that the small Mafs also regulate batteries of genes that are not under Nrf2 regulatory influence. However, it does not exclude the possible involvement of Nrf2, because double or triple mutations including *nrf2* and other CNC transcription factor genes may recapitulate these phenotypes.

We have shown in this study that both *nrf2*-null mutant mice and small *maf* compound null mutant mice confer a similar rescue phenotype to *keap1*-null mutant mice, demonstrating that Nrf2-small Maf heterodimers play indispensable roles in keratinocytic gene expression. Considering the fact that excess small Mafs have been shown to repress transcription by forming inactive homodimers, keratinocytic overexpression of small Mafs would also be predicted to rescue *keap1*-null lethality. A recent study showed, using chromatin immunoprecipitation, that Nrf2 and small Maf are recruited to a MARE element in the mouse quinone reductase gene promoter when the gene is activated (43). This result is in very good agreement with the cooperative gene activation model executed by Nrf2 and small Maf that we propose here. In addition to oxidative stress-inducible genes, Nrf2 has been recognized as a critical regulator of other biological processes, including wound healing (49), endoplasmic reticulum stress response (40), inflammation resolution (50), and apoptosis (51). We suggest that the contribution of small Mafs to each of these Nrf2-dependent processes should be individually evaluated; some of the processes may depend on both Nrf2 and small Mafs, whereas the others may be independent of any small Maf contribution. Finally, the compound knockout–rescue approach exploited in the present study is an effective system for evaluating the *in vivo* contribution of test regulatory factors to Nrf2 activity, as long as mice deficient in these test factors are available. It would be interesting to apply this system to other candidate molecules that have been touted to be required for Nrf2 transcription activity, including other factors in the transcriptional machinery itself as well as specific signal transduction pathways.

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Nrf2 deficiency improves autoimmune nephritis caused by the *fas* mutation *lpr*

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Nrf2 deficiency improves autoimmune nephritis caused by the *fas* mutation *lpr*.

Background. Nrf2 is a basic leucine zipper transcriptional activator essential for the coordinate transcriptional induction of antioxidant and phase II drug metabolizing enzymes. We previously reported that Nrf2-deficient female mice develop lupus-like autoimmune nephritis (*Kidney Int* 60:1343–1353, 2001). The result suggested that *nrf2* is a possible candidate gene in determining susceptibility to autoimmune diseases. MRL/*lpr* mice, defective in Fas-mediated apoptosis, develop glomerulonephritis due to the production of autoantibodies.

Methods. To investigate the mechanism whereby Nrf2 contributes to the susceptibility to autoimmune diseases, we generated *nrf2 −/− lpr/lpr* mice.

Results. Unexpectedly, the lifespan of *nrf2 −/− lpr/lpr* female mice was markedly prolonged and these mice showed an improvement in nephritis compared to *nrf2+/+ lpr/lpr* female mice. Immunologic abnormalities and hypergammaglobulinemia were also alleviated in *nrf2−/− lpr/lpr* female mice. Furthermore, lymphadenopathy was suppressed as a result of increased apoptosis. To elucidate the molecular mechanism causing a stimulation of apoptosis, we analyzed the response made by *nrf2 −/− lpr/lpr* mice to death signals. We show that *nrf2−/− lpr/lpr* mice are sensitive to tumor necrosis factor-α (TNF-α)–mediated apoptosis. Since intracellular glutathione levels are decreased in Nrf2-deficient cells, it is probable that a prolonged depletion in glutathione levels leads to the enhancement in TNF-α–mediated apoptosis.

Conclusion. These results indicate that a deficiency in Nrf2 enhances TNF-α–mediated apoptosis which in-turn ameliorates the abnormal apoptotic response that arises from a mutation in the *lpr* gene. Therefore, Nrf2 deficiency acts as a suppressor of the autoimmune accelerating gene *lpr*.

Nrf2 [nuclear factor-erythroid 2 (NF-E2–related factor 2)] is a basic leucine zipper (b-Zip) transcriptional activator, originally identified as a protein capable of binding the NF-E2 promoter element [1, 2]. Nrf2 is essential for the coordinate transcriptional induction of antioxidant and phase II drug metabolizing enzymes through the antioxidant response element/electrophile response element (ARE/EpRE) [3, 4]. The ARE/EpRE sequence has been identified within the proximal regulatory sequences of antioxidant genes encoding glutathione S-transferase (GST) [5], NAD(P)H quinine oxidoreductase (NQO1) [6], heme oxygenase-1 (HO-1) [7], γ-glutamylcysteine synthetase [8], and cystine membrane transporter (system Xc^-) [9]. The ARE also regulates a wide range of metabolic responses to oxidative stress caused by reactive oxygen species (ROS) or electrophiles [9]. Understandably, targeted disruption of the *nrf2* gene in mice renders the animals sensitive to high levels of oxidative stress due to impairment in this stress response system [9–11].

