(DMVA)-induced rat leukemia (Osaka *et al*., 1997), and (e) Lck promoter-driven 17AA(+)KTS(−)WT1 isoform-transgenic mice showed block of differentiation in T lymphoid progenitor cells (Li *et al*., 2003).

The *WT1* gene is alternatively spliced at two sites: 17AA site, which consists of exon 5 of the *WT1* gene, and KTS site, which exists between zinc-fingers 3 and 4, and yields four isoforms: (17AA(+)KTS(+), 17AA(+)KTS(−), 17AA(−)KTS(+), and 17AA(−)KTS(−)), each of which is considered to have different functions. All of the four isoforms were expressed in primary human solid cancers, including lung cancer (Oji *et al*., 2002), HNSCC (Oji *et al*., 2003a), and sarcoma (Ueda *et al*., 2003) and human primary leukemia (Siehl *et al*., 2000). Among the four WT1 isoforms, WT1 17AA(+)KTS(+) isoform was dominantly expressed in all of the cancers examined. The results showing that constitutive expression of WT1 17AA(+)KTS(+) isoform rescued the growth inhibitory effect of WT1 antisense oligomers on cancer cells (Oji *et al*., 1999) indicated the contribution of WT1 17AA(+)KTS(+) isoform to the growth of cancer cells (Hubinger *et al*., 2001). As for the functions of WT1 17AA(+)KTS(−) isoform, the results showing that lck promoter-driven 17AA(+)KTS(−)WT1-transgenic mice blocked differentiation of T lymphoid progenitor cells, indicated the involvement of WT1 17AA(+)KTS(−) isoform in the tumorigenesis of lymphoid malignancy (Li *et al*., 2003). As for 17AA(−)KTS(−) isoform, the results showing that 17AA(−)KTS(−)WT1 isoform induced G1 arrest in osteosarcoma cell lines (Englert *et al*., 1997) and inhibited G1/S progression and accelerated differentiation in 32D cl3 murine myeloid progenitor in response to G-CSF (Loeb *et al*., 2003) suggested tumor-suppressor roles in some tumors. As for 17AA(−)KTS(+)WT1 isoform, its functions remain unclear. These results indicate that each of the WT1 isoforms has different functions and play important roles in leukemogenesis and tumorigenesis.

Cancers are characterized by abnormal control of proliferation. The *WT1* gene is involved in the promotion of cell cycle, as it was indicated by findings that suppression of WT1 expression induced G2/M or G1 block in human leukemia K562 cells and HER2/neu-overexpressing breast cancer cells, respectively (Yamagami *et al*., 1998; Tuna *et al*., 2005). Moreover, 17AA(+)KTS(−)WT1 isoform increased the expression levels of antiapoptotic gene Bcl-2 in G401 rhabdoid cells (Mayo *et al*., 1999), indicating that the *WT1* gene might be involved in suppression of apoptosis. However, there is neither a direct evidence of antiapoptotic function of the *WT1* gene nor analysis of precise mechanism of the function.

In the present study, we demonstrate that siRNA specific for 17AA(+)WT1 isoforms induces apoptosis through activation of the intrinsic apoptosis pathway in WT1-expressing leukemia cells and that this apoptosis was associated with activation of proapoptotic Bax. Furthermore, stable expression of 17AA(+)WT1 isoforms (17AA(+)KTS(+) and 17AA(+)KTS(−)) inhibited apoptosis induced by apoptosis-inducing agents, etoposide and doxorubicin, through inhibition of mitochondrial damages in leukemia cells.

Results

17AA(+)WT1-specific siRNA induces apoptosis in WT1-expressing leukemia cells

To examine the roles of 17AA(+)WT1 or 17AA(−)WT1 isoforms in protection of leukemia cells from apoptosis, 17AA(+)WT1- and 17AA(−)WT1-specific siRNA vectors were constructed. RT–PCR and Western blot analysis showed that transient expression of 17AA(+)WT1- and 17AA(−)WT1-specific siRNA vectors specifically suppressed expression of 17AA(+)WT1 and 17AA(−)WT1 isoforms, respectively, in K562 cells (Figure 1A). Three WT1-expressing leukemia cell lines (K562, HL-60, and Kasumi1) and one WT1-non-expressing lymphoma cell line (Daudi) were transfected with 17AA(+)WT1-specific siRNA, 17AA(−)WT1-specific siRNA, or control mock vector and incubated for 16 h. Annexin V-propidium iodide (PI) two-color flow cytometry showed that transient expression of 17AA(+)WT1-specific siRNA induced apoptosis in all the three WT1-expressing leukemia cell lines, but not in WT1-non-expressing lymphoma cell line (Figure 1B). On the other hand, transient expression of 17AA(−)WT1-specific siRNA did not induce apoptosis in any of the three WT1-expressing leukemia cell lines and in one WT1-non-expressing lymphoma cell line. Furthermore, the cells treated with the 17AA(+)WT1-specific siRNA were analysed for mitochondrial cytochrome *c* release and loss of mitochondrial membrane potential (MMP) by Western blot and flow cytometry, respectively. Transient expression of 17AA(+)WT1-specific siRNA induced mitochondrial cytochrome *c* release and loss of MMP in WT1-expressing K562 cells, but not in WT1-non-expressing Daudi cells (Figure 1C and D). Furthermore, 17AA(+)WT1-specific siRNA vector-transfected cells were labeled by expression of GFP protein using Gene Silencer pGSU6 shRNA Vector Kit system. As shown in Figure 1E, apoptosis was induced only in the 17AA(+)WT1-specific siRNA vector-transfected cells, but not in the cells that were not transfected with 17AA(+)WT1-specific siRNA vector. These results indicated the antiapoptotic roles of 17AA(+)WT1 isoforms in WT1-expressing leukemia cells.

Transfection of 17AA(+)WT1-specific siRNA induce apoptosis through activation of intrinsic but not extrinsic apoptosis pathway

17AA(+)WT1-specific siRNA vector was transiently expressed in K562 cells for 16 h in the presence or absence of caspase inhibitors. Annexin V-PI two-color flow cytometry showed that pan-caspase inhibitor zVAD-fmk inhibited the apoptosis, indicating that 17AA(+)WT1-specific siRNA induced apoptosis in a caspase-dependent manner (Figure 2A). Block of apoptogenic signals by a caspase-3 inhibitor Ac-DEVD-CHO, at a point where the intrinsic apoptosis pathway and the

extrinsic one meet, and of apoptogenic signals by caspase-9 inhibitor Ac-LEHD-CHO, at a point downstream of the mitochondria in the intrinsic pathway, inhibited apoptosis induced by 17AA(+)WT1-specific siRNA. On the other hand, block of apoptogenic signals by caspase-8 inhibitor Ac-IETD-CHO at a point in the extrinsic pathway did not inhibit the apoptosis.

To examine the activation of apoptosis pathway by 17AA(+)WT1-siRNA, activities of caspase-3, -8, and -9 were examined by fluorometric assay at various time points after transfection of 17AA(+)WT1-specific siRNA. Activities of caspase-3 and -9 were significantly increased in 17AA(+)WT1-specific siRNA-transfected K562 cells compared to mock vector-transfected K562 cells. However, activity of caspase-8 was not increased compared to mock vector-transfected K562 cells (Figure 2B). Moreover, to examine whether the extrinsic pathway was involved in the mitochondrial damages induced by 17AA(+)WT1-specific siRNA, mitochondrial cytochrome *c* release was analysed in the presence or absence of caspase inhibitors 16 h after the transfection of 17AA(+)WT1-specific siRNA (Figure 2C). Western blot analysis showed that block of apoptogenic signals by caspase-8 inhibitor, Ac-IETD-CHO, at a branching point from the extrinsic apoptosis pathway to the intrinsic one did not inhibit mitochondrial cytochrome *c* release, confirming that the extrinsic apoptosis pathway was not involved in mitochondrial damages induced by 17AA(+)WT1-specific siRNA.

These results indicated that 17AA(+)WT1-specific siRNA induced apoptosis through activation of the intrinsic apoptosis pathway but not through activation of the extrinsic pathway in leukemia cells, and thus that the 17AA(+)WT1 isoforms played antiapoptotic roles in the intrinsic apoptosis pathway, but not in the extrinsic one.

Transfection of 17AA(+)WT1-specific siRNA activates Bax

Since 17AA(+)WT1 isoforms were shown to play antiapoptotic roles in the intrinsic apoptosis pathway, the effect of suppression of 17AA(+)WT1 isoforms on activation of Bax, which is considered to act as a gateway for various apoptotic signals at the mitochondria, was analysed. The 17AA(+)WT1-specific siRNA vector was transiently expressed in WT1-expressing K562 leukemia cells for 16 h, and oligomerization of Bax was examined by Western blot analysis. As shown in Figure 2D, transfection of 17AA(+)WT1-specific siRNA induced dimerization and oligomerization of Bax in K562 leukemia cells. These results indicated that 17AA(+)WT1 protein acted to inhibit apoptosis at some point upstream of mitochondria in the intrinsic apoptosis pathway.

*Stable expression of 17AA(+)*WT1 *isoforms protects leukemia cells from apoptosis induced by apoptosis-inducing agents through stabilization of mitochondrial membrane potential*

To confirm that 17AA(+)*WT1* isoforms had antiapoptotic functions, K562 cell clones that stably expressed one each of four WT1 isoforms at high levels were isolated (Figure 3a). These cell clones were treated with apoptosis-inducing agents, etoposide or doxorubicin, for 24 h and analysed for apoptosis. Annexin V-PI two-color flow cytometric analysis showed that stable expression of 17AA(+)WT1 isoforms significantly inhibited etoposide-induced apoptosis in K562 cells (Figure 3b). On the other hand, stable expression of 17AA(−)WT1 isoforms (17AA(−)/KTS(+) and 17AA(−)/KTS(−)WT1 isoforms) did not inhibit etoposide-induced apoptosis. Similarly, stable expression of 17AA(+)WT1 isoforms significantly inhibited doxorubicin-induced apoptosis in K562 cells, but that of 17AA(−)WT1 isoforms did not (Figure 3c). These results confirmed the antiapoptotic functions of 17AA(+)WT1 isoforms in leukemia cells.

Since it was well known that apoptosis-inducing agents such as etoposide and doxorubicin initiated cell death primarily by triggering the mitochondrial (intrinsic) apoptosis pathway, we next examined whether or not stable expression of 17AA(+)WT1 isoforms protected mitochondrial membrane damages induced by etoposide or doxorubicin. K562 cell clones transduced with one each of four WT1 isoforms were treated with etoposide or doxorubicin for 24 h and analysed for mitochondrial cytochrome *c* release and loss of MMP by Western blot and flow cytometry, respectively. Stable expression of 17AA(+)WT1 isoforms significantly blocked etoposide-induced release of cytochrome *c* from mitochondrial membrane (Figure 4a) and inhibited loss of MMP in K562 cells (Figure 4b). On the other hand, stable expression of 17AA(−)WT1 isoforms inhibited neither mitochondrial cytochrome *c* release nor the loss of MMP induced by etoposide. Similarly, stable expression of 17AA(+)WT1 isoforms inhibited both doxorubicin-induced release of cytochrome *c* from mitochondrial membrane and loss of MMP, whereas stable expression of 17AA(−)WT1 isoforms did not (Figure 4c and d). These results indicated that stable expression of 17AA(+)WT1 isoforms stabilized mitochondrial membrane and protected leukemia cells from apoptosis induced by apoptosis-inducing agents.

