Apoptosis of nur77/N10-transgenic thymocytes involves the Fas/Fas ligand pathway

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ABSTRACT The orphan nuclear receptor Nur77/N10 has recently been demonstrated to be involved in apoptosis of T cell hybridomas. We report here that chronic expression of Nur77/N10 in thymocytes of transgenic mice results in a dramatic reduction of CD4+CD8+ double-positive as well as CD4+CD8- and CD4-CD8+ single-positive cell populations due to an early onset of apoptosis. CD4+CD8- double-negative and CD25+ precursor cells, however, are unaffected. Moreover, nur77/N10-transgenic thymocytes show increased expression of Fas ligand (FasL), while the levels of the Fas receptor (Fas) are not increased. The mouse spontaneous mutant gld (generalized lymphoproliferative disease) carries a point mutation in the extracellular domain of the FasL gene that abolishes the ability of FasL to bind to Fas. Thymuses from nur77/N10-transgenic mice on a gld/gld background have increased cellularity and an almost normal profile of thymocyte subpopulations. Our results demonstrate that one pathway of apoptosis triggered by Nur77/N10 in double-positive thymocytes occurs through the upregulation of FasL expression resulting in increased signaling through Fas.

The analysis of the molecular mechanisms leading to apoptosis is crucial to understand differentiation and developmental processes, in particular, in the immune system. During T cell development, most self-reacting immature thymocytes are eliminated by clonal deletion (negative selection) to establish immunological self-tolerance (1). This death occurs by apoptosis after an immature CD4+CD8+ double-positive T cell encounters antigen presented by self-major histocompatibility complex (1–3). The vast majority of apoptotic cells in the thymus, however, are probably a reflection of failure to undergo positive selection (4). Apoptosis in thymocytes induced by engagement of the T cell antigen receptor requires new gene transcription (5), but relatively little is known about the molecular mechanisms that mediate this response. Using subtractive hybridization, a differentially expressed cDNA, nur77/N10, has been recently isolated from libraries prepared from dying T cell hybridomas (6) or thymocytes (7). Moreover, blocking Nur77/N10 with a dominant negative (6) or antisense construct (7) and inhibition of the DNA binding activity of Nur77/N10 by the immunosuppressive drug cyclosporin A (8) prevent T cell receptor (TCR)-mediated apoptosis in hybridomas. Originally, nur77/N10 (also called NGFI-B in rat) was identified as an immediate-early gene that can be induced by a variety of stimuli in PC12 pheochromocytoma cells (9) and in fibroblasts (10, 11), suggesting that it has an important function in mediating responses to various cell stimulatory signals. Nur77/N10, a zinc finger transcription factor, shares structural features of the steroid/thyroid receptor superfamily, but because no ligand has been shown to regulate its transcriptional activity, it is referred to as an orphan nuclear receptor. N10/Nur77 is expressed in many tissues including thymus (11) and binds to the regulatory element 5'-AAAGTCA-3' as a monomer (12, 13). Recently, it has been demonstrated that it can also form heterodimers with the receptors for 9-cis-retinoic acid (14, 15). So far, genes that potentially are regulated by Nur77/N10 encode steroidogenic enzymes (16). However, mice with a targeted disruption of Nur77/N10 maintain normal steroidogenesis (17).

To investigate the role of this orphan nuclear receptor during T cell development further, we generated transgenic mice overexpressing a full-length nur77/N10 cDNA in the thymus. Our data show that chronic expression of Nur77/N10 in transgenic thymocytes is sufficient to induce apoptotic death of CD4+CD8+ double-positive cells. We also show that while expression of the Fas receptor is not increased, mRNA and protein levels of the Fas ligand (FasL) are upregulated. Indeed, crossing the nur77/N10-transgene into a FasL-mutant mouse strain efficiently reduces the apoptotic death of thymocytes and results in increased cellularity of double-positive cells.