We previously reported that aged Nrf2-deficient female mice presented with several immunopathologic features characteristic of human lupus, including splenomegaly, proteinuria, production of antinuclear antibodies, mesangial and capillary immune complex deposition with subsequent proliferative or crescentic change within glomeruli, and, eventually, renal failure and death [12]. These findings indicate a role for oxidative stress in the development of lupus, and also point to *nrf2* as a possible candidate gene in determining susceptibility to autoimmune diseases. A number of lupus models and mouse strains have been established and studied extensively with respect to disease pathogenesis, including MRL/Mp-*lpr/lpr* (MRL/*lpr*) [13], BXSB/MpJ*Yaa* [13], and NZB/NZW F1 [14, 15]. However, linkage of the *nrf2* gene to any of these lupus strains has not been described previously. MRL/*lpr* mice spontaneously develop lymphadenopathy and an autoimmune disorder characterized by immune complex glomerulonephritis, arthritis, vasculitis, and antinuclear antibodies [13]. The predominant cell type that occupies the lymphoid organ are phenotypically double negative ($CD4^-CD8^-$) with respect to T-cell surface markers. These cells also possess the B220 cell surface marker usually restricted to B cells. The

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double negative T cells also display hallmarks of mature T lymphocytes, including CD3 and the αβ-TCR [13]. The genetic defect that leads to lymphoproliferation *(lpr)* is a mutation in the gene coding Fas [16], a cell surface molecule that belongs to the tumor necrosis factor (TNF) receptor gene family. The development of lymphadenopathy and of autoimmune disease in *lpr* mice has been attributed to a defect in Fas-mediated apoptosis causing a failure in the activation-induced cell death of activated T cells [16, 17]. Thus, the *lpr* gene is believed to be the fundamental molecular abnormality in MRL/*lpr* mice. However, the spectrum of autoimmune manifestations is greatly affected by the host genetic background. For example, when the *lpr* is crossed on to the MRL/Mp strain, the disease is markedly accelerated, and most mice die by 6 months of age [18]. Whereas, MRL/*lpr*, *lpr* congenic mice of the C3H/HeJ, AKR or C57BL/6 background rarely develop nephritis or vasculitis [19–21]. These results indicate that the development of autoimmune diseases in MRL/*lpr* mice requires both an accelerating gene (*lpr*) and susceptibility genes (genes specific to the genetic background).

To further define the mechanism whereby Nrf2 determines the susceptibility to autoimmune diseases, we generated *nrf2−/− lpr/lpr* female mice and estimated the survival rate and the development of glomerulonephritis.

Unexpectedly, *nrf2−/− lpr/lpr* female mice had a significantly longer lifespan and less severe nephritis than *nrf2+/+ lpr/lpr* female mice. Detailed analyses revealed that, in *lpr* mice, a deficiency in Nrf2 enhanced the sensitivity of TNF-α–mediated apoptosis and suppressed the autoimmune acceleration effect of the *lpr* gene. These results demonstrate that Nrf2 deficiency may act as suppressor of the autoimmune accelerating gene *lpr*, instead of being a susceptibility gene in these mice.

METHODS

Generation of *nrf2* mutant *lpr* mice

The generation of Nrf2-deficient mice was previously described [4]. Genotypes of homozygous wild-type and Nrf2-deficient mice were confirmed by polymerase chain reaction (PCR) amplification of genomic DNA isolated from tails. PCR amplification was carried out by using three different primers, 5′-TGGACGGGACTATTGAA GGCTG-3′ (sense for both genotypes), 5′-CGCCTTTT CAGTAGATGGAGG-3′ (antisense for wild-type), and 5′-GCGGATTGACCGTAATGGGATAGG-3′ (antisense for LacZ). MRL/*lpr* mice were obtained from Japan SLC (Shizuoka, Japan). Intercrossing Nrf2-deficient mice with an ICR background and MRL/*lpr* mice generated *nrf2−/− lpr/lpr* mice and control *nrf2+/+ lpr/lpr* mice. The present study utilized female mice from the same litters. Mice were maintained in the Laboratory Animal Resource Center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals in University of Tsukuba.