17AA(+)WT1 isoforms require zinc-finger region for the antiapoptotic functions

To examine subcellular localization of four WT1 isoforms, vectors that expressed polyhistidine-tagged one each of four WT1 isoforms were constructed and stably expressed in K562 leukemia cells (Figure 5a and b). As shown in Figure 5c, it was confirmed that stable expression of polyhistidine-tagged 17AA(+)WT1 isoforms significantly inhibited etoposide-induced apoptosis but that of polyhistidine-tagged 17AA(−)WT1 isoforms did not. Then, localization of polyhistidine-tagged WT1 protein was analysed by immunocytochemistry using an anti His-tag antibody. Confocal microscopic analysis showed that all of the four polyhistidine-tagged WT1 isoforms were detected in the nucleus but not in the cytoplasm and mitochondria in K562 leukemia cells (Figure 5d). These results raised

the possibility that nuclear-localized 17AA(+)WT1 isoform proteins transcriptionally regulated some molecule(s) that directly acted on mitochondrial membrane and that let the mitochondria stabilize.

Furthermore, to examine whether or not zinc-finger motif (exons 7–10) of the 17AA(+)WT1 isoforms were required for its antiapoptotic functions, polyhistidine-tagged 17AA(+)WT1 protein lacking zinc-finger motif (WT1ΔZF) was stably expressed in K562 leukemia cells (Figure 5a and b). Immunocytochemical analysis showed that polyhistidine-tagged WT1ΔZF protein was detected in the nucleus as full-length WT1 proteins

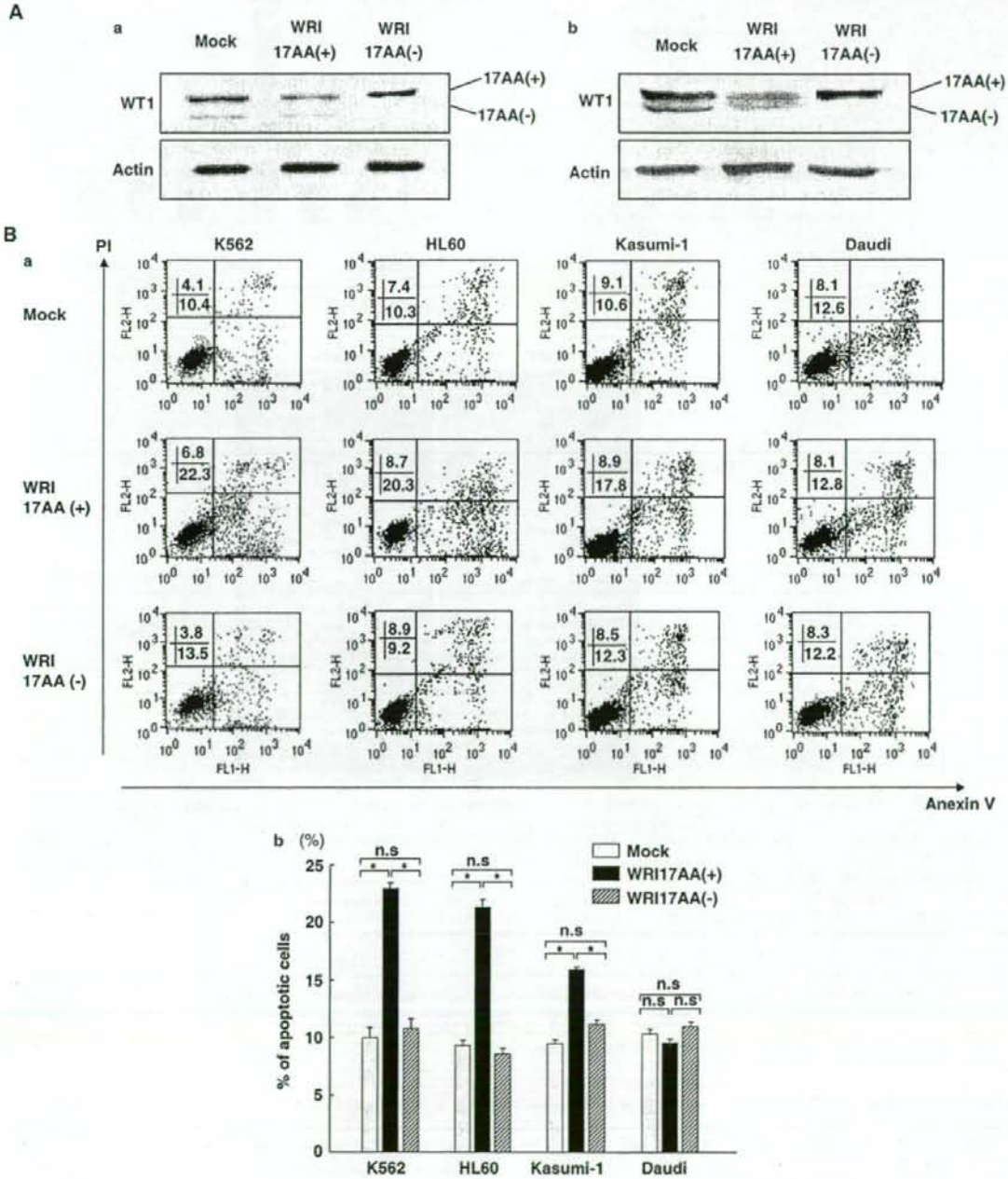

Figure 1 *Continued*

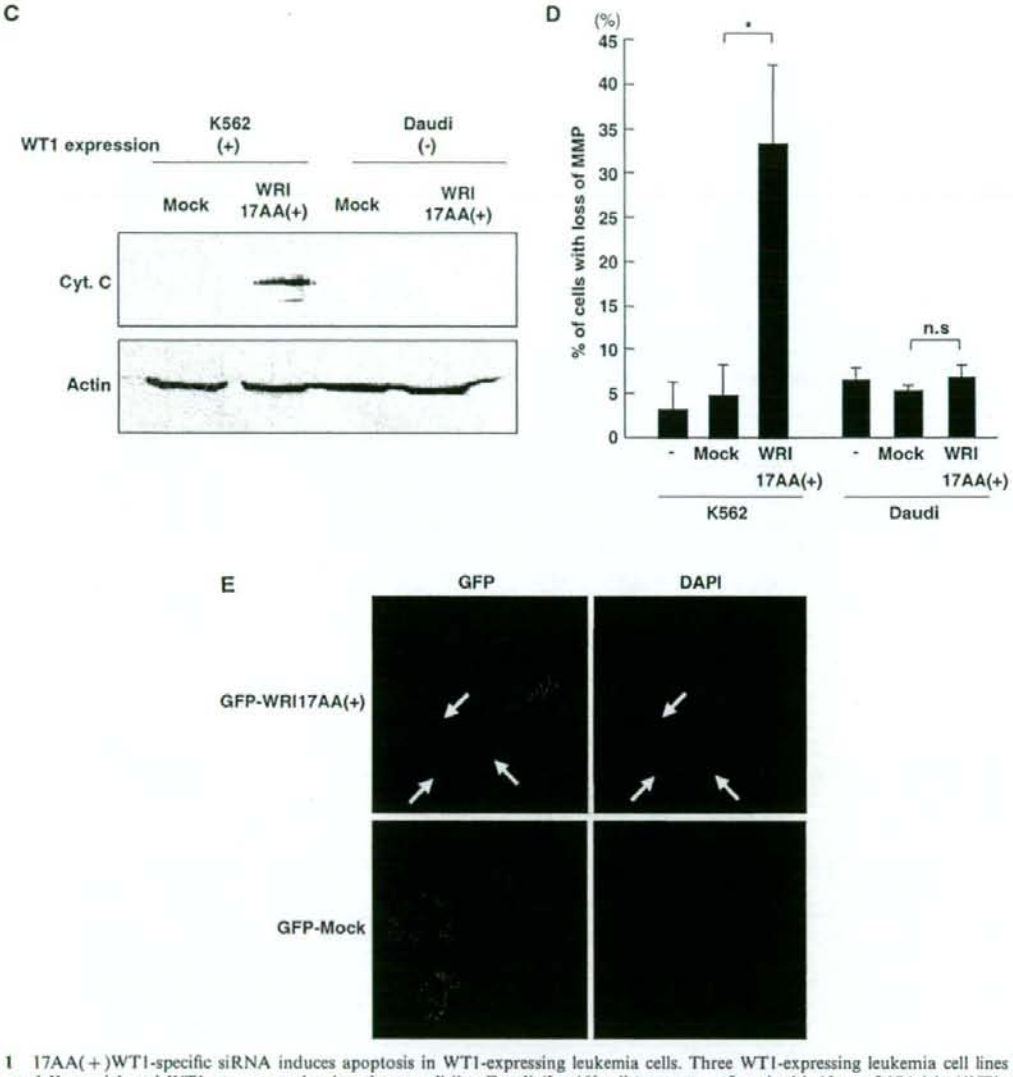

Figure 1 17AA(+)WT1-specific siRNA induces apoptosis in WT1-expressing leukemia cells. Three WT1-expressing leukemia cell lines K562, HL-60, and Kasumi-1 and WT1-non-expressing lymphoma cell line Daudi (2×10^6 cells) were transfected with 10 μg of 17AA(+)WT1-specific siRNA vector (WRI17AA(+)), 17AA(−)WT1-specific siRNA vector (WRI17AA(−)), or mock vector by electroporation and incubated for 16 h. Then, cells were collected for analysis. (**A**) Expression of 17AA(+) and 17AA(−)WT1 mRNA and of 17AA(+) and 17AA(−)WT1 protein in K562 cells transfected with 17AA(+)WT1-specific siRNA, 17AA(−)WT1-specific siRNA, or control vector were examined by RT–PCR using a PCR primer pair that jumped WT1 17AA coding sequences (a) and by Western blot analysis using anti-WT1 Ab (6FH2) (b), respectively. Actin was used as an internal control. (**B**) Apoptosis induced by 17AA(+)WT1- or 17AA(−)WT1-specific siRNA was analysed by Annexin V-PI two-color flow cytometry. WRI17AA(+), 17AA(+)WT1-specific siRNA treated; WRI17AA(−), 17AA(−)WT1-specific siRNA treated; Mock, and empty siRNA vector treated. (a) Representative results are shown in dot-plot. (b) Columns, means of percentages of apoptotic (Annexin V+PI−) cells from three independent experiments; bars, s.e. (**C**) Mitochondrial release of cytochrome *c* in K562 and Daudi cells. Representative results of Western blot analysis are shown. (**D**) The loss of MMP was examined by flow cytometry. Columns, means of percentages of cells with loss of MMP from three independent experiments; bars, s.e. (**E**) Specific induction of apoptosis in 17AA(+)WT1-specific siRNA-introduced WT1-expressing cells. K562 cells were transfected with 10 μg of 17AA(+)WT1-specific siRNA vector or control vector and incubated for 24 h. Representative results of confocal microscopic analysis are shown. Cells transfected with 17AA(+)WT1-specific siRNA vector or control vector were labeled with GFP expression (shown in green). DNA was stained with DAPI (shown in blue). Arrows indicate apoptotic cells.