MATERIALS AND METHODS

Mice. The coding region of the mouse nur77/N10 cDNA (11) was placed under the control of the mouse proximal lck promoter (3.2 kb; ref. 18). To obtain high translation efficiency, the initiation codon of nur77/N10 is preceded by the β-globin initiation signal. Human growth hormone gene sequences (2.1 kb) were added to confer stability to the mRNA transcripts, and a 2.0-kb fragment encompassing the human CD2 gene locus control region (LCR; ref. 19) was inserted at the 3' end (CD2 3'-LCR). Generation of transgenic mice and PCR genotyping of tail DNA was performed as described (20). Mice carrying the autosomal recessive mutation gld (B6Smm.C3H-Fasl−/−) were obtained from The Jackson Laboratory. To discriminate mice carrying the wild-type Fas ligand gene or the mutated gld allele, a PCR reaction using tail DNA was performed (20 cycles: 1 min at 95°C, 1 min at 44°C, 1 min at 72°C; primer sequences: 5'-CAACATATCCTCAACT-3' and 5'-AAGACTCTCATTCAAGAC-3') followed by a ligation chain reaction (21) with specific primers for the two genotypes (15 cycles: 1 min at 95°C, 4 min at 57°C; wild-type-specific primer, 5'-AATTGAGGAATCTAGA-CCT-3'; gld-specific primer, 5'-AATTGAGGAATCTACCGAC-3'; ligation partner for both reactions, 5'-TTTTCG-GCTTGTATAGGTTTA-3'). Wild type- and gld-specific primers were end-labeled at their 5' end with [γ-32P]ATP, and separate reactions together with the 5'-phosphorylated ligation partner were performed. Reaction products were separated in 15% denaturing polyacrylamid gels (ratio 1:20 N,N'-methylene-bisacrylamide/acrylamide, 8 M urea, 90 mM Tris, 90 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

Abbreviation: TCR, T cell receptor.

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**RNA and Protein Analysis.** Northern analysis using 10 \( \mu \)g of total RNA was performed as described previously (11). Purified probes were \(^{32}P\)-labeled using a nick translation kit from Amersham. RNA loading was controlled by analysis of expression of the glyceraldehyde-3-phosphate dehydrogenase (\( GAPDH \)) gene. Labeling of thymocytes with \(^{35}S\)labeled methionine and immunoprecipitation of lysates using Nur77/N10-specific antisera was performed as described previously (11, 20). For electrophoretic mobility shift assays, nuclear extracts from thymocytes were prepared (22), incubated with a \(^{32}P\)-labeled oligodeoxynucleotide (5'-TCGAGTTTTAAAAGGTCAT-GCTCAATT-3') encompassing the Nur77/N10 recognition sequence, and analyzed in a native 5% polyacrylamide gel as described (12, 13). Western blots using equal amounts of cytoplasmic extracts prepared from control and Nur77/N10-transgenic thymocytes were probed with FasL-specific antisera (Santa Cruz Biotechnology) as described previously (22).

**Histopathology and Immunohistochemistry.** Mice were sacrificed, and thymus and spleen were immersion-fixed in 10% buffered formalin. Tissues were embedded in paraffin blocks and processed by routine methods, sectioned at 4-6 \( \mu \)m thickness, stained with hematoxylin and eosin, and examined by light microscopy. DNA fragmentation in apoptotic cells was detected in situ by the TUNEL (23) procedure using paraffin tissue sections stained with the ApopTag kit from Oncor.

**Flow Cytometry.** Flow cytometry was done using a Coulter Epics Profile II flow cytometer and cell sorter. Single cell suspensions were prepared and analyzed for surface expression of CD4 (clone H129.19), CD8 (clone 53-6.7), CD25 (clone 7D4), TCR\( \beta \) (clone H57-597), Thy-1.2 (clone 53-2.1), and B220 (clone RA3-6B2) as described previously (24). Monoclonal antibodies were obtained from Gibco/BRL and Pharmingen. Apoptotic thymocytes with subdiploid DNA levels were analyzed after propidium iodide staining as described (25). Fas (clone Jo2) and FasL (rabbit polyclonal antiserum, Santa Cruz Biotechnology) expression on thymocytes was analyzed using fluorescein isothiocyanate- and phycoerythrin-conjugated secondary antibodies (Amersham, Boehringer Mannheim). Thymocyte subpopulations were analyzed for Fas and FasL surface levels by gating on CD8\(^{+}\) and CD25\(^{+}\) cells.

