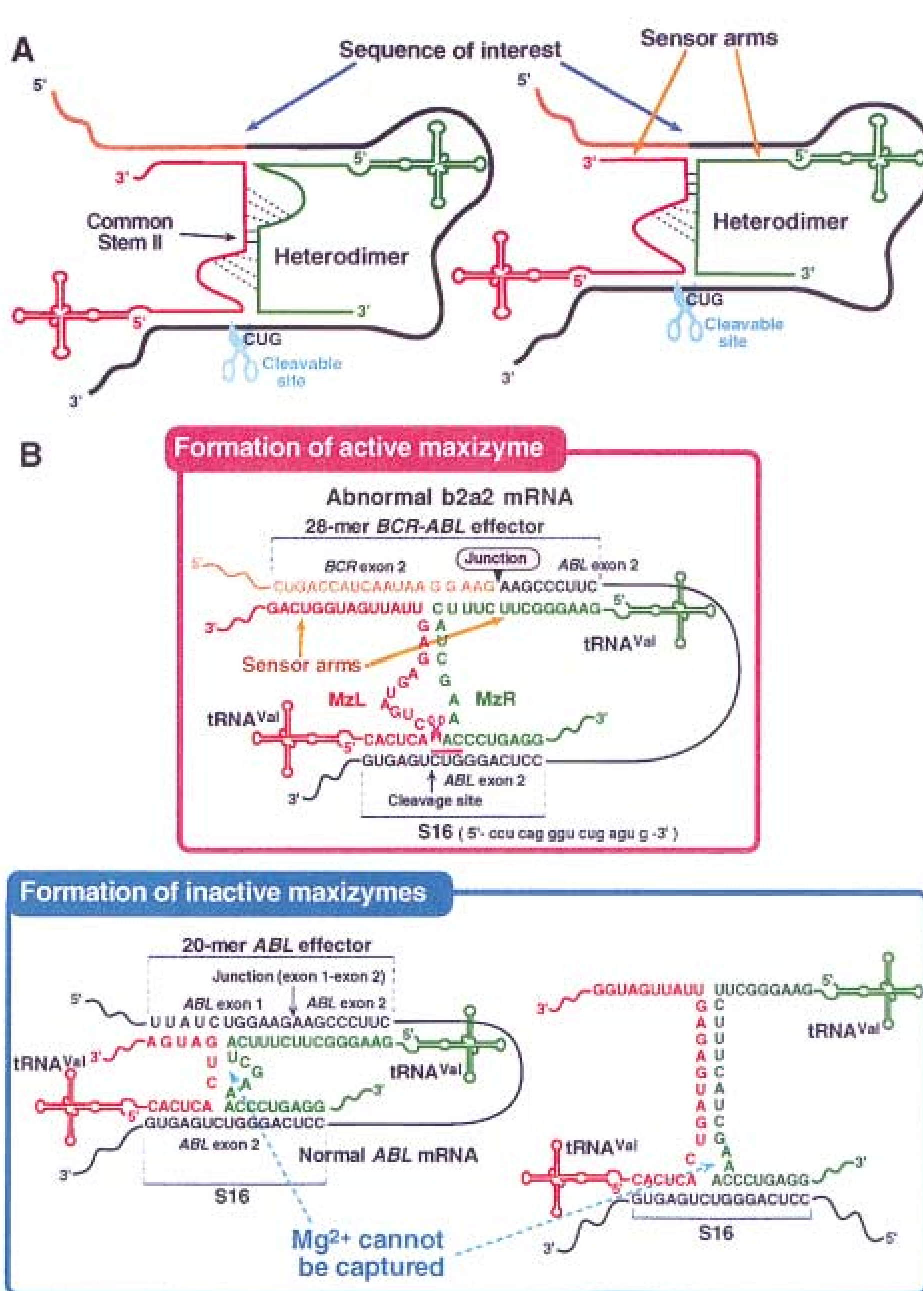

Figure 1. Design of the Novel Maxizyme (reprinted from ref. 3)
(A) Schematic representation for the specific cleavage of chimeric mRNA by the tRNAVal-driven maxizyme. The heterodimer (MzL [maxizyme left; red] and MzR [maxizyme right; green]) can generate two different binding sites: one is complementary to the sequence of a substrate, and the other is complementary to a second substrate (left structure). One of the catalytic cores of the heterodimer can be deleted completely to yield the even smaller maxizyme (right structure) in that the substrate recognition sequences recognize the abnormal chimeric junction, acting as sensor arms.
(B) Formation of an active or inactive maxizyme. In order to achieve high substrate specificity, the tRNAVal-driven maxizyme should be in an active conformation only in the presence of the abnormal *BCR-ABL* junction (upper panel), while the conformation should remain inactive in the presence of normal *ABL* mRNA or in the absence of the *BCR-ABL* junction (lower panel).