(Figure 5d). However, stable expression of polyhistidine-tagged WT1ΔZF did not inhibit etoposide-induced apoptosis in K562 cells (Figure 5c). These results indicated that zinc-finger region of 17AA(+)WT1 isoforms was needed to exert its antiapoptotic functions in leukemia cells.

Expression of proapoptotic Bcl-2 family member Bak was decreased by constitutive expression of 17AA(+)KTS(−)WT1 isoform

To examine the mechanisms by which 17AA(+)WT1 isoform exerts its antiapoptotic functions, the protein expression levels of a set of known apoptotic-related genes such as CDK inhibitors p21 and p27, proapoptotic Bcl-2 familiy members Bax and Bak, antiapoptotic Bcl-2 family members Bcl-2 and Bcl-XL, and caspase-9 were analysed by Western blot in K562 leukemia cells transduced with one each of four WT1 isoforms. As shown in Figure 6a and b, the expression levels of proapoptotic Bak protein were significantly decreased in 17AA(+)KTS(−)WT1 isoform-transduced K562 cells compared to other three WT1 isoform-transduced ones. On the other hand, expression levels of Bax, Bcl-2, Bcl-XL, p21, p27, and caspase-9 were not different among the four WT1 isoform-transduced K562 cells. These results showed that constitutive expression of 17AA(+)KTS(−)WT1

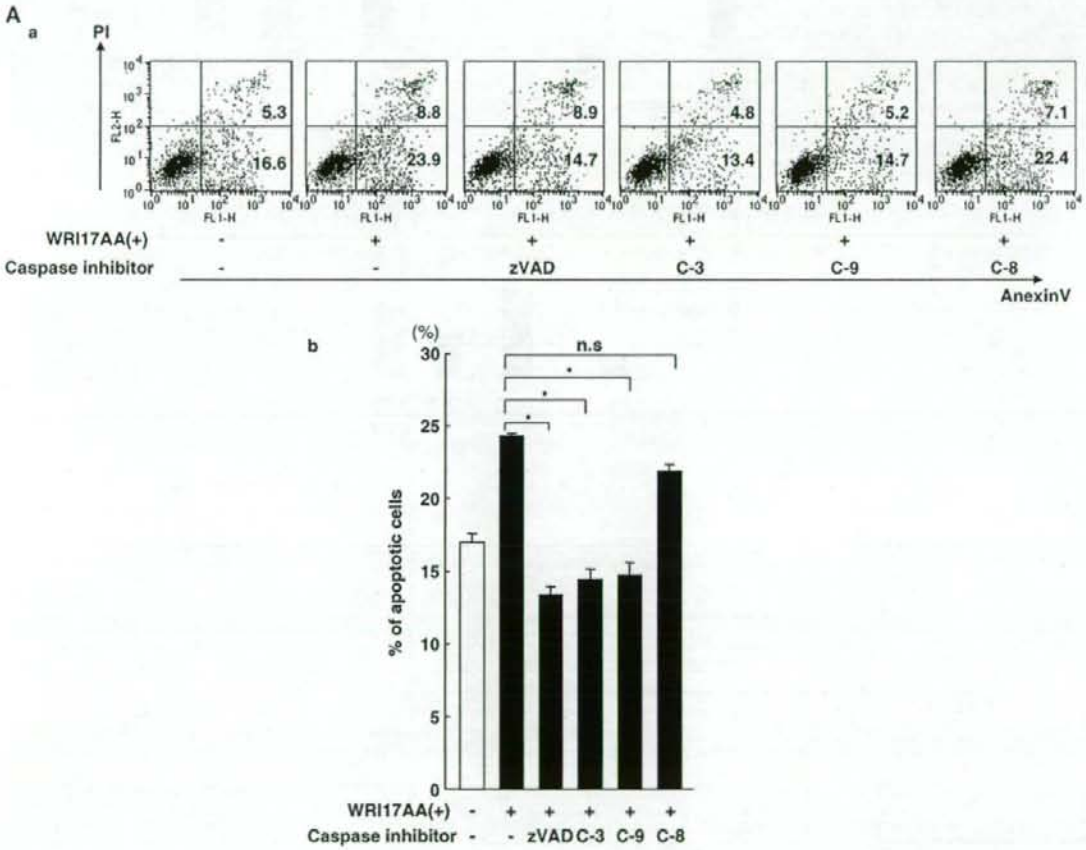

Figure 2 Transfection of 17AA(+)WT1-specific siRNA activates the intrinsic but not extrinsic apoptosis pathway. (**A**) K562 cells were transfected with 17AA(+)WT1-specific siRNA vector (WRI17AA(+)) and incubated for 16 h in the presence or absence of broad caspase inhibitor (zVAD-fmk), capsase-3 inhibitor (Ac-DEVD-CHO), capsase-9 inhibitor (Ac-LEHD-CHO), and capsase-8 inhibitor (Ac-IETD-CHO). Then, cells were analysed for apoptosis by Annexin V-PI two-color flowcytometry. zVAD, pan caspase inhibitor zVAD-fmk; C-3, caspase-3-specific inhibitor Ac-DEVD-CHO; C-9, caspase-9-specific inhibitor Ac-LEHD-CHO; and C-8, caspase-8-specific inhibitor Ac-IETD-CHO. (a) Representative results are shown in dot-plots. (b) Columns, means of percentages of apoptotic (Annexin V + PI−) cells from three independent experiments; bars, s.e. $*P<0.05$. (**B**) Activation of caspases by 17AA(+)WT1-specific siRNA. K562 cells (2×10^6 cells) were transfected with 10 μg of 17AA(+)WT1-specific siRNA vector (WRI17AA(+)), or empty siRNA vector by electroporation, incubated, and collected at the indicated time points. Caspases 3-, 8-, and 9-like activities were measured by fluorometric assay using Ac-DEVD-AFC, FAM-LEHD-FMK, and IETD-pNA, respectively, as substrates. Open column, empty siRNA vector-treated and closed column, 17AA(+)WT1-specific siRNA-treated. zVAD: As a negative control for assays, K562 cells were transfected with WRI17AA(+)-specific siRNA and incubated with broad caspase inhibitor zVAD-fmk (100 μM) for 24 h. ETP: As a positive control for caspases 3- and 9-like activities, K562 cells were treated with etoposide (100 μM) for 24 h. TRAIL: As a positive control for caspase-8-like activity, K562 cells were treated with TRAIL (500 ng/ml) for 5 h. Experiments were independently performed three times. bars, s.e. (**C**) Mitochondrial release of cytochrome *c* by 17AA(+)WT1-specific siRNA. K562 cells were transfected with 17AA(+)WT1-specific siRNA vector (WRI17AA(+)) and incubated for 16 h in the presence or absence of caspase inhibitors. Representative results of Western blot analysis for mithochondrial release of cytochrome *c* are shown. zVAD, pan caspase inhibitor zVAD-fmk; C-3, caspase-3-specific inhibitor Ac-DEVD-CHO; C-9, caspase-9-specific inhibitor Ac-LEHD-CHO; and C-8, caspase-8-specific inhibitor Ac-IETD-CHO. (**D**) Bax activation by the transfection of 17AA(+)WT1-specific siRNA was examined by Western blot analysis. *, monomer; **, dimer; and ***, oligomer of Bax protein.

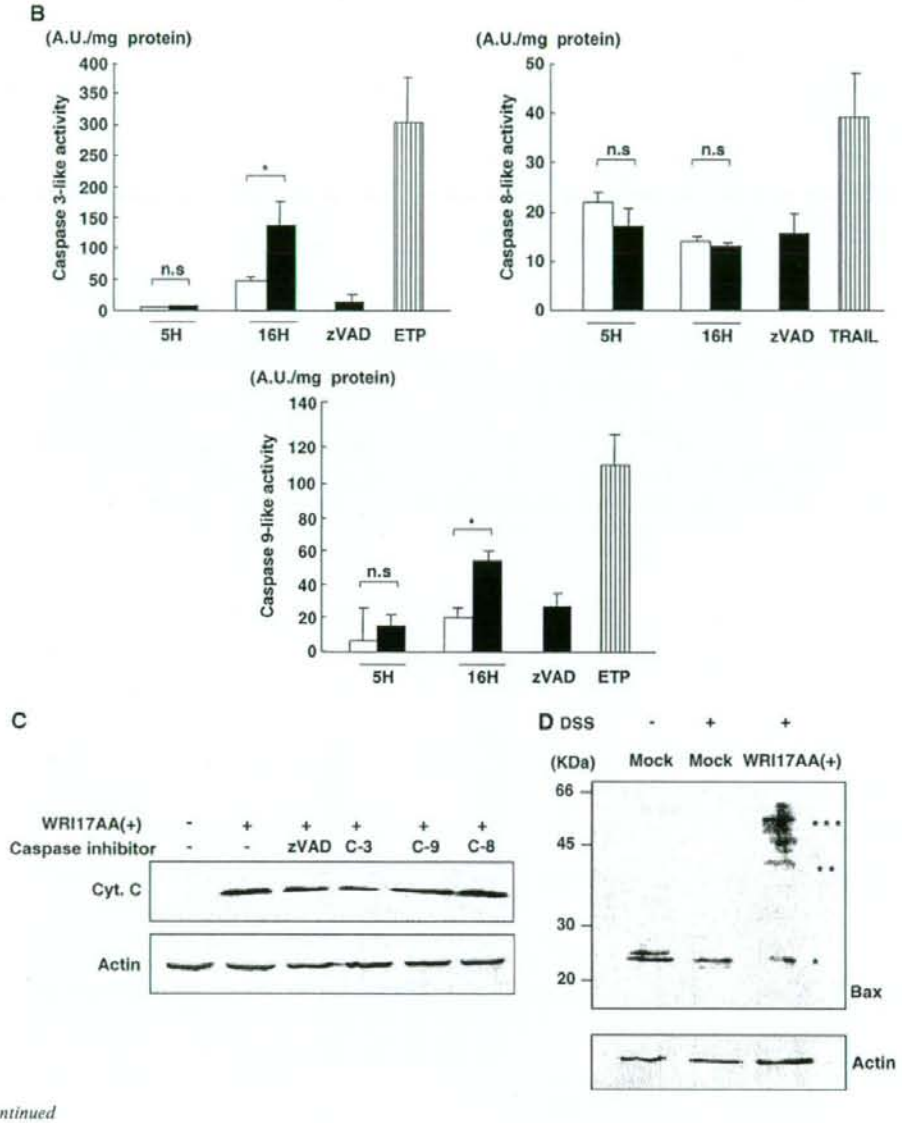

Figure 2 *Continued*

isoform decreased expression levels of proapoptotic Bcl-2 family member Bak.