Measurement of urinary protein, serum creatinine, and blood urea nitrogen

The urine of each mouse was collected in individual metabolic cages over a 24-hour period from *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* 20-week-old female mice. The amount of proteinuria was assessed by measuring the turbidity obtained with 3% sulphosalicylic acid. Protein values more than 2 mg/24 hours were considered abnormal. The concentration of serum creatinine and blood urea nitrogen (BUN) were measured by an automated analyzer for routine laboratory tests (Dry-Chem 3500) (Fuji Film, Inc., Tokyo, Japan).

Histopathologic analysis of renal tissues

Each mouse was bled while under ether anesthesia. Sera were stored at −80°C until use. At autopsy, organs were fixed with 10% formalin in 0.01 mol/L phosphate buffer (pH 7.2) and embedded in paraffin. Sections were stained with hematoxylin and eosin for histopathologic examination under light microscopy. For semiquantitative histologic analysis, more than 20 glomeruli from each kidney section were examined. The degree of glomerular lesion was estimated using a scale of 0 to 3 based on the severity and extent of histopathologic changes, as described previously [19]. The index of glomerular lesion is the mean value of more than 20 observed glomeruli. Sections frozen for immunofluorescent analysis were stained using fluorescein isothiocyanate (FITC)-labeled antimouse IgG, IgG1, IgG2a, IgG3, IgM, and C3 (ICN Pharmaceuticals, Inc., Aurora, OH, USA). Quantitative estimation of immunofluorescent staining was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Relative fluorescent intensity was described as the mean fluorescent intensity of *nrf2+/+ lpr/lpr* mice equals 1.0.

Immunohistochemical examination

Kidneys that were to be used for immunoperoxidase staining were snap-frozen in optimal cutting temperature (OCT) (Miles Scientific, Naperville, IL, USA) and stored at −80°C. We identified macrophage (F4/80), CD4-, CD8-, and B220-positive cells using monoclonal antibodies as described by Iwata et al [22]. Anti-CD4, CD8, and B220 antibodies were purchased from BD Biosciences (San Jose, CA, USA) and anti-F4/80 antibody from Serotec (Oxford, UK). These cells were identified by avidin-biotin complex immunoperoxidase technique using Histofine Kit (Nichirei, Tokyo, Japan). We counted macrophage, CD4-, CD8-, and B220-positive cells in periglomerular areas of 10 randomly selected glomeruli

and have expressed the results as cells/glomerulus. Interstitial CD4-, CD8-positive T cells and B220-positive cells were counted in 10 randomly selected fields of cortical interstitium using a light microscope (magnification, ×400) and described as/fields.

Cytokine expression

We evaluated renal colony stimulating factor 1 (CSF-1), monocyte chemoattractant protein 1 (MCP-1), TNF-α, transforming growth factor 1 (TGF-β1) and interferon γ (IFN-γ) transcripts by reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA was extracted from the kidney using TRIzol (Invitrogen, Carlsbad, CA, USA). RT was performed using the SuperScript First-Stranded Synthesis System for RT-PCR (Invitrogen). The complementary DNA product was amplified by PCR. Primers (CSF-1, 5′ primer AAA GCCACTCTTGGGGCATT, 3′ primer TCGATGGCT CCACTTCC; MCP-1, 5′ primer AGCAGGTGTCCCA AAGAAGC, 3′ primer ACAAAGTTTACCCATTCA TC; TNF-α 5′ primer AGGTTCTCTTCAAGGGAC AA, 3′ primer TCACAGAGCAATGACTCCAA; TGF-β1, 5′ primer GGACCTGGGTTGGAAGTGGA, 3′ primer GCGACCCACGTAGTAGACGA; IFN-γ, 5′ primer CAAGTGGCATAGATGTGGAA, 3′ primer G TTGTTGACCTCAAACTTGG; GAPDH, 5′ primer CCCCTTCATTGACCTCAACTACATGG, 3′ primer GCCTGCTTCACCACCTTCTTGATGTC) were used for PCR. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for PCR controls.

