International Immunology, Vol. 15, No. 6, pp. 741–749
doi: 10.1093/intimm/dxg070
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Critical function of T cell death-associated gene 8 in glucocorticoid-induced thymocyte apoptosis

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Keywords: apoptosis, dexamethasone, development, T lymphocyte, thymus

Abstract

Transcriptional expression of a gene or genes is absolutely required for induction of glucocorticoid-induced thymocyte apoptosis. We have previously shown that expression of T cell death-associated gene 8 (TDAG8) is quickly induced exclusively in the thymus after dexamethasone (DEX) treatment. Here, we present data that TDAG8 expression is induced prior to induction of DEX-mediated apoptosis. In contrast, TDAG8 expression in thymocytes was not induced in the process of γ-irradiation-mediated apoptosis. TDAG8 expression accelerated only DEX-induced, but not TCR-mediated or γ-irradiation-induced, thymocyte apoptosis in transgenic mice overexpressing TDAG8. Interestingly, these effects were specifically detected in $CD4^+CD8^+$ double-positive thymocytes. Moreover, activation of caspase-3, -8 and -9 was enhanced in thymocytes of TDAG8 transgenic mice after DEX stimulation. In conclusion, TDAG8 expression is involved in glucocorticoid-induced signals to activate caspase-9, -8 and -3 for subsequent apoptosis induction in $CD4^+CD8^+$ double-positive thymocytes.

Introduction

Glucocorticoids are hormones, small lipophilic compounds, which profoundly affect the immune system by binding to an intracellular glucocorticoid receptor (GR). After ligand stimulation, the GR is translocated to the nucleus and regulates gene transcription through homo-dimerization or interaction with other transcription factors, such as AP-1, NF-κB, CREB, STAT3 or STAT5 (1–8). The most recognized biological glucocorticoid effect on peripheral T cells is anti-inflammation and immunosuppression, due to the inhibition of cytokine induction, inhibition of chemokine synthesis and repression of genes encoding cell-surface receptors (9). The peripheral T cell repertoire is determined by positive and negative selection of immature thymocytes during thymic development. This process is thought to be mediated by apoptosis (10). Glucocorticoids are reported to induce apoptosis in immature thymocytes. Apoptosis is induced in immature thymocytes even at physiological glucocorticoid concentrations in the blood that are achieved during a stress response (11–13). Recent studies reported that glucocorticoids play an important role in positive and negative selection of immature thymocytes during thymic development (10,14,15).

Despite the enormous advances made in our understanding of regulated cell death, the molecular mechanism of glucocorticoid-induced cell death is still unknown. Glucocorticoid-induced thymocyte apoptosis is mediated via the mitochondrial pathway: requiring Apaf-1 and caspase-9, and inhibited by Bcl-2 and Bcl-x_L (16–21). Apaf-1 and caspase-9 exist as dormant proenzymes in healthy cells. Once glucocorticoids trigger the apoptotic process, mitochondrial cytochrome *c* is translocated into the cytosol and binds to Apaf-1, forming an oligomeric Apaf-1–cytochrome *c* complex (apoptosome) in the presence of dATP or ATP (22). This interaction leads to pro-caspase-9 activation through proteolysis. On the other hand, Bcl-2 and Bcl-x_L inhibit the release of cytochrome *c*, resulting in inhibition of glucocorticoid-induced apoptosis (23–27). Since GR is a transcriptional regulator, it has been

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Transmitting editor: T. Hamaoka

Received 24 January 2003, *accepted* 20 March 2003

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hypothesized that glucocorticoid-induced thymocyte apoptosis requires GR-mediated gene expression. This hypothesis is supported by reports that glucocorticoid-induced apoptosis requires dATP (28) and it is prevented by protein synthesis inhibitors (29,30). Recently, it was reported that a GR point mutation (A458T) was generated and utilized to establish $GR^{dim/dim}$ mice by gene targeting using the Cre/loxP system (31). This mutation impairs GR dimerization and subsequent glucocorticoid response element-dependent transcriptional activity, while functions that require cross-talk with other transcription factors, such as trans-repression of AP-1 or NF-κB-driven genes, remain intact. Generation of $GR^{dim/dim}$ mice demonstrated that glucocorticoid-mediated thymocyte apoptosis requires DNA binding of the GR. However, glucocorticoid-induced genes that mediate cell death were still elusive.

Many attempts have been made to isolate glucocorticoid-induced genes that mediate cell death (32–37). Unfortunately, to date there is no convincing data that any of the current candidates play such a role. We have previously reported the isolation of a dexamethasone (DEX)-induced gene 1, which is expressed in the thymus during DEX-mediated cell death and was found to be identical to the T cell death-associated gene 8 (TDAG8) (38). TDAG8 encodes a putative G protein-coupled receptor containing seven transmembrane segments. Expression of TDAG8 mRNA was limited to lymphoid organs, such as the thymus, spleen and lymph nodes, and greatly induced only in the thymus immediately after DEX treatment (38). To investigate the role of the TDAG8 molecule in DEX-induced thymocyte apoptosis, we created transgenic mice specifically overexpressing TDAG8 in immature thymocytes. Thymocytes from these animals exhibited an increase in the activation of caspase-9, -8 and -3 in response to DEX, resulting in an increase of DEX-induced apoptotic cells.

Methods

Mice

C57BL/6 mice (females, 5 weeks of age) were obtained from Japan SLC (Hamamatsu, Japan) and were kept in our animal faculty for at least 1 week before use. C57BL/6 or TDAG8 transgenic mice (7–10 weeks of age) were treated with various stimulators as described in the figure legends, and were sacrificed and used for each assay.