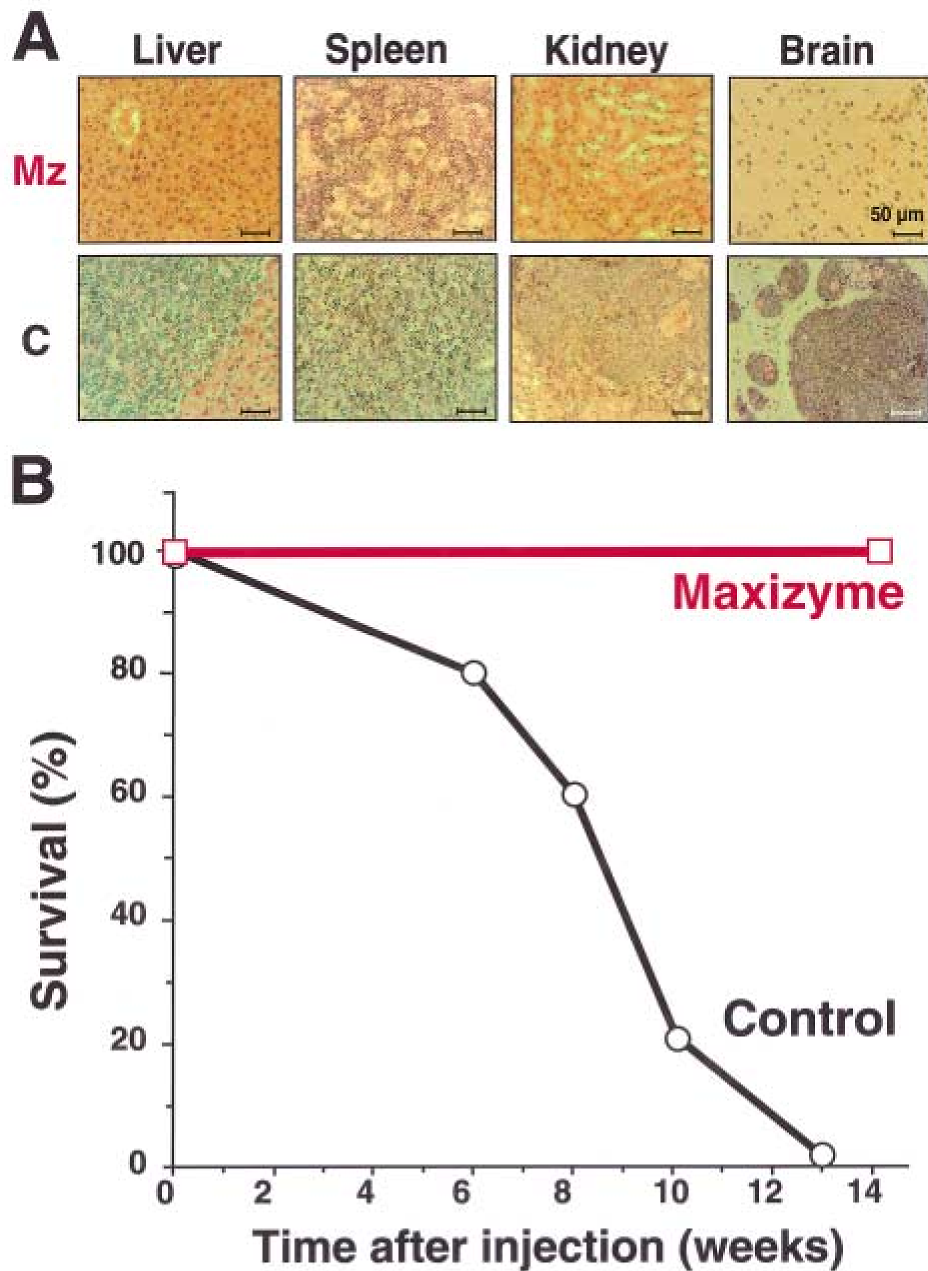

Figure 2. The antitumor effects of the maxizyme in a murine model of chronic myelogenous leukemia. (reprinted from ref. 9)
(A) Infiltration by BV173 leukemic cells, as revealed by hematoxylin-and-eosin staining of livers, spleens, kidneys and brains of NOD-SCID mice injected with BV173 cells that had been transduced either with a control vector or with the maxizyme-expressing vector. In most cells stained with hematoxylin and eosin, the nucleus is blue and the cytoplasm pink.
(B) Reduction of tumorigenicity of BV173 cells *in vivo*. A total of 2 106 BV173 cells transduced with either control vector or maxizyme were selected by incubation with puromycin and injected into the tail vein of NOD-SCID mice (two independent experiments, with 4-8 animals per group in each experiment). The survival of animals was monitored daily for more than 20 weeks after inoculation: all control mice died within 13 weeks, whereas maxizyme-treated mice remained disease-free for the entire period of the investigation. Scale bars, 50 mm.

vector is produced at the appropriate concentration to support dimerization *in vivo*. Also, the maxizyme apparently functions successfully in animals, cleaving *BCR-ABL* mRNA with exceptional efficiency.

At present, allogeneic transplantation is the only effective therapy for this type of leukaemia[10),11)], with only half of all patients on average being eligible for this treatment because of limited donor availability and age restrictions. Our results raise the possibility that this maxizyme could be useful for purging bone marrow in cases of CML treated by autologous transplantation, when it would presumably reduce the incidence of relapse by decreasing the tumorigenicity of contaminating CML cells in the transplant[12)].

We have also constructed five other maxizymes that successfully target other chimeric genes[13)], suggesting that maxizymes could be a new class of powerful gene-inactivating agents that can cleave any type of chimeric mRNA.

Acknowledement

The author would like to express to Professor Kazunari Taira and Professor Shigetaka Asano my deepest gratitude for advising on my study.

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