Total glutathione (GSH) level of kidneys

Kidneys from *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* 20-week-old female mice were homogenized and suspended in 5% sulfosalicylic acid. Subsequently, total GSH level was measured by an enzyme recycling method using the Total Glutathione Quantification Kit (Dojindo, Kumamoto, Japan).

Measurement of serum immunoglobulin

Serum immunoglobulin was determined by enzyme-linked immunosorbent assay (ELISA) as previously described [12]. Briefly, Nunc-Immunoplates (Nunc A/S, Roskilde, Denmark) were coated with goat antimouse immunoglobulin (ICN Pharmaceuticals, Inc.). The plates were kept at room temperature for 1 hour and were then washed with 0.1 mol/L phosphate-buffered saline (PBS). After washing, the plates were blocked with 0.5% bovine serum albumin (BSA) in a PBS solution. Serial dilutions of test serum samples were applied and incubated at room temperature for 1 hour. After washing with PBS, the plates were treated with alkaline phosphatase-conjugated goat antimouse IgM, IgG, IgG1,and IgG2a (Sigma Chemical Co., St. Louis, MO, USA) at room temperature for 1 hour. After additional washes, alkaline phosphatase substrate (Sigma Chemical Co.) solution was added and allowed to develop. Absorption at 405 nm was measured using an immuno-plate reader (BenchMark) (Bio-Rad, Hercules, CA, USA). In order to measure IgG3 levels, single radial immunodiffusion (SRID) was performed. One percent agalose gels in 0.1 mol/L PBS, pH7.2, which contained anti-Ig isotype serum (rabbit antimouse IgG3), was purchased from Miles Laboratories Inc. (Elkhart, IN, USA) were prepared and spread on a glass plate. Samples (4 μL) were applied to punched holes of 1.5 mm in diameter and the slides were incubated at room temperature for 48 hours. Precipitation rings were measured after staining with AminoBlock 10B.

Anti-double-stranded DNA antibody assay

ELISA was used to determine the titer of anti-double stranded DNA antibody in sera as described previously [19]. Nunc-Immunoplates were coated with protamine sulfate (Sigma Chemical Co.). Subsequently, a solution of double-stranded DNA was added to the plates. The double-standed DNA was prepared from calf thymus DNA treated with S1 nuclease (Sigma Chemical Co.) to eliminate single-stranded DNA according to the published method [23]. The plates were kept at 4°C overnight and then washed with PBS. Afterward, the plates were blocked with 0.5% BSA in PBS solution. Serial dilutions of test serum samples were added and incubated at room temperature for 1 hour. After washing with PBS, the plate was treated with alkaline phosphatase-conjugated goat antimouse IgG (Sigma Chemical Co.) and processed as described previously in this article. The titer of anti-double-stranded DNA antibodies in pooled sera from four 20-week-old MRL/*lpr* female mice was set at 100 units.

Lymphadenopathy scoring

Arbitrary clinical scoring of lymphnodes was assigned by an observer blinded to group identities, using a scale of 0–4 (0 = none; 1 = a single node anywhere; 2 = bilateral axillary; 3 = femoral, or cervical nodes; and 4 = massive generalized adenopathy) [24].

Fluorescence-activated cell sorter (FACS) analysis

Single-cell suspensions were prepared from the lymphnodes of each mouse and blocked with anti-FcγR antibody (2.4G2) for 10 minutes on ice to inhibit the interaction of staining reagents with the cell surface. Multicolor flow cytometry analysis was performed using LSR and Cellquest software (Becton Dickinson,

Franklin Lakes, NJ, USA) on viable cells as determined by forward light scatter intensity and propidium iodide exclusion. The following phycoerythrin (PE), FITC, or peridinin chlorophyll protein (perCP)-labeled monoclonal antibodies were used: anti-CD4-PE, anti-CD8-FITC, anti-CD3-perCP (BD Biosciences) and anti F4/80-PE (Serotec). Bilateral cervical and axillary lymphnodes were obtained from *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice. Total cell numbers of each lymphocytes and macrophages were counted.

Terminal deoxynucleotidyl transferase (TdT) nick end-labeling (TUNEL) assay

Apoptotic cells were estimated by the TUNEL assay, which relies on incorporation of labeled deoxyuridine triposphate (dUTP) at sites of DNA breaks. For the TUNEL procedure, all reagents, including buffer, were part of a kit (In situ Takara Apoptosis Kit; Takara, Inc., Otsu, Japan). Procedures were carried out according to the manufacturer's instructions. Apoptosis was examined in 5 μm thick sections of the spleens, kidneys, and livers of *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice. For quantitative histologic analysis, the degree of apoptosis was estimated using a scale based on the mean number of TUNEL-positive cells per 20 microscopic fields, or per field (magnification, ×400).