Cell culture

Thymus was obtained from C57BL/6 mice (6 weeks of age). Thymocytes (3.5×10^6 cells/ml) were suspended in 10 ml of RPMI supplemented with 10% heat-inactivated FCS (Sigma-Aldrich, St Louis, MO) and 50 μM 2-mercaptoethanol, and were cultured in culture dishes (Falcon3003; BD Falcon, Franklin Lakes, NJ). The cells were stimulated with various concentrations of DEX (Nippon Zenyaku, Koriyama, Japan), 20 μg/ml immobilized anti-mouse CD3 antibody (145-2C11; BD PharMingen, San Diego, CA), 20 μg/ml immobilized anti-mouse CD3 and 50 μg/ml immobilized anti-mouse CD28 antibody (BD PharMingen) or γ-irradiated at 10 Gy. After 2 and 18 h, the treated and untreated cells were harvested, and used for Northern blot analysis and apoptosis assay.

Generation of transgenic mice

The expression vector for the TDAG8 transgene was constructed as follows. The *lck* proximal murine promoter was used to express murine TDAG8 mainly in immature thymocytes *in vivo* (39–41). A fragment (1.5 kbp) which includes 100 bp of the 5′-untranslated region, the entire TDAG8 coding region and 400 bp of 3′-untranslated region sequence was blunt-ended and ligated to the blunted *Bam*HI site of the p1017 vector containing the *lck* promoter plus human growth hormone gene (Fig. 3A). A *Not*I fragment (6.8 kbp) of this construct was microinjected into the pronucleus of fertilized eggs at the one-cell stage of C57BL/6 × C57BL/6 F_2 mice.

Northern blot analysis

RNA was extracted from the indicated tissues of C57BL/6 mice or TDAG8 transgenic mice using TRIzol (Gibco/BRL, Rockville, MD). Total RNA (5 or 10 μg) was subjected to Northern blot analysis on a 1% agarose/formaldehyde gel. The RNA was transferred to Hybond-N (BD Amersham, Piscataway, NJ) by capillary action and probed with TDAG8 cDNA (950-bp *Sty*I fragment) or human growth hormone gene (1.4-kbp *Bam*HI–*Bgl*II fragment) for detection of endogenous or exogenous TDAG8 respectively.

Flow cytometry

For detection of apoptosis, freshly isolated thymocytes were stained with FITC–Annexin-V (Roche Diagnostics, Indianapolis, IN) and propidium iodide (PI; Sigma-Aldrich, St Louis, MO). For flow cytometric analysis, cells were stained with phycoerythrin (PE)–anti-CD4 (clone RM4-4; BD PharMingen) and FITC–anti-CD8 (clone 53-6.7; BD PharMingen). Flow cytometry was done on a FACScan and analyzed with CellQuest software (Becton Dickinson, Mountain View, CA).

TUNEL histochemistry

For TUNEL histochemistry, an *in situ* apoptosis detection kit (Takara Shuzo, Tokyo, Japan) was used. Briefly, paraffin-embedded sections were dewaxed and rehydrated in PBS according to standard protocols. Sections were then incubated with proteinase K (20 μg/ml) for 15 min at 37°C. The endogenous peroxidase activity in tissue sections was quenched with H_2O_2 (0.3%). The terminal deoxyribonucleotide transferase reaction was performed *in situ* with FITC-labeled nucleotides and subsequently treated with peroxidase-conjugated anti-FITC antibody. Labeled nuclei were then visualized by treating the section with diaminobenzidine tetrahydrochloride (10 μg) in 20 ml Tris–HCl, pH 7.6, plus 20 ml H_2O_2. Counterstaining was done using a modified methylgreen procedure.

Western blot analysis

Isolated thymocytes were lysed in ice-cold lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na_3VO_4 and 10 mM NaF) supplemented with protease inhibitor cocktail (Roche Diagnostics). Cell homogenates were spun at 14,000 *g* for 5 min and supernatants were collected. Protein concentrations from cell lysate were determined using a Bio-Rad protein

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assay kit (Bio-Rad, Hercules, CA). Equal amounts of cell lysate (20 μg/lane) were loaded onto SDS–PAGE (10–20%) gels, transferred on to PVDF transfer membranes (Perkin Elmer, Boston, MA) and blotted with the appropriate antibodies. Antibodies reactive to caspase-3 (46; Transduction, Lexington, KY), cleaved caspase-3 (D175; Cell Signaling Technology, Beverly, MA), caspase-9 (5B4; MBL, Nagoya, Japan) and β-actin (AC-40; Sigma-Aldrich) were used in this study. Western blot analysis was carried out according to standard procedures using Western Blot Chemiluminescence Reagent (Perkin Elmer).

Caspase cleaved assay

TDAG8 transgenic and non-transgenic littermates (7–10 weeks of age) were treated i.p. with DEX (100 μg/100 μl) or PBS (100 μl). After 6 h, thymocytes were isolated and used for the assay of caspase-3, -8 or -9 activity. Caspase-3, -8 and -9-like enzyme activity was measured using the CPP32/caspase-3 colorimetric protease assay kit (MBL), FLICE/caspase-8 colorimetric protease assay kit (MBL) or caspase-9/Mch6 colorimetric protease assay kit (MBL) respectively. The chromophore *p*-nitroanilide (*p*NA) light emission after cleavage from labeled DEVD-*p*NA, IETD-*p*NA or LEHD-*p*NA was measured at 400 nm using a microtiter plate reader.