Discussion

Accumulating findings indicate that the *WT1* gene plays oncogenic roles in tumorigenesis of various kinds of cancers. As for the mechanisms by which the *WT1* gene exerts its oncogenic functions, two possible mechanisms, promotion of cell-cycle progression and suppression of apoptosis, may be raised. We and others have reported that suppression of WT1 expression induced G2/M or G1 block in human leukemia K562 cells and HER2/neu-overexpressing breast cancer cells, respectively (Yamagami *et al.*, 1998; Tuna *et al.*, 2005). These results indicated the involvement of the *WT1* gene in cell-cycle progression in cancer cells. Suppression of apoptosis by the *WT1* gene was indicated by the findings that targeted disruption of the *WT1* gene resulted in enhanced apoptosis and embarrassed normal development of organs, including kidney (Kreidberg *et al.*, 1993; Davies *et al.*, 2004), retina (Wagner *et al.*, 2002), and spleen (Herzer *et al.*, 1999) in mice. However, whether or not the *WT1* gene could suppress apoptosis in leukemia cells

and what were precise mechanisms if it had an antiapoptotic function remained unknown. In the present study, we demonstrated a novel oncogenic function of the *WT1* gene to stabilize MMP and to inhibit apoptosis in human leukemia cells and showed that the antiapoptotic function of the *WT1* gene was exerted by 17AA(+)WT1 isoforms (17AA(+)KTS(+) and 17AA(+)KTS(−)) among the four WT1 isoforms. Thus, the *WT1* gene exerted an oncogenic function via both promotion of cell-cycle progression and suppression of apoptosis.

It is important to determine whether or not 17AA(+)WT1 proteins acted at the mitochondria in leukemic cells to understand the mechanism by which 17AA(+)WT1 isoforms block the apoptotic mitochondrial permeabilization. Polyhistidine-tagged 17AA(+)WT1 proteins localized in the nucleus and inhibited etoposide-induced apoptosis. These results raised the possibility that nuclear-localized 17AA(+)WT1 isoform proteins transcriptionally regulated some molecule(s) that directly acted on mitochondrial membrane and let the mitochondria stabilize. This was supported by the findings that proapoptotic Bcl-2 family member Bax, which was activated at a point upstream of the mitochondria, was activated by suppression of expression of 17AA(+)WT1 isoforms in leukemic cells. Therefore, protein expression levels of a set of known apoptosis-related genes such as CDK inhibitors, proapoptotic Bcl-2 family members, antiapoptotic Bcl-2 family members, and caspase-9 were examined in K562 cells transduced with one each of four WT1 isoforms. Expression of proapoptotic Bcl-2 family member Bak was significantly decreased in 17AA(+)KTS(−)WT1 isoform-transduced K562 cells compared to other three WT1 isoform-transduced and control vector-transduced ones (deleted). Since Bak was considered to act as a gateway for various apoptotic signals at the mitochondria, decreased expression of Bak might be one of the mechanisms by which 17AA(+)KTS(−)WT1 isoform exerted its antiapoptotic functions in leukemia cells. As for antiapoptotic Bcl-2 family genes, it was reported that 17AA(+)KTS(−)WT1 isoform functioned as a transcription factor in G401 rhabdoid cells and increased the expression levels of antiapoptotic gene Bcl-2 (Mayo *et al*., 1999). However, both expression levels of Bcl-2 and Bcl-XL in 17AA(+)KTS(−)WT1 isoform-transduced K562 cells were not different from those in control vector-transduced ones.

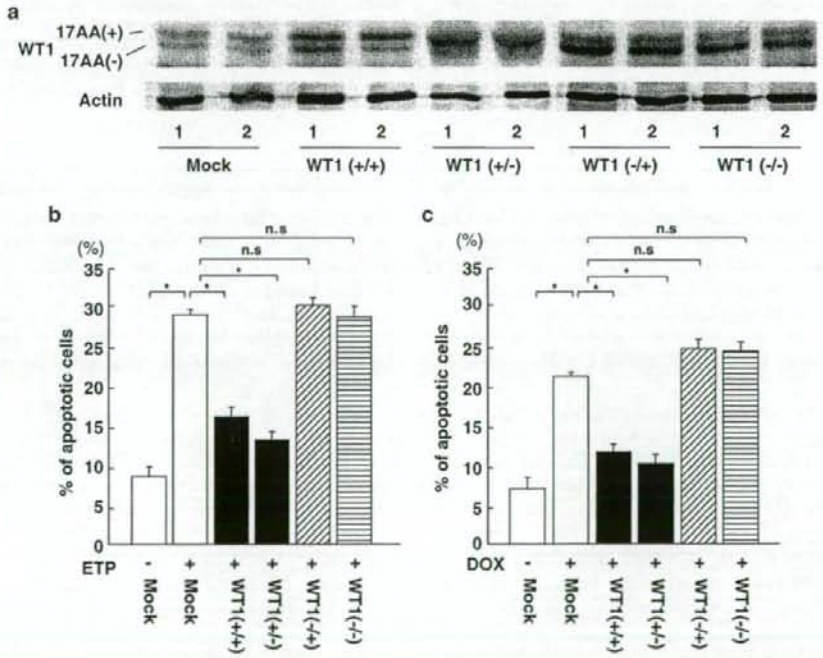

Figure 3 Stable expression of 17AA(+)WT1 isoforms protects leukemia cells from apoptosis. K562 cell clones that stably expressed one each of four WT1 isoforms at high levels were isolated. The cell clones were treated with etoposide (100 μM) or doxorubicin (100nM) for 24 h. Then, cells were analysed for apoptosis by Annexin V-PI two-color flow cytometric analysis. (**a**) Expression of 17AA(+) and 17AA(−)WT1 proteins in K562 cell clones established. Representative results of Western blot analysis are shown. (**b**, **c**) Apoptosis induced by etoposide (**b**) or doxorubicin (**c**) was analysed by Annexin V-PI two-color flow cytometry. Columns, means of percentages of apoptotic cells in three different K562 cell clones that stably expressed the transduced WT1 isoforms; bars, s.e. Experiments were independently performed three times for each cell clones. (**a**–**c**) WT1(+/+), WT1 17AA(+)KTS(+) isoform; WT1(+/−), WT1 17AA(+)KTS(−) isoform; WT1(−/+), WT1 17AA(−)KTS(+) isoform; and WT1(−/−), WT1 17AA(−)KTS(−) isoform. *$P<0.05$.

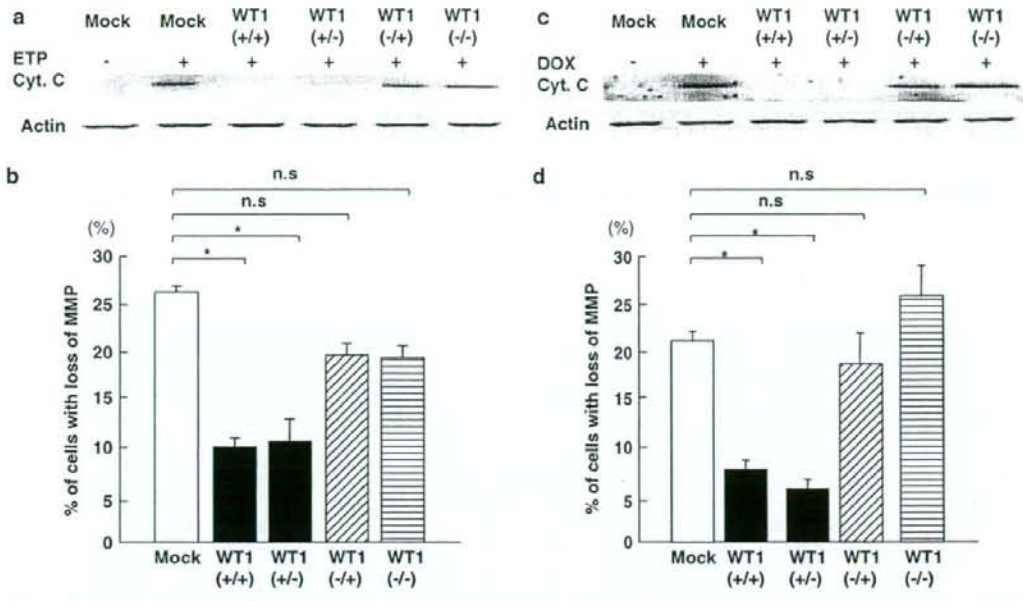

Figure 4 Stable expression of 17AA(+)WT1 isoforms protects mitochondria from membrane damages induced by apoptosis-inducing drugs. K562 cell clones transduced with one each of four WT1 isoforms were treated with etoposide (100 μM) or doxorubicin (100nM) for 24 h. Then, mitochondrial damages were examined by Western blot analysis for mitochondrial release of cytochrome *c* and by flow cytometric analysis for loss of mitochondrial membrane potential (MMP). (**a**) Mitochondrial release of cytochrome *c* in K562 clones treated with etoposide. (**b**) Induction of loss of MMP by etoposide. (**c**) Mitochondrial release of cytochrome *c* in K562 clones treated with doxorubicin. (**d**) Induction of loss of MMP by doxorubicin. (**b**) and (**d**) Columns, means of percentages of cells with loss of MMP in three different K562 cell clones that stably expressed the transduced WT1 isoforms; bars, s.e. Experiments were independently performed three times for each cell clones. $^*P<0.05$.

In the present study, it was shown that wild-type 17AA(+)WT1 isoforms inhibited apoptosis induced by apoptosis-inducing agents, whereas a mutant 17AA(+) WT1 isoform lacking zinc-finger region (exons 7–10) did not. These results indicated that zinc-finger region was essential for the antiapoptotic functions of 17AA(+) WT1 isoforms. Since zinc-finger region was DNA-binding site of the *WT1* gene, these results indicated that the antiapoptotic function of WT1 17AA(+) isoforms operated through transcriptional regulation of the other genes. As for downstream targets of 17AA(+)WT1 isoforms, proapoptotic Bak might be a direct or indirect target of 17AA(+)KTS(−)WT1 isoform as shown in the present study. As for the downstream target of 17AA(+)KTS(+)WT1 isoform, we could not determine it. However, since this isoform has an insertion of three amino acids (lysine, threonine, and serine (KTS)), by which binding of 17AA(+) KTS(+)WT1 isoform to a consensus sequence as that for 17AA(+)KTS(−)WT1 isoform was abrogated, 17AA(+)KTS(+)WT1 isoform may transcriptionally regulate other gene(s) than one(s) regulated by 17AA(+)KTS(−)WT1 isoforms (Reynolds *et al*., 2003). Comprehensive studies, including microarray analysis are being planned to identify the targets of 17AA(+) KTS(+)WT1 isoform. Interestingly, both the KTS(+) and KTS(−)WT1 isoforms required 17AA(+) region for their antiapoptotic functions, suggesting the necessity of the interaction with some molecule(s) through this region for their antiapoptotic functions. Taken together, these results may indicate that 17AA(+) KTS(+) and 17AA(+)KTS(−)WT1 isoforms recruit some molecule(s) through the 17AA region, bind to their different target sequences through their zinc-finger regions, and regulate the transcription of target genes to play antiapoptotic roles.