Induction of splenocyte apoptosis by TNF-α

Splenocytes were isolated from 8-week-old *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice. The cells were washed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.5 mmol/L 2-mercaptoethanol, 2 mmol/L glutamine, 1 mmol/L *N*-2-hydroxyethylpiperazine-*N'*−2-ethanesulfonic acid (HEPES) (pH 7.4) and antibiotics (all from Invitrogen Inc.). The cells were again washed in medium before use in subsequent tissue culture experiments. The cells (1×10^6/mL) were incubated with various concentrations of TNF-α for 12 hours. TNF-α was purchased from BD Biosciences. Cell viability was determined by trypan blue exclusion. The cells were preincubated with glutathione ethyl monoester (GSH-OEt) (2 mmol/L for 15 to 20 minutes) to increase the intracellular pool of GSH. GSH-OEt was obtained from Wako (Osaka, Japan).

Hepatocellular apoptosis induction by administration of anti-Fas antibody and TNF-α in mice

Agonistic antimouse Fas antibody (Jo-2) (BD Biosciences) (2 mg/mouse) was administered to 20-week-old female mice intraperitoneally in sterile saline solution. Human recombinant TNF-α (BD Biosciences) (0.5mg/mouse) was also administered intraperitoneally. To increase the TNF-α sensitivity, D-galactosamine hydrochloride (GalN) (Nacalai Tesque, Tokyo, Japan) was preinjected intravenously 30 minutes before TNF-α injection. To assess the importance of the intracellular GSH level, GSH-OEt (20 mg/mouse) was preinjected intravenously 2 hours before TNF-α injection and the same experiments performed. Hepatocellular injury was monitored biochemically by measuring serum alanine aminotransferase (ALT) activity using an automated analyzer (Dry-Chem 3500) (Fuji Film). At the end of the experimental period mice were sacrificed for necropsy. The liver was excised, fixed with 10% buffered formalin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin for examination by light microscope.

Statistical analysis

Results were expressed as means ± SEM. Multiple data comparisons were performed using the one-way analysis of variance (ANOVA) routinely with the Bonferroni correction. Significant differences between the groups of mice were analyzed using the Wilcoxon test for paired samples, and P values less than 0.05 were considered statistically significant. Comparisons of survival rate were done by the Kaplan-Meier method.

RESULTS

Survival rate

nrf2−/− lpr/lpr female mice had a markedly prolonged lifespan with 50% of the mice having survival times twice as long as *nrf2+/+ lpr/lpr* female mice (Fig. 1). The 50% survival weeks of *nrf2−/− lpr/lpr* female mice were 45.4 ± 2.2 weeks in comparison to 23.2 ± 6.1 weeks for the *nrf2+/+ lpr/lpr* female mice. The 50% survival weeks of MRL/*lpr* mice was 6 months [18], which was almost the same as that of *nrf2+/+ lpr/lpr* mice.

Renal function and urinary protein

MRL/*lpr* mice are known to die from renal failure caused by autoimmune kidney disease [13]. We evaluated renal function in *nrf2−/− lpr/lpr* mice by measuring serum creatinine and BUN, as an index of renal function, in a time series experiment. The levels of serum creatinine and BUN were decreased in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice at 20 weeks old, but only significantly in the case of BUN levels. We also performed other studies, including urinary protein analysis, at 20 weeks of age (Table 1). The urinary protein levels of *nrf2−/− lpr/lpr* female mice were significantly lower than that of *nrf2+/+ lpr/lpr* female mice at 20 weeks of age.