Data analysis

Values were expressed as mean ± SE. Statistical analyses were carried out by analysis of variance (two-way ANOVA).

Results

Induction of TDAG8 expression by DEX stimulation preceded DEX-induced thymocyte apoptosis

We reported previously that TDAG8 expression was detected only in lymphoid organs and greatly induced only in the thymus immediately after DEX treatment (38). If TDAG8 is involved in glucocorticoid-induced signaling for thymocyte apoptosis, TDAG8 expression should be induced before the occurrence of apoptosis. Therefore, the kinetics of TDAG8 expression after DEX treatment was studied by Northern blot analysis (Fig. 1A). To confirm whether apoptosis was induced under this condition, cells were stained by PI and Annexin-V, and apoptotic cells were defined as Annexin-V$^+$ cells (Fig. 1B). To quantitate the expression level of TDAG8 mRNA, the density of bands detected by Northern blot analysis was measured using a bio-imaging analyzer Bas1000 and the results are shown in Fig. 1(C). After DEX stimulation, TDAG8 expression was maximally induced at 2 h and started to decrease to pre-treatment levels at 16–24 h. On the other hand, apoptotic cells were clearly detected at 8 h and their population reached a peak level at 16 h (Fig. 1C). Therefore, induction of endogenous TDAG8 expression by DEX stimulation preceded DEX-induced thymocyte apoptosis.

Correlation of induction of TDAG8 expression with DEX-induced apoptosis

We have previously shown that apoptosis of murine T cell hybridoma, BD5-8, was induced by DEX, anti-CD3 and

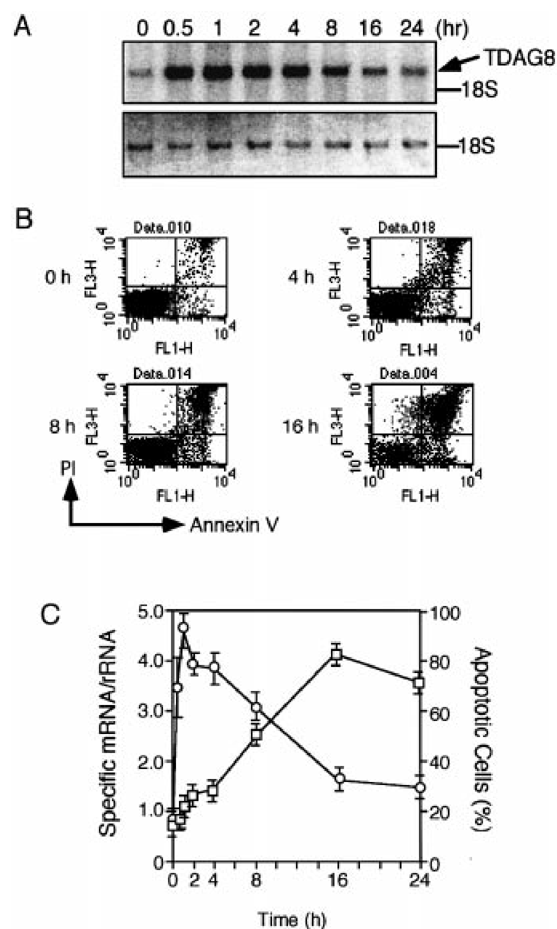

Fig. 1. TDAG8 expression and induction of apoptosis in the thymus of DEX-treated mice. C57BL/6 mice (6 weeks of age) were treated i.p. with DEX (500 μg/500 μl). Mice were sacrificed at the indicated time. Total RNA (5 μg) extracted from the thymus was subjected to Northern blot analysis and probed with TDAG8 cDNA (950-bp *Sty*I fragment) (A). The density of each band was analyzed by a bio-imaging analyzer Bas1000 (C, open circle). At each time point, freshly isolated thymocytes were stained with FITC–Annexin-V and PI, and analyzed by flow cytometry (B and C, open squares). Four mice were examined and typical data are presented in (A) and (B). Data of (C) represents mean ± SE.

γ-irradiation treatment, but TDAG8 expression was detected only after DEX treatment (38). It is shown that TDAG8 was induced by anti-TCR antibody treatment in primary cultured thymocytes (45). However, TDAG8 induction by DEX was not examined. At this time, we analyzed the correlation between TDAG8 expression and induction of apoptosis after DEX, anti-CD3 or γ-irradiation treatment using primary cultured thymocytes. The treatment with 10^{-9} or 10^{-8} M DEX did not induce thymocyte apoptosis (Fig. 2A) (14). Under these conditions, induction of TDAG8 expression was not detected in thymocytes by DEX stimulation (Fig. 2A). Apoptosis was markedly induced by DEX at the concentration of 10^{-7} M and TDAG8 expression was highly induced at this concentration. These results suggest that apoptosis was tightly associated with the expression of TDAG8 by DEX stimulation. Next, we determined whether TDAG8 expression is induced by other

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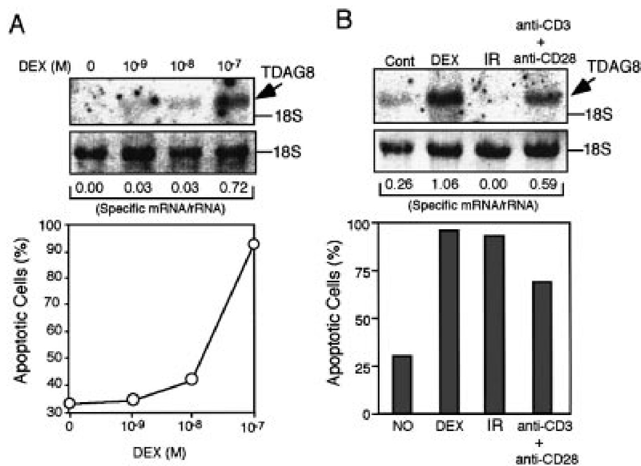