Our data showed that 17AA(+)WT1 isoforms exert antiapoptotic functions in leukemia cells through stabilization of mitochondrial membrane potential. Most chemotherapeutic drugs are considered to initiate cell death primarily by triggering the mitochondrial apoptosis pathway (Shimizu *et al*., 1996). Constitutive expression of 17AA(+)WT1 isoforms resulted in resistance of K562 leukemic cells to the apoptosis-inducing chemotherapeutic reagent such as etoposide and doxorubicin. Thus, antiapoptotic function of 17AA(+)WT1 isoforms through stabilization of MMP may contribute to chemotherapy resistance in leukemia. Recently, we analysed the expression of the WT1 isoforms in 36 primary leukemias and found that 17AA(+)WT1 isoforms were dominantly expressed in all of these leukemias examined regardless of disease subtypes. Therefore, since suppression of the expression of 17AA(+)WT1 isoforms should improve the sensi-

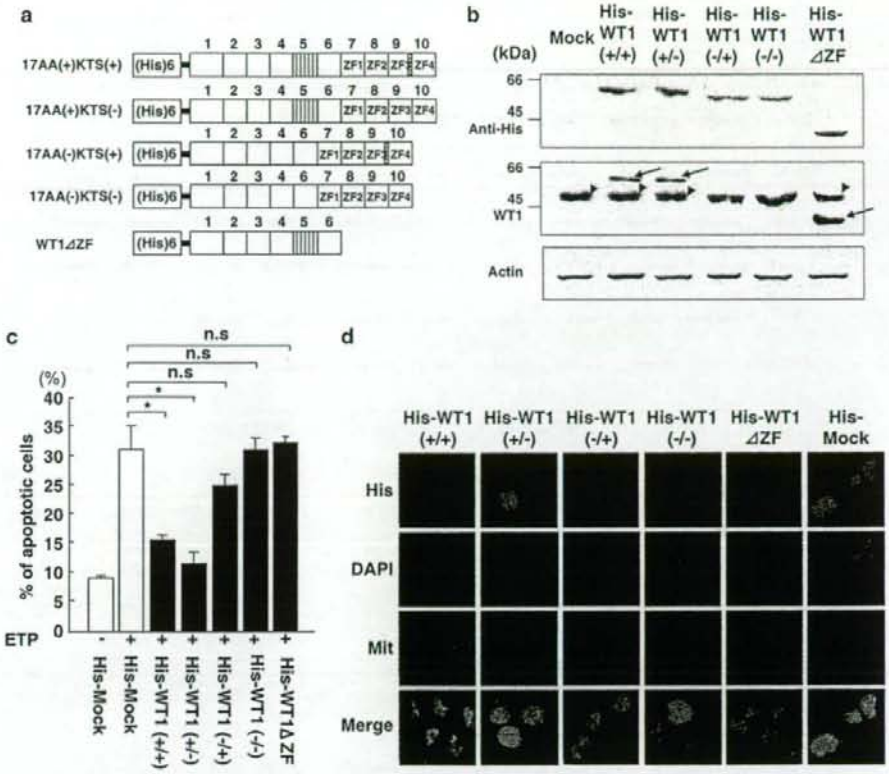

Figure 5 Requirement of zinc-finger region for the antiapoptotic functions of 17AA(+)WT1 isoforms. Polyhistidine-tagged one each of four WT1 isoforms or 17AA(+)WT1 lacking zinc-finger region (WT1ΔZF) was stably expressed in K562 leukemia cells. (**a**) Construction of four polyhistidine-tagged WT1 isoforms and WT1ΔZF. (**b**) Expression of four polyhistidine-tagged WT1 isoform proteins and WT1ΔZF protein were examined. Representative results of Western blot analysis using anti-polyhistidine tag Xpress (upper), anti-WT1 6F-H2 (middle), and anti-actin (lower) antibodies are shown. In the middle panel, arrows indicate exogeneous polyhistidine-tagged WT1 proteins. Arrowheads indicate endogenous WT1 protein. In polyhistidine tagged-17AA(−)WT1 isoform-transduced cell clones, exogenous polyhistidine-tagged WT1 proteins were not distinguished from endogenous WT1 proteins because molecular weights of these proteins were similar. (**c**) The K562 cell clones were treated with etoposide (100 μM) for 24 h and analysed for apoptosis by Annexin V-PI two-color flow cytometry. Columns, means of percentages of apoptotic cells in three different K562 cell clones that stably expressed the polyhistidine-tagged WT1 isoforms or WT1ΔZF; bars, s.e. Experiments were independently performed three times for each cell clones. $^*P<0.05$. (**d**) Localization of polyhistidine-tagged one each of four WT1 isoforms and WT1ΔZF proteins were examined by immunocytochemistry using anti-His tag Xpress antibody (shown in green). DNA was stained with DAPI (shown in blue). The mitochondria was stained with Mitotracker Red 580 (shown in red).

tivity of leukemia cells to chemotherapeutic drugs, 17AA(+)WT1 isoforms should be a novel molecular target for treatment of leukemia.

Materials and methods

Cell lines and culture conditions

Three highly WT1-expressing leukemia cell lines, chronic myeloid leukemia cell line K562, acute myeloid leukemia cell line Kasumi-1, and acute promyelocytic leukemia cell line HL-60, and one WT1-non-expressing Burkit lymphoma cell line Daudi were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS).

Antibodies

Monoclonal anti-bak (Oncogene Research Products, Boston, MA, USA), anti-WT1 (Dako, Carpinteria, CA, USA), anti-actin (Chemicon, Temecula, CA, USA), anti-Bcl-XL (Chemicon, Temecula, CA, USA), anti-p21 (Oncogene Research Products, Boston, MA, USA), anti-caspase-9 (R&D Systems, Inc., Minneapolis, MN), anti-p27 (BD Biosciences, Pharmingen, San Jose, CA), polyclonal anti-bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cytochrome *c* (Pharmingen) antibodies, and goat anti-rabbit or anti-mouse IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology) were used as secondary antibodies in Western blot analysis. For immunocytochemistry, monocolonal anti-His tag (anti-Xpress) (Invitrogen, Carlsbad, CA, USA) and rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate isomer1 (FITC) (DAKO, A/S, Denmark) were used.

Reagents

zVAD-fmk (broad-caspase inhibitor), Ac-LEHD-CHO (inhibitor for caspase-9), Ac-DEVD-CHO (inhibitor for caspase-3),

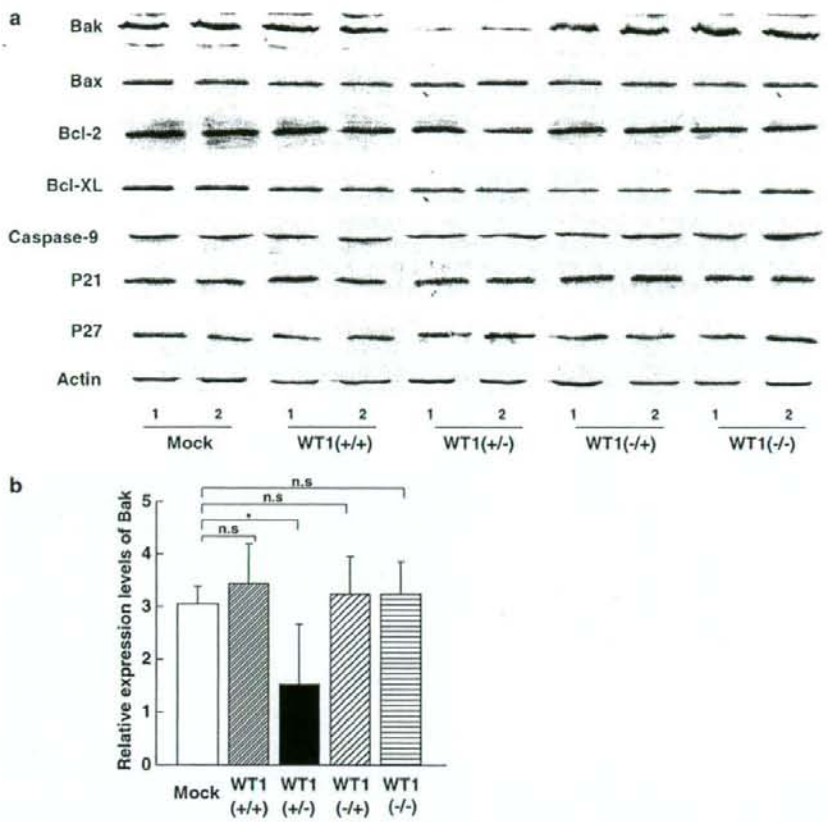

Figure 6 Expression of apoptotic-related proteins in K562 cell clones transduced with one each of four WT1 isoforms. (**a**) Expression levels of a set of known apoptotic-related proteins in K562 leukemic cells transduced with one each of four WT1 isoforms were examined by Western blot analysis. Three different K562 cell clones transduced with one each of four WT1 isoforms were independently analysed more than two times. Representative results are shown. (**b**) The density of Bak band divided by that of actin band to normalize the difference in protein loading for Western blot analysis in individual samples are shown as relative expression levels. Columns, means of relative expression levels of Bak protein in three different K562 cell clones that stably expressed the transduced WT1 isoforms; bars, s.e. $*P<0.05$.

and Ac-IETD-CHO (inhibitor for caspase-8) (Peptide Institute Inc., Osaka, Japan) were used to inhibit caspase activity at the concentration of 100 μM. Etoposide (Wako, Osaka, Japan) and Doxorubicin (Sigma Chemical Co., Steinheim, Germany) were used to activate the intrinsic apoptosis pathway and induce apoptosis at the concentration of 100 μM and 100 nM, respectively. Soluble TRAIL (PEPROTECH EC, London, UK) was used to activate the extrinsic apoptosis pathway and induce apoptosis at the concentration of 500 ng/ml. The enzyme substrate Ac-DEVD-AFC (BIOBOL, Tebu, France), FAM-LEHD-FMK (Serologicals, Norcross, GA, USA), and IETD-pNA (BioVision Lab, Palo Alto, CA, USA) were used to detect caspase-3-like, caspase-9-like, and caspase-8-like activities, respectively. The crosslinkers disuccinimidyl suberate (DSS) (Pierce Biotechnology, Rockford, IL, USA) was used in the detection of dimerization or oligomerization of Bax protein.

Vector construction

A pcDNA 3.1(+) (Invitrogen, Carlsbad, CA, USA) containing one each of four human WT1 isoforms (17AA(+)/KTS(+), 17AA(+)/KTS(−), 17AA(−)/KTS(+), and 17AA(−)/KTS(−)) was constructed and used for expression of one each of WT1 isoforms in K562 cells. The sequences of one each of four human WT1 isoforms and WT1 lacking zinc-finger region that were cloned in a pUC119 vector were PCR-amplified using Pfx *Taq* polymerase (Invitrogen, Carlsbad, CA, USA) and integrated into pcDNA 3.1/His vector (Invitrogen, Carlsbad, CA, USA). All the PCR-amplified sequences were confirmed for the absence of mutation by direct sequencing using BigDye Terminator V.1.1 cycle sequencing kit (Applied Biosystem, Branch berg, NJ, USA).