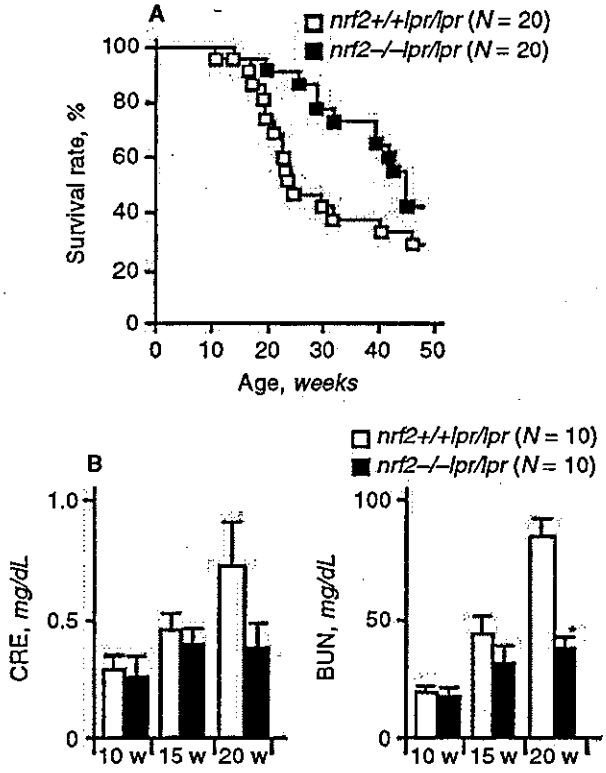

Fig. 1. Survival rate and renal function of *lpr* female mice. (*A*) Survival rate of *nrf2−/− lpr/lpr* ($N = 20$) and *nrf2+/+ lpr/lpr* ($N = 20$) female mice using Kaplan-Meier method. The difference in survival rate between *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice was significant ($P < 0.05$). (*B*) Serum creatinine (CRE) and blood urea nitrogen (BUN) were decreased in *nrf2−/− lpr/lpr* mice ($N = 10$) compared to *nrf2+/+ lpr/lpr* mice ($N = 10$) at 20 weeks old, but only BUN showed a significant decrease ($^*P < 0.05$) (serum creatinine, $P = 0.08$). Each bar represents the mean± SEM.

Table 1. Clinical and serologic manifestations in *nrf2 −/− lpr/lpr* and *nrf2 +/+ lpr/lpr* female mice at 20 weeks of age[a]

	nrf2 −/− lpr/lpr	*N*	*nrf2 +/+ lpr/lpr*	*N*	*P* value
Urinary protein *mg/day*	1.2 ± 0.3	15	3.6 ± 1.0	16	<0.05
IgM *mg/dL*	469.4 ± 52.1	10	624.9 ± 111.2	10	NS
IgG *mg/dL*	2821.5 ± 353.1	10	8526.1 ± 788.9	10	<0.05
IgG1 *mg/dL*	199.8 ± 35.1	10	524.3 ± 88.4	10	<0.05
IgG2a *mg/dL*	1815.2 ± 318.4	10	6226.7 ± 1475.0	10	<0.05
IgG3 *mg/dL*	651.2 ± 86.3	10	913.4 ± 104.2	10	NS
Anti-dsDNA IgG *units*	31.5 ± 10.3	10	103.3 ± 22.7	10	<0.05
Lymphadenopathy score[b]	0.9 ± 0.3	20	1.5 ± 0.5	20	<0.05

ds DNA is double-stranded DNA.
[a]Results are shown as means ± SEM; [b]Using a scale of 0 to 4 described in the Methods section.

Histologic analysis

Renal histopathologic studies were performed in *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* female mice at 20 weeks of age. In *nrf2+/+ lpr/lpr* female mice, glomerulonephritis with inflammation, sclerosis, and crescent formation were observed (Fig. 2A). By contrast, glomerular lesions were improved in *nrf2−/− lpr/lpr* female mice (Fig. 2B). The average of indices of glomerular lesions (as described in the **Methods** section) in *nrf2+/+ lpr/lpr* and *nrf2−/− lpr/lpr* female mice were 2.8 ± 0.3 ($N = 10$) and 2.0 ± 0.2 ($N = 10$), respectively ($P < 0.05$). IgG, IgM, and C3 deposits were observed by immunofluorescence staining in the kidney of both *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice. IgG and C3 deposits in the mesangial regions and the capillary walls of glomeruli were decreased in *nrf2−/− lpr/lpr mice* compared to *nrf2+/+ lpr/lpr* mice ($P < 0.05$). From the isotype staining of IgG, IgG2a and IgG3 deposits were found to be decreased in *nrf2−/− lpr/lpr mice* compared to *nrf2+/+ lpr/lpr* mice, significantly ($P < 0.05$) (Fig. 2C and D).