**RESULTS**

**Characterization of nur77/N10-Transgenic Lines.** To investigate the role of the mammalian transcription factor Nur77/N10 during T cell development, we generated several independent transgenic lines overexpressing a mouse nur77/N10 cDNA clone under the control of the \( \text{Ick} \) proximal promoter and CD2 3'-locus control region (Fig. 1a). These regulatory sequences confer copy number-dependent and integration site-independent expression of the transgene to all thymocyte subsets beginning at the earliest stages of T cell development (26, 27). Total thymus RNA from F1-transgenic animals was analyzed in Northern blots, and lines 2 and 14 with the highest levels of transgene mRNA expression were selected for further analyses (Fig. 1b). The results described here were observed in both lines 2 and 14 without any significant differences, whereas lines 18 and 19 showed no or only a very mild phenotype, respectively. Thymocytes from lines 2 and 14 also had increased Nur77/N10 protein levels compared with control littermates (Fig. 1c). Since DNA binding of Nur77/N10 in vivo may depend on a specific ligand or other factors, we examined whether increased Nur77/N10 levels result in increased nuclear DNA binding activity. In contrast to control cells, nuclear extracts from transgenic thymocytes presented strong binding to a Nur77/N10 target sequence, which was blocked by Nur77/N10 antiserum (Fig. 1d).

**Thymic Atrophy and Thymocyte Apoptosis in nur77/N10-Transgenic Mice.** The thymuses from nur77/N10-transgenic animals were dramatically reduced in size (Fig. 2A), and

![Fig. 1](image-url)
Fig. 2. Thymic atrophy and increased apoptosis in nur77/N10-transgenic mice. (A) Gross appearance of thymuses from adult control and transgenic (lines 2 and 14) mice. (B) Pyknotic cells with condensed chromatin in a hematoxylin and eosin stained thymus section from a 8-day-old nur77/N10-transgenic mouse (×100). (C and D) Early onset of apoptosis and decreased cellularity in newborn nur77/N10-transgenic thymus. Peroxidase-staining of fragmented DNA in thymus sections from control (C) and transgenic (D) mice (×40).

Histological examination revealed a decreased cellularity and a reduced medullary compartment (Table 1 and data not shown). We also observed an increase in small cortical thymocytes with condensed chromatin (Fig. 2B). Increased numbers of apoptotic cells in the thymic cortex of nur77/N10-transgenic mice were detected by the TUNEL procedure (23). Thymic atrophy and increase in apoptotic thymocytes were consistently observed in fetal, newborn, and adult transgenic animals (Fig. 2 C and D and data not shown). The spleen showed decreased cellularity in the periarterial lymphatic sheath, a mature T cell area. The B cell areas like the outer region of the white pulp, lymphatic follicles, and the marginal zone appeared normal (data not shown).

Flow cytometric analysis revealed that already 8–10 days after birth, CD4+CD8−, CD4−CD8+ and CD4−CD8− thymocyte populations were reduced in nur77/N10-transgenic mice (Table 1 and data not shown). This change in CD4 and CD8 expression was even more evident in 7-week-old transgenic animals (Fig. 3 a and b). Also, mature T cells expressing high levels of the T cell receptor α and β chains (TCRαβ) were absent in transgenic thymus. The TCRαβ population was reduced, and a relative increase in TCR− cells was observed.

Table 1. Decreased cellularity in nur77/N10-transgenic thymuses

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<tr>
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<th>Control</th>
<th>Transgenic</th>
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<tr>
<td>Mean total cellularity (cells × 10⁶)</td>
<td>125 ± 21.9</td>
<td>9.0 ± 2.1</td>
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<tr>
<td>CD25-positive cells (cells × 10⁶)</td>
<td>5.5 ± 1.2</td>
<td>5.4 ± 1.0</td>
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<td>Trypan blue-positive cells (%)</td>
<td>6.9 ± 1.4</td>
<td>21.5 ± 2.8</td>
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CD25-positive thymocytes were calculated from subpopulation percentages and total thymocyte counts. Values are means from at least four 10-day-old animals ± SE.

