Functional analysis of the pro-apoptotic factor Bax using hammerhead ribozymes

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ABSTRACT
A pro-apoptotic protein Bax is a Bcl-2 family member and forms homodimers and also heterodimerizes with death antagonists, Bcl-2 and Bcl-XL. To elucidate the detail of function of Bax in cells, we constructed a hammerhead ribozyme targeted to the Bax mRNA. The level of Bax protein in Hela-K cells expressing Bax-ribozyme was decreased compared with that of wild type Hela-K cells. Therefore, the Bax-ribozyme should be useful for the future investigations of the details of apoptosis pathway.

INTRODUCTION
Apoptosis is the process of programmed cell death in vertebrates that plays a central role in development and homeostasis (1,2). Transmembrane receptor molecules including TNF, Fas and TRAIL are known to activate apoptotic signaling pathways following ligand binding. TNF, Fas and TRAIL are known to induce the release of cytochrome c from the mitochondria during apoptosis. The release of cytochrome c from the mitochondria during apoptosis was regulated by a Bcl-2 family. The Bcl-2 family displays either positive or negative regulatory on apoptosis. For example, Bcl-2 and Bcl-XL function as an anti-apoptotic gene during many apoptosis pathways.

In contrast, Bax acts as a death agonist within a common apoptotic pathway (3). Then, Bax suppresses the ability of Bcl-2 to block apoptosis. Moreover, Bax-deficient mice display lymphoid hyperplasia consistent with a role for Bax in the promotion of apoptosis (4). However, it was reported that Bax-induced cell death did not apparently require ICE-like proteases (5). Since activation of ICE-like proteases is an essential step in apoptosis signaling, unknown function of the Bax may exist.

In this report, to elucidate function of the pro-apoptotic factor Bax, we tried knock-down of the Bax using hammerhead ribozymes.

RESULTS AND DISCUSSION
To elucidate details of function of Bax, we constructed a hammerhead ribozyme targeted to the Bax mRNA (Bax-ribozyme). Hammerhead ribozymes are smallest catalytic RNAs and can cleave any RNAs which have NUX triplet, where N is A, U, G, or C; and X is A, U, or C. Therefore, the ribozymes have great potential to be used as effective therapeutic agents and powerful tools of functional analysis of unknown gene products in vivo (6-8).

In order to construct the Bax-ribozyme, we
chose a human tRNA Val promoter as expression system of the ribozyme (6). This system is suitable for the expression of short RNAs and the level of expression is at least from 10 to 100-fold higher than that expressed by the pol II dependent expression system. Then, Hela cells expressing Fas gene (Hela-K cells) were transfected with ribozyme expression plasmids. Ribozyme-transfected Hela-K cells were selected by incubation with G418 for 3 weeks. The expression of the ribozyme was confirmed using the RT-PCR analysis.

Next, we checked the level of Bax mRNA in cells expressing Bax-ribozymes using the RT-PCR analysis. As a result, the level of Bax mRNA was reduced drastically in Hela cells that expressed the Bax-ribozyme compared with that of untransfected (WT) Hela-K cells. We next examined the level of Bax protein in Hela-K cells expressing Bax-ribozyme using the Western blot analysis. In Hela cells expressing Bax-ribozymes, the level of Bax protein was significantly reduced compared with that of WT Hela-K cells. The level of CBP protein as a control did not change in all kinds of cells. Moreover, in order to demonstrate that the observed inhibitory effects were actually due to a ribozyme-mediated cleavage, we used an inactive Bax-ribozyme ("Inactive Bax-ribozyme ", with a single G-to-A mutation at the catalytically important conserved nucleotide). As a result, an inactive Bax-ribozyme did not affect the level of Bax mRNA.

Since the level of Bax protein was decreased in cells expressing the Bax, we examined whether the change of phenotype appears in cells expressing the Bax. Then we checked viability of cells expressing Bax-ribozyme during the Fas-induced apoptosis. We counted viable cells at 24 hours after a treatment with α-Fas antibody. As results, wild type cells induced apoptosis after the treatment with α-Fas antibody (100%). In contrast, the percentage of apoptosis in cells expressing the Bax-ribozyme was decreased compared with that of WT Hela-K cells (40.3%). Similar results were obtained by in situ staining using a 4',6-Diamidino-2-phenylindole dihydrochloride n-hydrade (DAPI). Therefore, the Bax-ribozyme with high level activity should be useful for the future investigations of the details of Fas-induced apoptosis pathway.

ACKNOWLEDGMENT
We thank Dr. Yonehara for gift of the Fas expression plasmid.

REFERENCES