To prepare 17AA(+)WT1- and 17AA(−)WT1-specific siRNA vector, oligonucleotides encoding dsRNA directing (5′-AGCTCCAGCTCAGTGAAATGGACAGAAGGG-3′) in 17AA(+)WT1 mRNA and the sequence corresponding to the ligated sequences flanking the 17AA region in 17AA(−)WT1 mRNA were chemically synthesized, respectively (Japan BioScience, Saitama, Japan), annealed, and inserted into tRNA-shRNA expression vector piGENE tRNA Pur (Clontech, CA, USA) or Gene Silence pGSU6 shRNA Vector (Gene Therapy Systems, Inc., San Diego, CA, USA) which co-expressed GFP.

Transient expression of siRNA vectors
For transient transfection of siRNA vectors, K562, HL-60, Kasumi-1, and Daudi cells (2×10^6 cells) were washed three times and incubated with 10 μg plasmid DNA in 300 μl medium (FBS-) in a 4-mm cuvette. Transfection was performed by electroporation (150 V, 1000 μF) using Gene Pulser Xcell™ system (BioRad, CA, USA).

Stable expression of vectors
Mammalian expression vectors were linealized with *Pvu*I and introduced into K562 cells by electroporation using Gene Pulsor II (BioRad, CA, USA). The cell clones that stably expressed the vectors were isolated using the corresponding selective antibiotics.

Analysis of apoptosis by flow cytometry
To assess apoptotic cells, 1×10^5 cells were washed with PBS, and stained with Annexin V-FITC and PI at room temperature for 15 min in the dark using MEBCYTO Apoptosis Kit (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) according to the manufacturer's instructions. Then, the stained cells were analysed by FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Apoptosis was shown as percentages of apoptotic cells to the total number of counted cells.

Determination of cytochrome c release
To assess the release of cytochrome *c* from mitochondria to cytoplasm, cells were washed once with PBS, lysed in ice-cold STE buffer (250 mM sucrose, 25 mM Tris, and 1 mM EDTA, pH 6.8), and immediately centrifuged at 15000 *g* for 15 min. The supernatants were mixed with an equal volume of 2 × Laemmeli's SDS sample buffer for Western blot analysis and stored at −20°C until use.

Analysis of mitochondrial membrane potential loss
Changes in MMP following induction of apoptosis were assessed using MitoLight apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. In brief, after induction of apoptosis, cells were incubated at 37°C for 15 min in reaction buffer containing MitoLight mitochondrial dye that stained mitochondria in living cells in a membrane potential-dependent fashion. Then, the status of mitochondrial membrane potential was analysed using a FACScan flow cytometer in the FL1 channel.

RNA isolation and RT–PCR
Total cellular RNA was isolated using ISOGEN (WAKO, Osaka, Japan). Total RNA (2 μg) was reverse transcribed using murine Maloney leukemia virus (M-MLV) reverse transcriptase according to the manufacturer's protocols (Promega, Madison, WI, USA). PCR was performed in a total volume of 20 μl with 1 μl cDNA synthesis mixture for 25 cycles of 94°C denaturation (1 min), 60°C annealing (1 min), and 72°C extension (1.5 min). PCR primer was as follows. WT1: forward primer, 5′-GACCTGGAATCAGATGAA-3′, reverse primer, 5′-GAGAACTTTCGCTGACAAGTT-3′; Actin: forward primer, 5′-CCCAGCACAATGAAGATCAA GATCAT-3′, reverse primer, 5′-ATCTGCTGGAAGGTGGA CAGCGA-3′.

Western blot analysis
Cells were washed twice with PBS and lysed with 2 × Laemmeli's SDS sample buffer. Proteins were separated by SDS–PAGE and transferred to Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). After blocking of non-specific binding, immunoblots were incubated with primary antibody followed by incubation with the appropriate anti-rabbit or anti-mouse IgG antibody conjugated with alkaline phosphatase, and visualized using BCIP/NBT kit (Nacalai Tesque, Kyoto, Japan).

Activities of caspases
Caspases 3-like, 9-like, and 8-like activities were measured as described previously (Shimizu *et al.*, 1996). Briefly, the cells were collected at various time points, lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% Triton-X) on ice for 30 min and centrifuged. The supernatant was stored at −20°C until use. Concentration of proteins was determined using a Bio-Rad protein assay reagent by Bradford method. Then, the cytosol containing 50 μg of proteins was suspended in reaction buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA) containing 10 μM of the enzyme substrate Ac-DEVD-AFC (for caspase-3-like activity), FAM-LEHD-FMK (for caspase-9-like activity), or IETD-pNA (for caspase-8-like activity) and incubated at 37°C for 1 h. The fluorescence at 485/535 nm (for caspases 3- and 9-like activities) or 405 nm (for caspase-8-like activity) was measured using a Spectra-Max Gemini XS fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Detection of oligomerization of Bax
To detect oligomerization of Bax, 2×10^6 K562 cells were collected, washed three times with PBS, incubated with PBS containing 1 mM of DSS at room temperature for 30 min, and then incubated with 100 mM Tris buffer (pH 7.4) for 15 min to quench the crosslinker.

Immunocytochemistry
K562 cells attached onto glass slides by cytocentrifugation were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Then, cells were permeabilized with methanol at room temperature for 5 min. After blocking (2% BSA, 0.1% NaN_3, 0.2% Tween 20, 6.7% glycerol in PBS) for 45 min, the cells were stained with monoclonal anti-His tag (anti-Xpress) and rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate isomer 1 (FITC) (DAKO, A/S, Denmark). Nucleic acid stain was preformed by using 100 ng/ml DAPI (4′,6-amido-2-phenylindol) (Chemicon, Temecula, CA, USA). For staining of the mitochondria, K562 cells were incubated in the medium containing 100 nM Mitotracker Red 580 (Molecular Probe, Eugene, OR) at 37°C for 30 min. After washes with cell culture medium, cells were observed by laser confocal microscopy, LSM 510 META (Carl Zeiss Inc., Oberkochen, Germany).

Statistical analysis
One-way analysis of variance followed by Fisher's PLSD was used to determine the statistical significance of apoptosis employing the STATVIEW software (Abacus Concepts, Inc., Berkeley, CA, USA).

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Role of the mitochondrial membrane permeability transition in cell death

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Abstract In recent years, the role of the mitochondria in both apoptotic and necrotic cell death has received considerable attention. An increase of mitochondrial membrane permeability is one of the key events in apoptotic or necrotic death, although the details of the mechanism involved remain to be elucidated. The mitochondrial membrane permeability transition (MPT) is a Ca^{2+}-dependent increase of mitochondrial membrane permeability that leads to loss of $\Delta\psi$, mitochondrial swelling, and rupture of the outer mitochondrial membrane. The MPT is thought to occur after the opening of a channel that is known as the permeability transition pore (PTP), which putatively consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), cyclophilin D (Cyp D: a mitochondrial peptidyl prolyl-*cis*, *trans*-isomerase), and other molecule(s). Recently, significant progress has been made by studies performed with mice lacking Cyp D at several laboratories, which have convincingly demonstrated that Cyp D is essential for the MPT to occur and that the Cyp D-dependent MPT regulates some forms of necrotic, but not apoptotic, cell death. Cyp D-deficient mice have also been used to show that the Cyp D-dependent MPT plays a crucial role in ischemia/reperfusion injury. The anti-apoptotic proteins Bcl-2 and Bcl-x_L have the ability to block the MPT, and can therefore block MPT-dependent necrosis in addition to their well-established ability to inhibit apoptosis.

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Keywords Apoptosis · Necrosis · Mitochondria · Cyclophilin D · Cyclosporin A · Membrane permeability transition · Cytochrome c · Ischemia

Introduction

Apoptosis is a form of programmed cell death and an outline of the relevant signaling pathways at the molecular level is now well established. Mammalian cells possess two major apoptotic signaling pathways, which are known as the intrinsic pathway and the extrinsic pathway [1]. The intrinsic pathway involves an increase of outer mitochondrial membrane permeability that leads to the release of various proteins from the intermembrane space into the cytoplasm, including apoptogenic molecules such as cytochrome *c*, Smac/Diablo, HtrA2 (Omi), AIF, and DNaseG [1, 2]. In the presence of ATP (dATP), cytochrome *c* binds to Apaf-1 and triggers its oligomerization, after which pro-caspase-9 is recruited and undergoes autoactivation. The protein complex comprising cytochrome *c*, Apaf-1, and caspase-9 is called the "apoptosome". In short, an increase of outer mitochondrial membrane permeability is central to apoptosis [3, 4], and mitochondrial membrane permeability is directly regulated by the Bcl-2 family of proteins [4, 5] (see Fig. 1). However, the detailed mechanisms underlying the increase of outer mitochondrial membrane permeability during apoptosis and how this process is controlled by Bcl-2 family members are still to be determined. The model that was initially developed to explain the apoptotic increase of mitochondrial membrane permeability was based on the "mitochondrial membrane permeability transition" (MPT) [6], an event which has been appreciated for some time among investigators studying the mitochondria. This review summarizes recent progress with

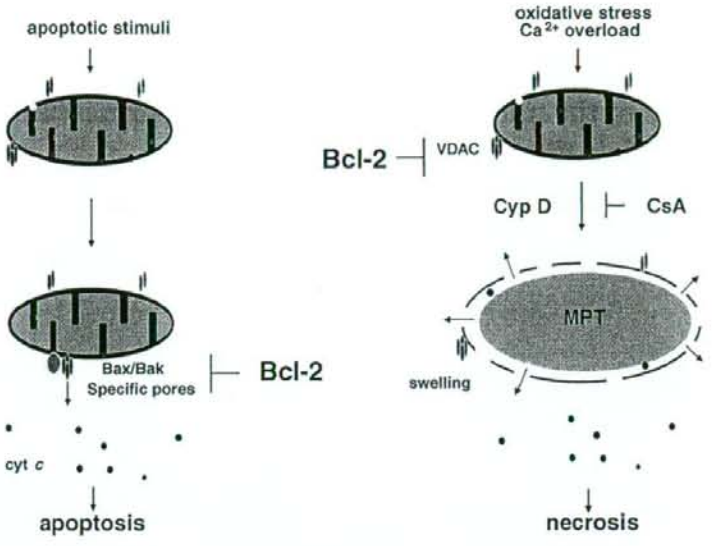

Fig. 1 Role of the mitochondria in apoptosis and necrosis. An increase in the permeability of the outer mitochondrial membrane is crucial for apoptosis to occur and is regulated by multidomain pro-apoptotic members of the Bcl-2 family (Bax and Bak), resulting in the release of several apoptogenic factors into the cytoplasm. In contrast, the Cyp D-dependent MPT involves an increase in the permeability of both the outer and inner mitochondrial membranes, and leads to necrosis induced by Ca^{2+} overload and oxidative stress. Both types of mitochondrial membrane permeability change are inhibited by anti-apoptotic members of the Bcl-2 family (Bcl-2 and Bcl-x_L)

regard to our understanding of the role of the MPT in cell death.