In contrast, we detected a dramatic relative increase in double-negative CD4−CD8− thymocytes and early CD25+ precursor cells (Fig. 3 a and b as well as e and f). However, the absolute number of CD25+ cells per thymus was comparable with controls (Table 1). Flow cytometric analysis of cell DNA content revealed a strong increase in thymocytes with subdiploid DNA levels in nur77/N10-transgenic animals (Fig. 3 g and h). Trypan blue staining of thymocytes also showed increased numbers of dead cells in transgenic mice (Table 1). Examination of splenocyte populations showed a marked reduction in the number of Thy-1.2+ T cells and a relative increase of B220+ B cells (Fig. 3 i and j). We did not detect any significant expression of CD3, CD4, CD8, or TCRαβ on transgenic splenocytes, whereas the number of IgM+ B cells was increased. Thy-1.2+ cells were also absent from peripheral blood (data not shown). These results correlate with the histological changes observed in the spleen of nur77/N10-transgenic animals.

Increased Expression of Fas Ligand in nur77/N10-Transgenic Thymocytes. It has been shown that Nur77/N10 can be activated by T cell receptor engagement (6–8), but the mechanisms underlying the apoptotic death of thymocytes are still largely unknown. Recently, it has been demonstrated that binding of Fas (CD95/APO-1) to its ligand FasL (APO-1L, ref. 28) is required for programmed cell death after T cell activation (29–31). We therefore examined the expression of Fas and FasL in nur77/N10-transgenic thymocytes. Fas is expressed in mouse thymus (32, 33), and we observed no increased Fas mRNA levels in transgenic thymocytes (data not shown). In contrast to Fas, Fasl mRNA expression in normal thymus is very low but increases strongly after lymphocyte activation (34, 35). While we also detected only very low expression of Fasl in control mice, nur77/N10-transgenic thymuses showed high Fasl mRNA (Fig. 4 a) and protein levels (Fig. 4 b).

Flow cytometric analysis confirmed the increased FasL expression on transgenic thymocytes (Fig. 4 c). To examine whether this was a consequence of altered thymocyte popu-
lations in nur77/N10-transgenic animals, we analyzed CD25+ and CD8+ thymocyte subpopulations for FasL expression. Interestingly, CD25+ cells from control mice, representing 2–3% of the total thymocyte population, expressed FasL, whereas CD8+ thymocytes were FasL−. CD25+ thymocytes from transgenic animals showed similar levels of FasL expression. In contrast to control cells, however, transgenic CD8+ thymocytes expressed FasL (Fig. 4 c). Expression of Fas on transgenic CD8+ thymocytes was slightly reduced compared with control cells (Fig. 4 c). This could be due to receptor internalization after signaling and/or apoptotic death of cells expressing high levels of Fas. Similar results were obtained when CD4+CD8+ thymocytes were analyzed for Fas and FasL expression (data not shown). These results indicate that both the relative increase in CD25−FasL+ cells and the up-regulation of FasL expression in double-positive thymocytes account for the dramatic increase in FasL mRNA and protein in nur77/N10-transgenic thymocytes.

**Decreased Apoptosis in nur77/N10-Transgenic Mice Carrying a Mutant FasL.** The mouse spontaneous mutant gld (generalized lymphoproliferative disease) carries a point mutation in the extracellular domain of the FasL gene that abolishes the ability of FasL to bind to Fas (36, 37). To genetically link Nur77/N10-induced apoptosis to the Fas/FasL pathway, we crossed nur77/N10-transgenic with gld-mutant animals. Thymuses from nur77/N10-transgenic, nontransgenic gld/gld, and nur77/N10-transgenic mice on a gld/gld background were analyzed by flow cytometry. CD4+CD8+, CD4+CD8−, CD4−CD8+, and CD4−CD8− thymocytes from nur77/N10-transgenic animals showed almost normal distribution in the presence of a mutated FasL molecule (Fig. 5). In addition, CD25+ precursor cells showed a relative decrease compared with nur77/N10-transgenic animals with a functional FasL gene (data not shown). The total cellularity was also increased (8-fold), demonstrating that the apoptosis in nur77/N10-transgenic mice is reduced in mice with a defective Fas/FasL pathway.

**DISCUSSION**

Here we report that the orphan receptor Nur77/N10 up-regulates expression of FasL, but not Fas, leading to apoptotic death of transgenic thymocytes. Recently, a 400-bp sequence upstream of the ATG codon of the FasL gene was cloned, and NF-κB, IRF-1, and SP-1 binding sites were identified (38). However, their functional significance and whether Nur77/N10 directly or indirectly participates in the transcriptional regulation of the FasL gene requires further investigation.