Fig. 2. Induction of TDAG8 gene expression and apoptosis in thymocytes treated with various stimuli. Thymocytes from C57BL/6 mice (6–8 weeks of age) were treated with indicated concentrations of DEX, anti-CD3 (20 μg/ml) and anti-CD28 antibodies (50 μg/ml) or γ-irradiation (10 Gy; IR). At 2 h, thymocytes were harvested and total RNA was extracted. Total RNA (10 μg) was subjected to Northern blot analysis and probed with TDAG8 cDNA (950-bp *Sty*I fragment). The density of each band was analyzed by a bio-imaging analyzer Bas1000 and marked as specific mRNA/rRNA. At 18 h, thymocytes were harvested, stained with FITC–Annexin-V and PI, and analyzed by flow cytometry. One representative of three independent experiments is shown.

apoptotic stimuli. Although γ-irradiation induced apoptosis in thymocytes, this treatment failed to induce TDAG8 expression (Fig. 2B). On the other hand, stimulation with anti-CD3 and anti-CD28 antibodies induced apoptosis in the majority of thymocytes, and TDAG8 expression by these stimuli was clearly detected, but weaker than that in DEX-treated thymocytes.

Establishment of TDAG8 transgenic mice

To further explore the functional role of TDAG8 on glucocorticoid-induced thymocyte apoptosis, transgenic mice overexpressing the TDAG8 gene specifically in immature thymocytes were generated. As shown in Fig. 3(A), *lck*^{Pr}-TDAG8 contained a full-length mouse TDAG8 cDNA downstream of the proximal murine promoter of the p56^{lck} gene, which is mostly active in immature thymocytes and is almost silent in mature peripheral T cells (39–41). The 3′-untranslated portion of this construct contained introns, exons and poly(A) addition sites from the human growth hormone gene. Six C57BL6 founder animals were identified and three lines (b, c and e) bearing the *lck*^{Pr}-TDAG8 construct were established. RNA expression levels of exogenous TDAG8 in their thymi were variable among the three lines by Northern blot analysis using a probe for the detection of exogenous TDAG8 (Fig. 3B). The result of quantification of each band for exogenous TDAG8 is shown in Fig. 3(C). The expression level of exogenous TDAG8 is highest in line b and lowest in line e within the three lines. The expression of the transgene was predominantly detected in the thymus by Northern blot analysis for various tissues in line b of transgenic mice (Fig. 3D).

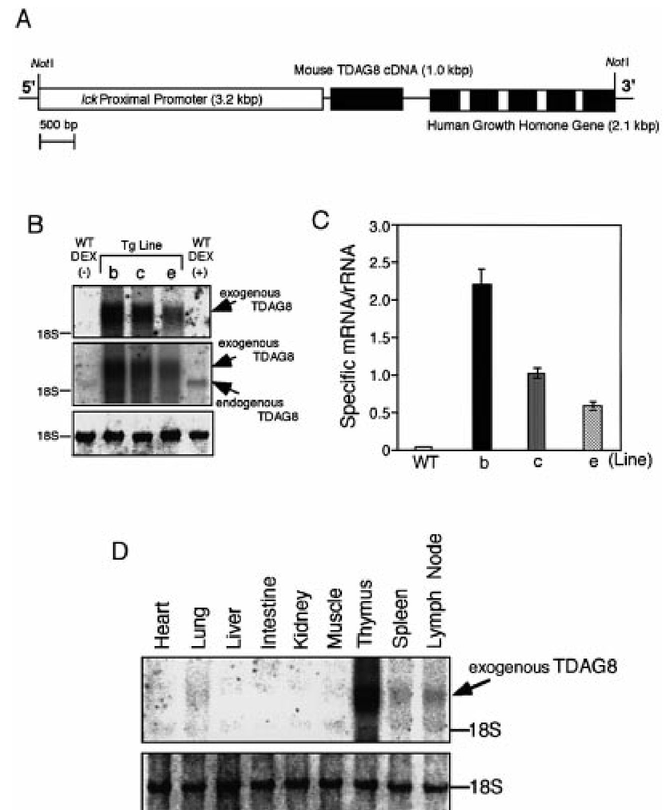

Fig. 3. TDAG8 transgene construct and expression of the TDAG8 transgene. (A) The 1.5-kbp fragment containing the coding region of mouse TDAG8 cDNA (1 kbp) (closed box) was inserted at the *Bam*HI site 3′ to the 3.2-kbp *lck*^{pr} (open box). Introns and exons (hatched boxes) of the human growth hormone constitute the 3′-untranslated region. (B) Total RNA was extracted from the thymus of three independent TDAG8 transgenic (Tg) founder mice and non-transgenic (WT) littermates (5–7 weeks of age). Total RNA (5 μg) was subjected to Northern blot analysis and probed with human growth hormone gene (1.4-kbp *Bam*HI–*Bgl*II fragment) or TDAG8 cDNA (950-bp *Sty*I fragment) for detection of exogenous (upper panel) or endogenous TDAG8 (middle panel) respectively. Four mice of each line were examined and typical data are presented. (C) The density of each band of exogenous TDAG8 in three lines of transgenic mice was analyzed by a bio-imaging analyzer Bas1000. Four mice of each line were examined and data represent mean ± SE. (D) Total RNA was extracted from the indicated tissues of line b TDAG8 transgenic mice. Total RNA (5 μg) was subjected to Northern blot analysis and probed with the human growth hormone gene (1.4-kbp *Bam*HI–*Bgl*II fragment) for detection of exogenous TDAG8. Four mice were examined and typical data are presented.