MPT

Mitochondria isolated from a variety of sources can show a sudden increase in the permeability of the inner mitochondrial membrane to solutes with a molecular mass of less than 1,500 Da, which results in the loss of $\Delta\psi$, mitochondrial swelling, and rupture of the outer mitochondrial membrane [7, 8] (see Fig. 1). This process is called the mitochondrial membrane permeability transition (MPT). The MPT can be induced under various conditions, such as exposure of mitochondria to Ca^{2+} together with inorganic phosphate. Although the molecular mechanisms of the MPT are largely unknown, the most widely accepted model (working hypothesis) is that it occurs after the opening of a channel complex that has been termed the permeability transition pore (PTP), which is thought to consist of the voltage-dependent anion channel (VDAC: outer membrane channel), the adenine nucleotide translocator (ANT: inner membrane channel), cyclophilin D (Cyp D), and possibly other molecule(s) [9] (see Fig. 2). However, it still remains uncertain whether the PTP really exists and what its exact nature is. Moreover, several experimental findings are difficult to explain by this model (see the introduction of [10]). A role of the ANT in the MPT is supported by MPT inhibition and activation by bonkrekic acid and atractyloside, respectively, which are ANT ligands [11]. Cyp D is a mitochondrial member of the cyclophilin family, which possesses peptidyl prolyl-*cis*, *trans*-isomerase (PPIase) activity and has a crucial role in protein folding [12]. The putative role of Cyp D in regulating the MPT is based on the observation that cyclosporin A (CsA), a specific inhibitor of the cyclophilin family, blocks the MPT [13]. Since CsA inhibits PPIase and the MPT at similar concentrations, PPIase activity may be critical for the MPT to occur. Cyp D resides in the mitochondrial matrix, but becomes associated with the inner mitochondrial membrane during the MPT. Based on the enzymatic activity of Cyp D as a PPIase, it may induce a conformational change of an inner membrane channel such as the ANT that leads to an increase of inner membrane permeability. In addition to the CsA-sensitive and Ca^{2+}-dependent ("regulated") MPT, the existence of a CsA-insensitive ("unregulated") MPT has also been suggested, although its mechanism and relationship to the CsA-sensitive MPT are totally unknown [10].

In the mid-1990s, the MPT attracted the attention of investigators in the cell death field, because it was reported that at least some forms of apoptosis could be inhibited by CsA, suggesting a role of the CsA-sensitive MPT in this process of cell death [9, 14]. A possible role of the MPT in apoptosis is also supported by the finding that apoptosis can sometimes be inhibited by bonkrekic acid [11, 15]. The CsA-sensitive MPT has also been implicated in remodeling of the mitochondrial cristae and mobilization of cytochrome *c* stores from the cristae during apoptosis, which promotes the complete release of cytochrome *c* [16]. However, the overall role of the MPT in apoptosis was still controversial, because there have been a number of reports that apoptosis

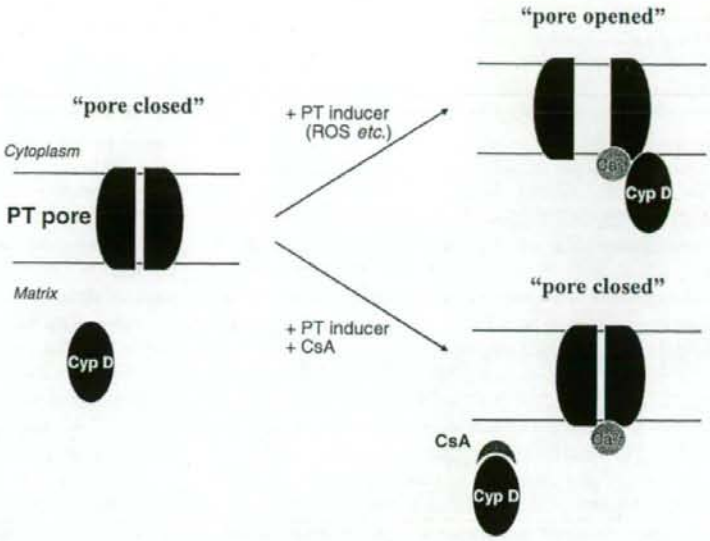

Fig. 2 Model of the MPT pore Under normal conditions, Cyp D is localized to the mitochondrial matrix, and the MPT pore is closed. In the presence of permeability transition inducers, Cyp D is considered to bind to and induce a conformational change of a channel in the inner membrane, resulting in opening of the MPT pore. Cyclosporin A (CsA) binds to and inhibits Cyp D to prevent MPT pore opening

is not inhibited by CsA [17]. Also, it has been demonstrated that $\Delta\psi$ occurs after cytochrome *c* release in at least some types of apoptosis, suggesting that the MPT is not always the trigger for cytochrome *c* release and cell death. This issue was recently been solved by studies performed in Cyp D-deficient mice, as discussed later.

Are the VDAC, the ANT, and Cyp D essential for the MPT?

It has long been thought that the VDAC, the ANT, and Cyp D play an essential role in the MPT, but convincing evidence was lacking until very recently.

An important role of the VDAC in the MPT has been supported by the following findings: (1) the electrophysiological properties of the PTP are strikingly similar to those of the VDAC incorporated in planar phospholipids bilayers [18, 19]; (2) various factors that alter VDAC channel properties, such as addition of NADH, Ca^{2+}, or glutamate, as well as binding to hexokinase II [20–24], also modulate PTP activity [25–27]; and (3) chromatography of mitochondrial extracts on a Cyp D affinity column leads to purification of the VDAC associated with the ANT [28].

The most convincing evidence about involvement of the VDAC in the MPT should theoretically be obtained by studies employing VDAC-deficient cells. Such a study was recently performed with mitochondria isolated from VDAC1-deficient cells, and it was found that VDAC1-deficient mitochondria still undergo the MPT normally, suggesting that VDAC1 is not important for this process. However, this result could have been due to compensation for VDAC1 deficiency by other isoforms, including VDAC2 and VDAC3. So far, experimental evidence for a direct role of the VDAC in the MPT has been provided by studies using specific anti-VDAC antibodies [29]. Two polyclonal anti-VDAC antibodies, which recognize different VDAC epitopes and inhibit its activity in liposomes [29], have been shown to inhibit the Ca^{2+}-induced MPT [29], supporting a crucial role for the VDAC in this process.

The ANTs (ANT1 and 2 in mice and ANT1, 2, and 3 in humans) are also considered to be important for the MPT. It has been demonstrated that Cyp D interacts directly with the ANT, although it is not known whether CsA inhibits this interaction [28, 30]. Regarding the role of the ANT in the MPT, considerable progress was made recently because it was shown that liver mitochondria from mice lacking both ANT1 and ANT2 still underwent the MPT, although the triggering Ca^{2+} concentration was slightly increased [31]. This finding suggests that ANT1/2 only play a limited role, if any, in the MPT or else that deficiency of ANT1/2 was compensated by other channel(s). The lack of an important role for the ANT in the MPT would be consistent with the observation that mitochondria isolated from yeast lacking the ANT still undergo MPT-like changes, including loss of membrane potential and swelling in response to ethanol, which are very similar events to those occurring in mammalian mitochondria during the MPT [32]. However, it is unknown whether

yeast mitochondria undergo a real MPT, because swelling of these mitochondria and loss of membrane potential in response to ethanol are not inhibited by CsA, although this inability of CsA to inhibit MPT-like events might be due to its inability to inhibit a Cyp D counterpart in yeast mitochondria. If the ANT is not involved in the MPT, the other channel(s) that are actually involved might be ANT-like inner membrane channels, because the MPT is modulated by ANT ligands such as bonkrekic acid or atractyloside and is accompanied by loss of $\Delta\psi$ (i.e., increased permeability of the inner mitochondrial membrane). Identification of one or more channels in the inner mitochondrial membrane that are directly involved in the MPT and might be targets of Cyp D would be an important step forward.

The role of Cyp D in the MPT was initially suggested by the finding that the MPT is blocked by CsA, which is known to inhibit the PPIase activity of cyclophilins. This finding has recently been confirmed by studies performed employing Cyp D gene (*ppif*)-deficient mice [33–36]. It has been demonstrated that Cyp D-deficient mitochondria isolated from the livers of these mice do not undergo the CsA-sensitive MPT in response to a variety of inducers, including Ca^{2+}, atractyloside, and H_2O_2. Because the MPT does not occur, these mitochondria accumulate a much higher concentration of Ca^{2+} than control mitochondria [33, 36]. However, the CsA-insensitive MPT (with loss of $\Delta\psi$ and swelling) can still occur when these Cyp D-deficient mitochondria are exposed to high concentrations of Ca^{2+} [33, 35]. In addition, Cyp D-deficient mitochondria show a normal response to reagents like ubiquinone and thiol oxidants that cause the CsA-insensitive MPT [35]. The CsA-sensitive MPT and CsA-insensitive MPT might share a common mechanism, because both forms of MPT are inhibited by ubiquinone 0 [35]. This finding might also suggest that Cyp D only sensitizes the mitochondria to the Ca^{2+}-induced MPT, although these two forms of MPT might be mediated by different mechanisms. This issue will only be solved by identification of the essential players involved in the MPT. In any case, it has been confirmed that Cyp D has a specific role in the CsA-sensitive MPT.

Although it is now clear that the Cyp D is an essential component of the CsA-sensitive MPT, there are still many questions to be answered. More studies are needed to elucidate the molecular nature of the MPT pore complex. Assuming that Cyp D interacts as a PPIase with other molecules essential for the MPT that probably reside in the inner mitochondrial membrane, a promising approach would be the isolation of a protein complex containing Cyp D and the VDAC. Another issue would be investigation of the relationship between the Cyp D-dependent MPT and the unregulated MPT. Furthermore, does the unregulated MPT have a role in apoptosis or other forms of cell death?

Role of the MPT

For a long time, it has been unclear whether the CsA-sensitive MPT plays an important role in the apoptotic increase of mitochondrial membrane permeability. However, studies of Cyp D-deficient mice have finally solved this issue. Various cells isolated from Cyp D-deficient mice, such as thymocytes, embryonic fibroblasts (MEFs), and hepatocytes, undergo apoptosis normally in response to various stimuli, including etoposide, stautosporine, and tumor necrosis factor-α [33–36]. Small intestinal cells from Cyp D-deficient mice are also as sensitive to X ray-induced apoptosis as cells from control mice [33]. These results provide the most compelling evidence that the CsA-sensitive MPT is not essential for apoptosis. Of course, it remains possible that some forms of apoptosis might be mediated by the CsA-sensitive MPT, and thus inhibited by CsA. However, the inhibitory effect of CsA on apoptosis might need to be more carefully evaluated because it is usually studied at relatively high CsA concentrations that could inhibit other targets, including cytoplasmic cyclophilins involved in transcriptional regulation, thus having a secondary effect on apoptosis. Accordingly, it may be necessary to re-evaluate CsA-dependent inhibition of apoptosis by using Cyp D-deficient cells or by silencing Cyp D to assess the real effect of CsA.

Several studies have indicated that overexpression of Cyp D protects cells against some forms of apoptosis. For example, the overexpression of CypD inhibits apoptosis induced by overexpression of caspase-8 (but not Bax) or by exposure to arsenic trioxide [37, 38]. It may be possible that these forms of apoptosis are mediated by the MPT, which is somehow affected by Cyp D overexpression. However, studies of transgenic mice with myocardial expression of Cyp D have revealed that cardiac myocytes isolated from these mice show a tendency to undergo mitochondrial swelling and spontaneous death [34], suggesting that the effects of Cyp D expression might be cell type-specific.