Fas is not expressed on CD25+ precursors of double-positive thymocytes, and the onset of Fas expression parallels the

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**Fig. 4.** Increased FasL expression in nur77/N10-transgenic thymocytes. (a) Northern blot analysis of FasL mRNA levels in control and nur77/N10-transgenic thymuses. The filter shown in Fig. 1b was rehybridized with a 1-kb mouse FasL cDNA probe. (b) Western blot of thymocyte extracts probed with FasL-specific antiserum. (c) Flow cytometric analysis of Fas and FasL expression on thymocyte subpopulations from 10-day-old control and transgenic mice.
acquisition of CD4 and CD8 surface markers on thymocytes (ref. 32 and data not shown). Our finding that FasL is expressed on CD25+ and down-regulated on double-positive thymocytes was surprising, and its role during T cell development remains to be established. In contrast to thymocytes, transgenic CD4+CD8+ Fas+ thymocytes express membrane-bound FasL, suggesting a suicide mechanism in cis. Our data indicate that Nur77/N10 induces apoptosis by increasing FasL-mediated signaling in Fas-bearing thymocytes without affecting CD25+Fas- precursors. A similar mechanism has been proposed for the apoptotic death of peripheral activated T cells triggered by the HIV-1 Tat and gp120 proteins. These molecules also upregulate the cellular expression of FasL without affecting Fas mRNA levels (39).

Recently, it has been shown that thymic and peripheral T cell death is unimpaired in mice with a targeted disruption of Nur77/N10 (40). Thus, nur77/N10 is not a single gene uniquely required for the TCR-mediated death program. However, the removal of autoreactive thymocytes as well as peripheral clonal deletion and the elimination of activated T cells are crucial for a functional immune system. It is therefore likely that related members of the Nur77/N10 family such as Nurrl (41) and NOR-1 (42) or other factors compensate for the loss of Nur77/N10 function in these animals. In a gain of function approach using transgenic mice, we demonstrate here that overexpression of Nur77/N10 is sufficient to induce apoptosis in thymocytes.

DNA binding of Nur77/N10 is activated by TCR engagement on thymocytes (6), and our results that Nur77/N10 increases both mRNA and cell surface expression of FasL, leading to programmed cell death of transgenic thymocytes, link this orphan nuclear receptor to the Fas/FasL pathway of programmed cell death. Mice carrying the spontaneous mutations lpr (lymphoproliferation) and gld (generalized lymphoproliferative disease) develop lymphadenopathy and splenomegaly due to an abnormal accumulation of lymphocytes (28, 43). The lpr and gld mutations are loss of function mutations of Fas and FasL, respectively (44, 36, 37). Whereas peripheral clonal deletion and elimination of activated T cells are impaired in lpr mice, positive and negative selection appear to be normal in these animals (45, 46). Similarly, gld mice show defects in the clonal deletion of autoreactive T cells in the periphery (28, 43). However, the precise role of Fas/FasL interactions during thymocyte development is still unclear (32, 45, 47, 48). To examine whether the Nur77/N10-mediated apoptosis of thymocytes is dependent on FasL, we crossed the Nur77/N10 transgene onto a gld/gld mutant background. Our data show that the absence of a functional Fasl molecule rescues the nur77/N10-transgenic phenotype, although not completely. This finding genetically places Nur77/N10 upstream of FasL but also suggests that functional Nur77/N10-mediated signal transduction pathways resulting in apoptosis of thymocytes exist. We also observed a partial rescue of the nur77/N10-transgenic phenotype in mice homozygous for the lpr allele (data not shown), further supporting the importance of the Fas/FasL pathway in Nur77/N10-mediated thymocyte apoptosis. Recently, it has been demonstrated that tumor necrosis factor can mediate mature TCR-induced apoptosis through the p75 tumor necrosis factor receptor (49). Although it is unclear whether this mechanism is also operative in more immature thymic T cells, it will be interesting to examine the role of the p75 tumor necrosis factor receptor in nur77/N10-transgenic mice.

Note. Subsequent to the submission of this manuscript, an independent report by Calnan et al. (50) has also shown that constitutive expression of Nur77/N10 induced apoptosis in developing thymocytes.

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