Overexpression of TDAG8 in thymocytes augments induction of thymocyte apoptosis

We analyzed gross anatomical changes, specifically in the thymus and spleen, in transgenic mice at 20 h after PBS (control) or DEX treatment. Representative data is shown in Fig. 4(A). In transgenic mice, although the size of the thymus was slightly smaller than that in non-transgenic mice in the case of PBS treatment, it was clearly smaller than that in non-transgenic mice after i.p. injection of DEX solution (100 μg/100 μl) into mice. In contrast, the size of the spleen did not differ between non-transgenic and transgenic mice in the case of PBS or DEX treatment. As shown in Fig. 4(B), the decrease

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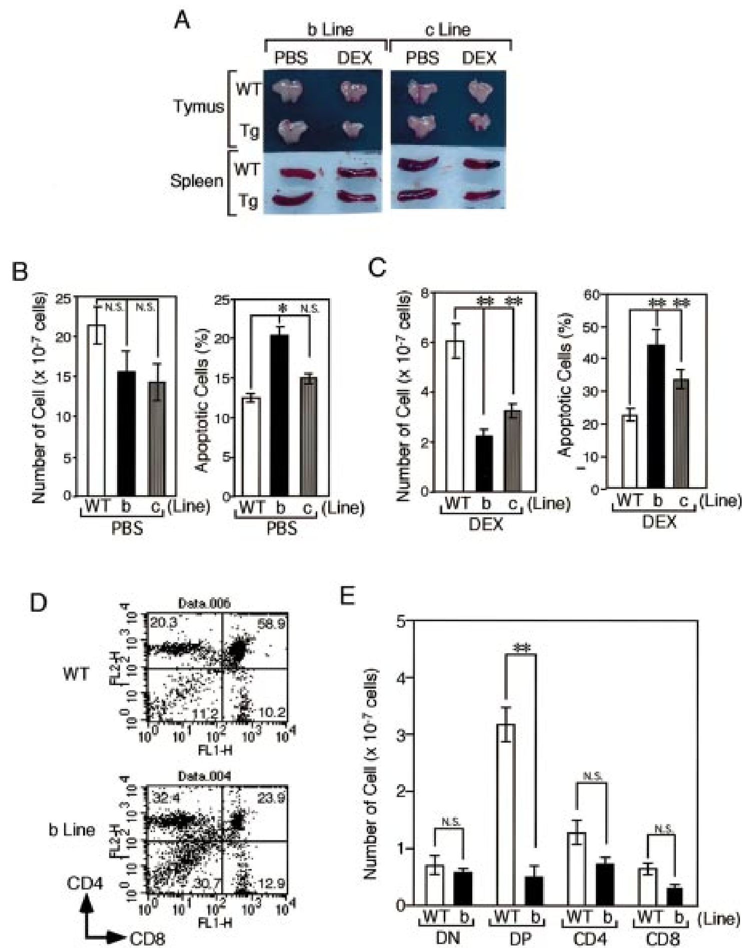

Fig. 4. Analysis of DEX-induced apoptosis in thymocytes of TDAG8 transgenic mice. TDAG8 transgenic (lines b and c; Tg) and non-transgenic (WT) littermates (7–10 weeks of age) were treated i.p. with DEX (100 μg/100 μl) or PBS (100 μl). After 20 h, thymus and spleen were isolated from DEX-treated mice (non-transgenic mice, n = 24; line b transgenic mice, n = 11; line c transgenic mice, n = 9) and PBS-treated mice (non-transgenic mice, n = 23; line b transgenic mice, n = 8; line c transgenic mice, n = 10). Gross morphological changes of the thymus and spleen (A), and total cell number and apoptotic cells of thymus were examined after PBS (B) or DEX (C) treatment. Isolated thymocytes were stained with FITC–Annexin-V and PI (B and C) or with FITC–anti-CD8 and PE–anti-CD4 antibodies (D), and analyzed by flow cytometry. The number of each population (DN, $CD4^-CD8^-$ double-negative cells; DP, $CD4^+CD8^+$ double-positive cells; CD4, CD4 single-positive cells; CD8, CD8 single-positive cells) was estimated from the total cell number and the ratio of each population (E). Typical data are presented in (A) and (D). Data of (B), (C) and (E) represent mean ± SE. *P < 0.01, **P < 0.005 and NS, not significant; two-way ANOVA when compared with non-transgenic mice.