Immunohistochemical examination

Abnormal renal function in MRL/*lpr* mice consists of leukocyte infiltration, including macrophage, CD4 and CD8 T cells, and B220-positive cells [22]. We evaluated the numbers of these infiltrates in the glomeruli and interstitium *nrf2−/− lpr/lpr* kidneys at 20 weeks old. The numbers of macrophage (F4/80), CD4, CD8-positive T cells, and B220-positive cells were significantly reduced in *nrf2−/− lpr/lpr* kidneys compared with *nrf2+/+ lpr/lpr* kidneys (Fig. 3A and B). It has been reported that B220-positive cells in the kidney are unique double negative T cells and are not B cells in MRL/*lpr* mice [25, 26]. This result suggests that the reduction in numbers of macrophages, CD4, CD8-positive and double-negative T cells would lead to an improvement of kidney disease in *nrf2−/− lpr/lpr* mice.

Influence of *nrf2 −/−* on the level of key cytokines

It was previously reported that CSF-1, MCP-1, TNF-α, TGF-β1, and IFN-γ transcripts are up-regulated with the onset of renal injury in MRL/*lpr* mice and are a causative factor in the progression of autoimmune kidney disease [27–31]. So we determined these cytokines transcript levels by RT-PCR method. In *nrf2−/− lpr/lpr* kidneys, CSF-1, MCP-1, TNF-α, TGF-β1, and IFN-γ transcripts were reduced compared to *nrf2+/+ lpr/lpr* kidneys. (Fig. 3C).

Level of GSH in renal tissue

It has previously been described that various antioxidant and detoxification enzymes which are subject to Nrf2 regulation are reduced in the liver and macrophages of Nrf2-deficient mice [5–9]. As a consequence, Nrf2 deficiency may lead to high levels of oxidative stress and a depletion of GSH synthetic enzymes. We hypothesized that a reduction in GSH in renal tissue could influence the pathogenesis of autoimmune kidney disease. Therefore we measured renal GSH levels in *nrf2−/− lpr/lpr*