In contrast to the lack of any influence of Cyp D deficiency on apoptosis, the Cyp D-dependent MPT plays an important role in some forms of necrotic cell death (see Fig. 1). Cyp D-deficient MEFs show significantly increased resistance to H_2O_2-induced necrosis [33, 34], and Cyp D-deficient hepatocytes display resistance to necrosis induced by a Ca^{2+} ionophore (A23187) or by H_2O_2 [33, 34]. Interestingly, when H_2O_2-induced and Ca^{2+} ionophore-induced necrosis is inhibited by Cyp D deficiency in these cells, apoptosis does not occur as an alternate death mechanism [33], suggesting that the H_2O_2/Ca^{2+}-triggered apoptotic signaling pathways are somehow blocked in these types of cells.

Another very interesting question concerns the biological significance of the MPT because it is conceivable that the MPT plays a role in some physiological processes. By

analyzing Cyp D-deficient mice and cells in more detail, some hints about the role of the MPT should be obtained.

Regulation of the MPT by Bcl-2

Anti-apoptotic members of the Bcl-2 family, such as Bcl-2 itself and Bcl-x_L, are known to inhibit the Bax/Bak-dependent apoptotic increase of mitochondrial membrane permeability by direct interaction with pro-apoptotic members of this family, and also inhibit the MPT itself [39, 40] (see Fig. 1). How do these proteins block the MPT? Given that Bax/Bak is not essential for the MPT [33], Bcl-2 (Bcl-x_L) might directly inhibit a component of the PTP complex. In fact, Bcl-2 (Bcl-x_L) is capable of blocking VDAC activity [39] and ANT activity in liposome systems [41]. As described above, the VDAC plays a role in the MPT [29], whereas the ANT might not be important [31], so Bcl-2 and Bcl-x_L possibly inhibit the MPT by blocking the VDAC or unknown channels similar to the ANT that are actually involved in the MPT.

Role of the Cyp D-dependent MPT in disease

The advent of Cyp D-deficient mice has provided compelling evidence that the Cyp D-dependent MPT plays a crucial role in ischemia/reperfusion injury affecting the heart [33, 34] and brain [36], suggesting that the Cyp D-dependent MPT is involved in ischemia/reperfusion-induced cell death and that Cyp D and other components of the MPT are promising therapeutic targets. However, there have been a large number of reports published on the death mechanisms of ischemia/reperfusion injury and investigation of therapeutic methods, making it evident that ischemia/reperfusion injury is a very complex phenomenon which might involve multiple death mechanisms, because such injury can be suppressed by various inhibitors of different forms of cell death. It has been shown that ischemia/reperfusion injury can be ameliorated by inhibiting apoptosis with caspase inhibitors [42–45], inhibiting necroptosis with Nec1 [46], or blocking the Ask 1 pathway [47]. In studies of model systems employing cell lines, the death mechanisms involving caspases, a Nec1 target, Ask1, and the Cyp D-dependent MPT do not seem to overlap with each other. Why are so many different potential mechanisms involved in ischemia/reperfusion injury? Different death mechanisms might operate in the same cell in a sequential manner or in parallel, meaning that the inhibition of one mechanism might have a protective effect. Alternatively, different death mechanisms might act on different cells during ischemia/reperfusion injury and the dying cells might trigger the death process in other cells. It is also possible that different cell death mechanisms are activated by different ischemic conditions. For further studies of ischemia/reperfusion injury, mice that lack certain cell death mechanisms, such as Cyp D-deficient mice and Bax/Bak-deficient mice, would be useful tools.

The Cyp D-dependent MPT might also be involved in other diseases. It has been reported that mitochondria isolated from the livers of MND2 mice with mutation of the omi gene are more susceptible to the MPT [48]. MND2 mice succumb to motoneuron disease [49], which might be caused by the MPT occurring at a lower threshold in neuronal mitochondria. Thus, future studies may unveil a role of the Cyp D-dependent MPT in the pathogenesis of various diseases.

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CASE HISTORY

Quantitation of sleep and spinal curvature in an unusually longevous owl monkey (*Aotus azarae*)

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Keywords
aging – captive – Cebidae – kyphosis – longevity – nocturnality – radiography

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Abstract

Background A table summarizing the primary literature on 19 species of longevous non-human primates, other than owl monkey, is presented.
Methods We prospectively quantitated the sleep of a longevous female owl monkey (*Aotus azarae*), aged > 30 years, longitudinally for 2 years and also evaluated the senility-induced change in spinal curvature.
Results The mean daily total sleep time (TST) of this monkey ranged between 790 and 1106 minutes, and was markedly higher in comparison with its female progeny (aged 16 years and used as a control) whose daily TST during the same experimental period ranged between 612 and 822 minutes.
Conclusion The calculated kyphotic index (KI) of 2.27 for this monkey, compared with the KIs 4.83 and 5.42, for its progeny and female grandprogeny (aged 1 year) respectively, confirmed the prominent spinal curvature.

Introduction

Although the longevity quantitation of non-human primates living in the wild is susceptible to higher degree of doubt and imprecision, in the past five decades more reliable quantitation of longevity among captive non-human primates have become feasible. Based on previously published longevity reports on captive primates [6, 27, 41], longevous status among the four major non-human primate groups can be arbitrarily fixed as, > 40 years for apes, > 30 years for Old World monkeys, > 20 years for New World monkeys and > 10 years for Prosimians. Table 1 provides a select list of original reports which have appeared since 1969 on 19 species of longevous non-human primates held in captivity. As one could expect, majority of these reports were retrospective studies representing clinicopathological investigations based on the postmortem specimens of tissues and bones. Prospective studies on longevous non-human primates have been sparse at best. In addition, reports on the longevous owl monkey (*Aotus*) have been lacking.

Among the more than 230 species of non-human primates, the owl monkey is unique in being the only nocturnally adapted Anthropoidean primate [4, 39, 44]. Owl monkeys are strictly arboreal and lead a monogamous family life, with a group size of two to five members consisting of a breeding pair and young progeny. Young members emigrate from the family group when they complete the subadult stage by the end of 3 years [59]. The life span of owl monkey in the wild remains yet to be clarified [16], though 12–20 years [22] and 26–30 years [16] have been noted as plausible longevity ranges for owl monkeys under captive conditions.

As of now, the only available report on aged owl monkeys [13] relates to histopathological examination of postmortem brain tissues, as a primate representative, on a comparative scan on the neuropathology of aging in the brains of 47 vertebrate species. Unfortunately, the ages of the two owl monkeys studied for the presence of lipofuscin pigment, argyrophilic plaques, neurofibrillary tangles and corpora amylacea have not been stated.

The owl monkey colony established at the Primate Research Institute (PRI), Inuyama, Japan, in mid-1970s currently consists of 16 subjects, among which 12 belong to *Aotus azarae* species. Among these, the

Table 1 A select list of studies on longevous non-human primates[1]

Primate[2]	Number	Age range (years)	Study Focus	Ref
Apes				
Chimpanzee[3]	5	40–59	Brain weight	[23]
Chimpanzee[3]	7	40–48	Reproductive function	[19]
Chimpanzee[3]	1	>40	Bone mineral density	[21]
Chimpanzee[3]	9	>40	Sociobehavioral manifestations	[25]
Chimpanzee[3]	9	40 (mean)	Behavior	[5]
Lowland gorilla[4]	1	44	Senile plaques	[31]
Lowland gorilla[4]	2	*c.* 41	Sexual behavior and estrus cycle	[2]
Siamang[5]	1	*c.* 40	General report	[45]
Old World monkeys				
Japanese macaque[6]	1	*c.* 40	Skeleton	[49]
Crab-eating macaque[7]	1	>35	Senile plaques	[40]
Rhesus macaque[8]	29	30–37	Age-related pathology	[52, 53]
Rhesus macaque[8]	7	31–36	Life span	[50]
Rhesus macaque[8]	1	34	Menopause	[56]
Rhesus macaque[8]	2	31	Hyperthyroidism	[7]
Rhesus macaque[8]	3	31	Behavior and pathology	[12, 35]
Rhesus macaque[8]	10	≥30	Serum dehydroepiandrosterone sulfate	[30]
Rhesus macaque[8]	1	>30	Degenerative joint disease	[14]
Rhesus macaque[8]	1	>30	Pathology	[32]
Assamese macaque[9]	1	>30	Pathology	[32]
Baboon, hamadryas[10]	5	>30	Aging	[33]
Baboon, hamadryas[10]	7	>30	Pathology	[32]
Vervet monkey[11]	1	30	Aging	[33]
New World monkeys				
Woolly monkey[12]	2	30–31	Reproductive function	[38]
Squirrel monkey[13]	6	22–27	β/A4 amyloid in brain	[57]
Squirrel monkey[13]	1	>20	Cerebral tumor	[26]
Capuchin monkey[14]	1	>40	General postmortem	[24]
Prosimians				
Fat-tailed dwarf lemur[15]	1	15	Brain iron and lipofuscin	[17, 18]
Grey lesser mouse lemur[16]	1	12	Brain iron and lipofuscin	[17, 18]
Ring-tailed lemur[17]	16	10–14	Fecundity, birth seasonality	[43]
Ring-tailed lemur[17]	5	13–22	Hemosiderosis	[47]
Black lemur[18]	6	11–25	Hemosiderosis	[47]
Brown lemur[19]	1	14	Hemosiderosis	[47]
Ruffed lemur[20]	2	11–13	Hemosiderosis	[47]
Ruffed lemur[21]	3	12–28	Hemosiderosis	[47]
Potto[22]	3	11–24	Reproductivity, life span	[10, 11]

[1]The arbitrarily fixed age levels for longevous status among non-human primate groups are, >40 yrs (apes), >30 yrs (Old World monkeys), >20 yrs (New World monkeys) and >10 years (Prosimians), based on previously reported longevity records in captivity [6, 27].
[2]Species names are as follows: [3]*Pan troglodytes*; [4]*Gorilla gorilla*; [5]*Hylobates (Symphalangus) syndactylus*; [6]*Macaca fuscata*; [7]*Macaca mulatta*; [8]*Macaca fascicularis*; [9]*Macaca assamensis*; [10]*Papio hamadryas*; [11]*Chlorocebus(Cercopithecus) aethiops*; [12]*Lagothrix lagotricha*; [13]*Saimiri sciureus*; [14]*Cebus apella*, [15]*Cheirogaleus medius*; [16]*Microcebus murinus*; [17]*Lemur catta*; [18]*Lemur macaco macaco*; [19]*Lemur fulvus*; [20]*Variecia variegata variegata*; [21]*Variecia variegata rubra*; [22]*Perodicticus potto*.

oldest member was wild born and has passed 28 years in captivity, as of September 2005. Although the owl monkey colony at our facility has been in existence for three decades, partly due to specific and focused interests of most primatologists in Japan and partly due to the labor needed to develop non-invasive protocols which satisfy the primate care protocol adopted by our Institute since 1980s, these Neotropical monkeys barely received research attention. However, the members of this owl monkey colony had received routine veterinary care and none of the living members had suffered from any maladies including pain and were not in need of specific clinical veterinary care, medications for alleviating maladies and medical interventions. Until 2002, only two short reports were published based on the genetic [29] and circadian

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