of thymocyte number in two transgenic lines reflected the increased number of apoptotic cells (line b; P = 0.0223). This phenomenon was more obvious after DEX treatment (number of cells: line b, P < 0.0001; line c, P = 0.0045; apoptotic cells: line b, P < 0.0001; line c, P = 0.0005; Fig. 4C). The induction of thymocyte apoptosis in transgenic mice was detected at 6 h after DEX treatment and increased in a dose–response manner for DEX treatment (data not shown). The population of $CD4^+CD8^+$ double-positive thymocytes in transgenic mice decreased significantly as compared with that of non-transgenic mice after DEX treatment, reflecting relative increases in CD4 single-positive, CD8 single-positive and $CD4^-CD8^-$ double-negative populations (Fig. 4D). The number of $CD4^+CD8^+$ double-positive thymocytes dramatically decreased (P < 0.0001), but CD4 single-positive and CD8 single-positive cells did not strongly decrease (Fig. 4E). To detect the place where apoptosis is induced and the number of apoptotic cells in histology, apoptotic cells in thymic sections were analyzed using the TUNEL assay. In non-transgenic mice, the width of the thymic cortex became thinner after i.p. injection of DEX, anti-CD3 antibody or γ-irradiation (Fig. 5A) and apoptotic cells were detected within these areas of the cortex (Fig. 5B). More importantly, the number of apoptotic cells in the cortex of the thymus increased dramatically after DEX treatment in transgenic mice. On the other hand, the degree of apoptotic cells in anti-CD3 antibody- and γ-irradiation-induced thymocyte apoptosis was comparable between non-transgenic mice and transgenic mice. The degree of anti-CD3 antibody- and γ-irradiation-induced thymocyte apoptosis as judged by the number of Annexin-V^+ cells was also not different between transgenic mice and non-transgenic littermates (anti-CD3 antibody: non-transgenic, 23.67 ± 2.73%; transgenic, 24.88 ± 3.36%; γ-irradiation: non-transgenic, 45.34 ± 1.29%; transgenic, 47.36 ± 1.94%).

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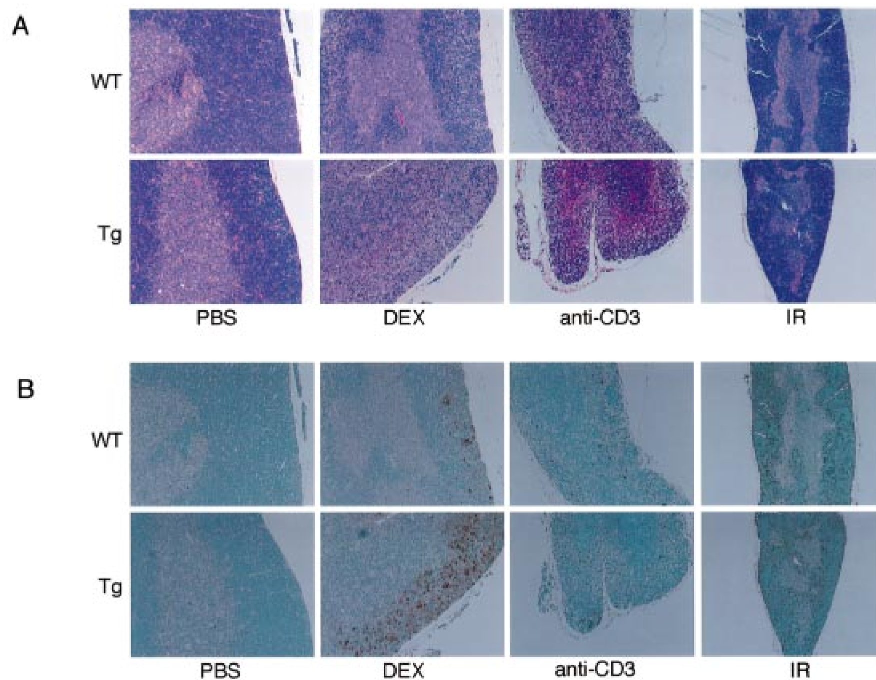

Fig. 5. Histological analysis of the thymus in DEX-treated TDAG8 transgenic mice. TDAG8 transgenic (line b; Tg) and non-transgenic (WT) littermates (7–10 weeks of age) were treated i.p. with DEX (100 μg/100 μl), anti-CD3 antibody (50 μg/100 μl) or PBS (100 μl), or with γ-irradiation (5 Gy; IR). Thymus was isolated from DEX or γ-irradiation-treated mice after 20 h, or anti-CD3 antibody-treated mice after 48 h, and stained with hematoxylin & eosin (A) or TUNEL (B). Three mice were examined and typical data are presented.

Caspase activation by expression of exogenous TDAG8 upon DEX treatment

Caspases, members of a family of cysteine-containing, aspartate-specific proteases, are the key apoptosis effectors (42–44). Recent studies have shown that glucocorticoid stimulation led to activation of caspase-3, -8, and -9 in thymocytes. If TDAG8 is involved in glucocorticoid-induced signaling for thymocyte apoptosis, TDAG8 transgene over-expression will enhance activation of these caspases in response to DEX. To test this hypothesis, we analyzed the cleavage of DEVD (Asp-Glu-Val-Asp)-, IETD (Ile-Glu-Thr-Asp)- or LEHD (Leu-Glu-His-Asp)-specific fluorescent substrates for caspase-3, -8 or -9 respectively, and also detected the active fragments of caspase-3 and -9 by Western blot analysis using cell lysates of thymocytes. TDAG8 transgenic, line b and non-transgenic littermates were treated with i.p. injection of PBS (100 μl) or DEX (100 μg/100 μl), and after 6 h thymocytes were isolated from these mice and used for analysis of caspase activation. Cleavage of DEVD-, IETD- or LEHD-specific substrates of caspases in the thymocytes of TDAG8 transgenic mice was significantly increased as compared with that of non-transgenic littermates in response to DEX (caspase-3, $P < 0.0001$; caspase-8, $P = 0.0001$; caspase-9, $P = 0.0008$; Fig. 6A). Moreover, as shown in Fig. 6(B and C), activation of pro-caspase-3 and -9 was indicated by the decrease of pro-enzyme forms and appearance of active fragments of caspases. The active fragments of caspase-3 and -9 in thymocytes of TDAG8 transgenic mouse were increased as compared with non-transgenic littermates in response to DEX.

Discussion

Glucocorticoid-induced thymocyte apoptosis requires the expression of genes that are critical for induction of apoptosis (28–31). There are several reports on the isolation of glucocorticoid-induced genes that mediate cell death (32–37). However, unfortunately, to date convincing data is lacking that shows that any of these candidates play such a role. In this study, TDAG8 was shown to be involved in glucocorticoid-induced signals to activate caspase-9, -8 and -3 for subsequent apoptosis induction in $CD4^+CD8^+$ double-positive thymocytes.

Using the differential display technique, we identified that TDAG8 is preferentially expressed in the thymus of DEX-treated mice (38). TDAG8 was cloned to be expressed in thymocytes from mice treated by anti-TCR antibody (45). Two independent studies demonstrated that the tissue distribution of TDAG8 expression was specific to lymphoid organs and abundantly expressed in the thymus (38,45). Apoptosis of the murine immature B cell lymphoma line, WEHI-231, was induced by anti-IgM antibody treatment, but TDAG8 expression was undetected (38). Therefore, it was also suggested that TDAG8 expression was specific for the thymocyte apoptosis process. It should be pointed out, however, that there was a difference between two independent studies with respect to the effect of TDA48 on TCR signaling or DEX-induced apoptosis. We have shown that DEX, anti-CD3 antibody and γ-irradiation treatment could induce apoptosis, as demonstrated by DNA fragmentation in murine T cell hybridoma, BD5-8. However, TDAG8 induction was detected only after DEX stimulation (38). Therefore, one important

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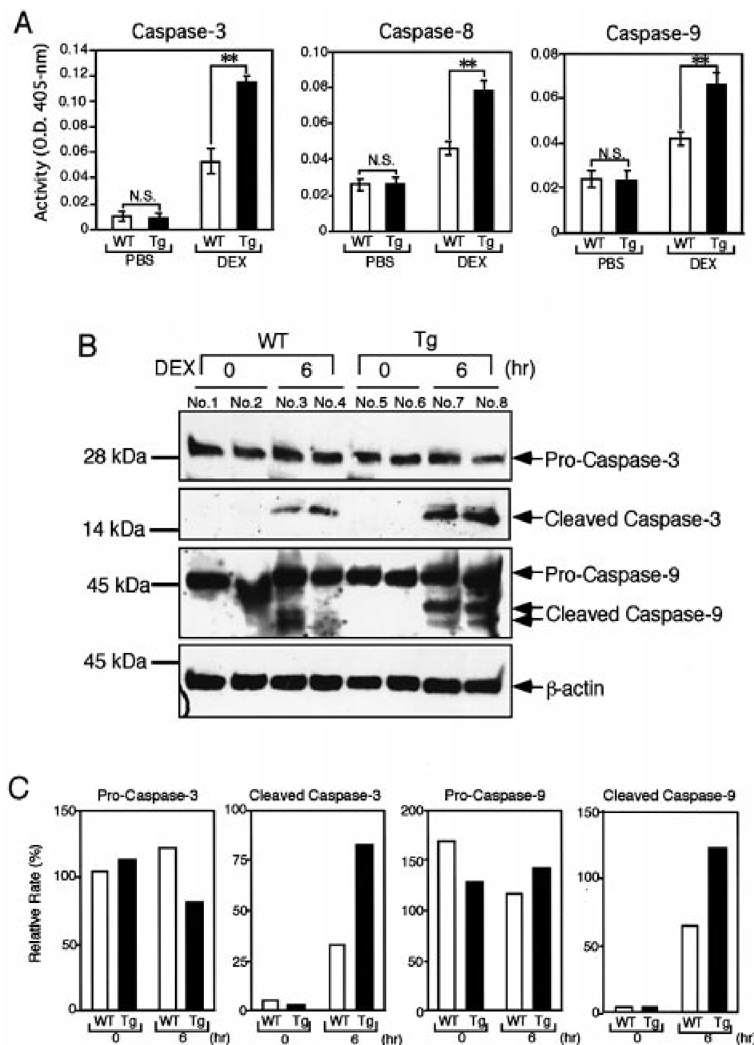

Fig. 6. Analysis of caspase activity in the thymocytes of DEX-treated TDAG8 transgenic mice. TDAG8 transgenic (line b; Tg) and non-transgenic (WT) littermates (7–10 weeks of age) were treated i.p. with DEX (100 μg/100 μl) or PBS (100 μl). After 6 h, thymocytes were isolated from PBS-treated non-transgenic mice (n = 4) and transgenic mice (n = 4) and DEX-treated non-transgenic mice (n = 7) and transgenic mice (n = 5), and used for assay of caspase-3, -8 or -9-like enzymatic activity (A), and Western blot analysis of caspase-3 and -9 (B). Caspase-3, -8 and -9-like enzymatic activity was measured as OD based on spectrophotometric detection of chromophore *p*NA light emission after cleavage from labeled DEVD-*p*NA, IETD-*p*NA or LEHD-*p*NA at 400 nm using a microtiter plate reader respectively. Data represent mean ± SE. *$P < 0.01$, **$P < 0.005$ and NS, not significant; two-way ANOVA when compared with non-transgenic mice. Western blot analysis was performed using anti-caspase-3, anti-cleaved caspase-3, anti-caspase-9 or anti-β-actin antibodies. At least four mice were examined and typical data are presented. Quantification of each band is expressed as percentage of density of β-actin counted in each band (C).

objective of this study was to clarify whether TDAG8 is critical for apoptosis induced by DEX treatment or anti-TCR antibody treatment.

It was previously shown that the TCR-mediated signal alone did not efficiently induce apoptosis of $CD4^+CD8^+$ double-positive thymocytes *in vitro* even when enhanced by co-engagement with CD4 or LFA-1 (46). The TCR-mediated signal delivers a potent apoptotic stimulus when combined with signals from the co-stimulatory molecule CD28 (46). To examine differences of stimuli in apoptosis and TDAG8 induction, thymocytes were treated with DEX, a combination of anti-CD3 and anti-CD28 antibodies or γ-irradiation *in vitro*. All these treatments induced thymocyte apoptosis as shown by Annexin-V staining (Fig. 2). However, γ-irradiation failed to induce TDAG8 expression, suggesting that TDAG8 is not involved in γ-irradiation-induced apoptosis. On the other hand, stimulation with a combination of anti-CD3 and anti-CD28 antibodies induced TDAG8 expression in thymocytes, but not in T cell hybridoma. These results suggest that TDAG8 may be involved in DEX- as well as TCR-mediated apoptosis in thymocytes. It was previously reported that thymocyte apoptosis induced by physiologic concentrations of glucocorticoid hormones was significantly inhibited by cross-linking with a proper concentration of anti-TCR or anti-CD3 antibody (10,14). Moreover, a recent study reported that thymocyte apoptosis induced by TCR activation was mediated by glucocorticoid *in vivo* (15). These results indicate that the signaling pathways triggered by TCR and GR-mediated

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apoptosis cooperate intricately for thymocyte development, and that the degree of each stimulation participates in negative and positive selection of the peripheral T cell antigen-specific repertoire of the adult animal.

In this study, we demonstrated that apoptosis was induced by DEX stimulation, which was shown by Annexin-V staining, and that TDAG8 expression was induced in thymocytes during this process in thymocytes. More importantly, TDAG8 expression preceded thymocyte apoptosis. To explore the role of the TDAG8 molecule in glucocorticoid-induced thymocyte apoptosis, we generated transgenic mice specifically overexpressing TDAG8 in immature thymocytes. The full-length mouse TDAG8 cDNA was inserted downstream of the *lck* proximal murine promoter, which is utilized extensively for overexpressing exogenous genes in immature thymocytes (19, 39–41,47–50). Expression of TDAG8 mRNA was detected in thymocytes from three transgenic animal lines by Northern blot analysis. The exogenous TDAG8 expression level was much stronger than that of endogenous TDAG8 (Fig. 3B and C). Moreover, expression of TDAG8 protein in COS7 cells transfected with the expression vector plasmid containing c-*myc* epitope fused with TDAG8 was detected by Western blot analysis using anti-c-*myc* antibody (data not shown).

Although the molecular mechanism of glucocorticoid-induced apoptosis is not fully clarified, glucocorticoid-induced thymocyte apoptosis is mediated via the mitochondrial pathway, which requires Apaf-1 and caspase-9, from analysis of Apaf-1 or caspase-9 null mice (16–18). It is known that caspase-8 and -3, in addition to caspase-9 are also activated by glucocorticoid stimulation (16–18). Although it is unknown whether caspase-8 is essential for glucocorticoid-induced thymocyte apoptosis, caspase-3 is not essential for glucocorticoid-induced thymocyte apoptosis since thymocytes from caspase-3 null mice undergo apoptosis with DEX treatment (16,17,51). In our results, after DEX treatment, activation of caspase-9, -8 and -3 was increased in thymocytes from TDAG8 transgenic mice as compared with non-transgenic littermates. Since caspase-3 is activated by caspase-9 or -8 (43,44), these results indicate that TDAG8 is critical for activation of caspase-9 or -8 in the signaling pathway of glucocorticoid-induced thymocyte apoptosis. Analysis of Apaf-1 null mice has shown that caspase-9 or -8 is likely located downstream of Apaf-1 in glucocorticoid-induced thymocyte apoptosis (17). Apaf-1 is activated by binding with ATP or dATP and cytochrome *c* translocated into the cytosol from mitochondria in cells undergoing apoptosis (22). It is known that the Bcl-2 family proteins are major regulators of the mitochondria-initiated caspase activation pathway (23–27). In the thymus, Bcl-x_L expression increases dramatically when T cells differentiate from double-negative thymocytes to double-positive thymocytes and is negligible in single-positive thymocytes (52). Recent studies have shown that Bcl-x_L can directly associate with caspase-8 and -9, and Apaf-1. In addition, caspase-9 activation is inhibited via Bcl-x_L association (53–55). Thus, there is a possibility that Bcl-x_L is involved in the regulation of caspase-9 and -8 activation in the thymocytes of TDAG8 transgenic mice by DEX treatment. Further work is required to show that Bcl-x_L is involved in TDAG8-mediated signaling. In this study, we demonstrate that TDAG8 is critical for activation of caspase-3, -8 and -9, and apoptosis induction in $CD4^+CD8^+$ double-positive thymocytes.

Acknowledgments

We would like to thank Dr H. Shima and Dr M. Morimatsu for their valuable advice on this manuscript. We also thank H. Murota and K. Yamaguchi for excellent technical support.

Abbreviations

TDAG8	T cell death-associated gene 8
DEX	dexamethasone
GR	glucocorticoid receptor
PE	phycoerythrin
PI	propidium iodide
*p*NA	*p*-nitroanilide

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