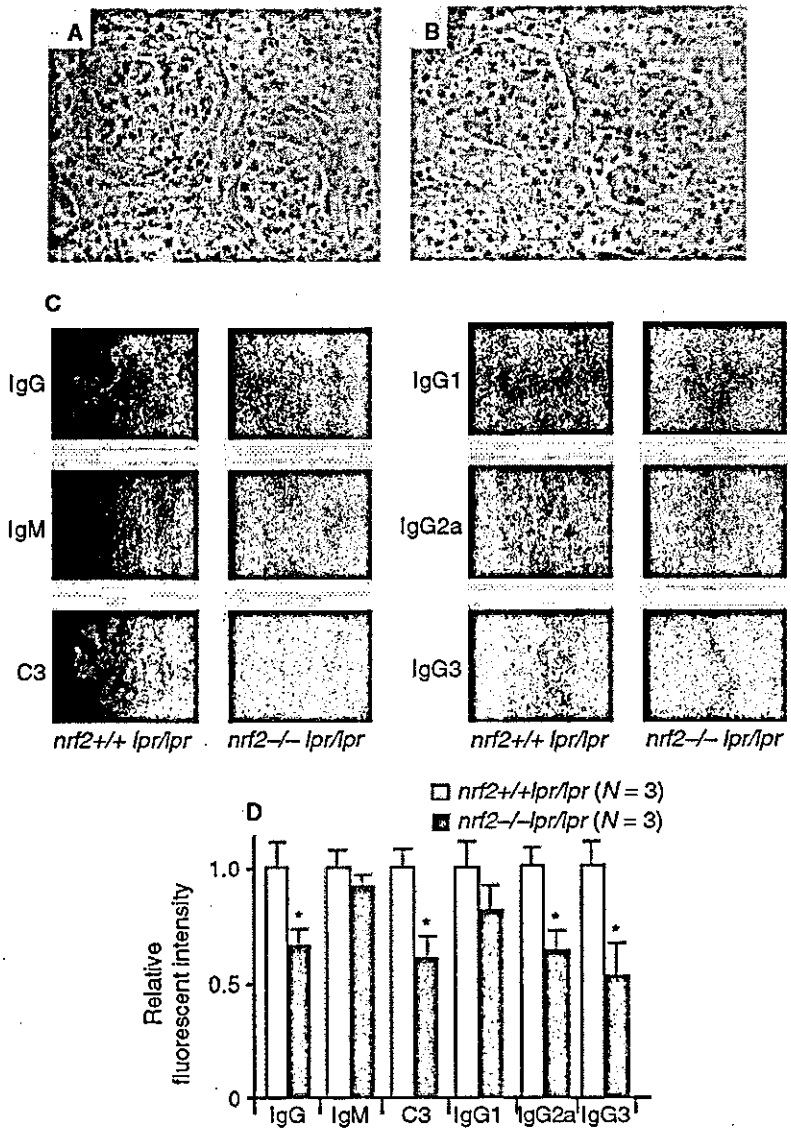

Fig. 2. Histopathologic analysis of renal tissues in *nrf2−/− lpr/lpr* mice. Renal section from an *nrf2−/− lpr/lpr* 20-week-old female mouse (*B*) showed the improvement of glomerular lesions compared to an age-matched *nrf2 +/+ lpr/lpr* mouse (*A*) (×100, hematoxylin and eosin stain). Immunofluorescent staining of IgG, IgM, C3, IgG1, IgG2a, and IgG3 (*C*). IgG, IgG2a, IgG3, and C3 deposits in the mesangial regions and the capillary walls of glomeruli were decreased in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice (×400). Quantitative analysis of immunofluorescent stainig revealed IgG, IgG2a, IgG3, and C3 deposits were significantly decreased in *nrf2−/− lpr/lpr* mice (*D*) (*$P < 0.05$). Each bar represents the mean ± SEM.

mice. Renal GSH was significantly reduced in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice (Fig. 3D).

Serum immunoglobulin and anti-double-stranded DNA antibodies

To evaluate immunologic abnormalities, the serum immunoglobulins in *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* female mice at 20 weeks of age were measured. There was no significant difference in serum IgM levels between *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* female mice (Table 1). Serum IgG levels of *nrf2−/− lpr/lpr* were lower than those of *nrf2+/+ lpr/lpr* female mice. Serum immunoglobulin levels of *nrf2+/+ lpr/lpr* female mice were almost comparable to those of MRL/*lpr* female mice as previously described [32]. The levels of IgG, IgG1, and IgG2a were also decreased in *nrf2−/− lpr/lpr* female mice compared to *nrf2+/+ lpr/lpr* female mice (Table 1). Serum IgG3 level in *nrf2−/− lpr/lpr* was also lower than *nrf2+/+ lpr/lpr* mice, but not significantly. There was not a significant difference in serum IgG3 level between *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice. However, in immunofluorescence staining, IgG3 deposition was decreased in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice. This result suggests IgG3 is one of the major factors responsible for the development of glomerulonephritis in *lpr/lpr* mice. The production of anti-double-stranded DNA autoantibody was significantly reduced in *nrf2−/− lpr/lpr* female mice when compared to *nrf2+/+ lpr/lpr* female mice ($P < 0.05$) (Table 1). Since the amount of anti-double-stranded DNA autoantibody in pooled sera from 20-week-old MRL/*lpr* female mice was set

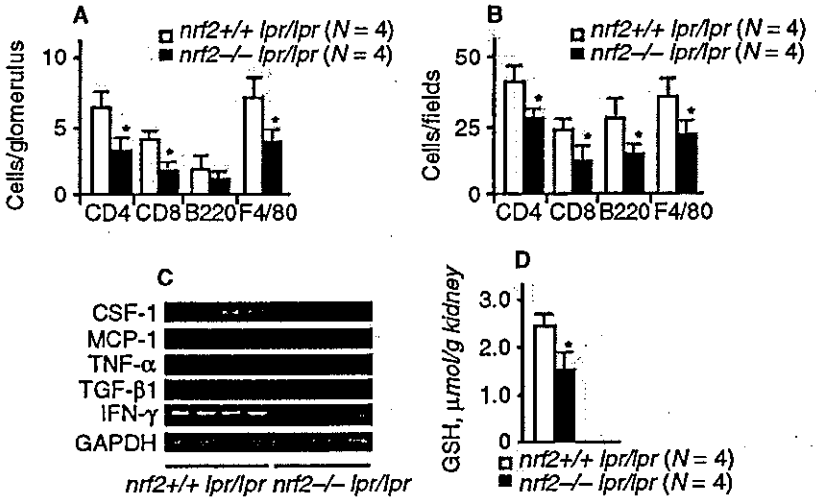

Fig. 3. Renal profile of infiltrated leukocytes and macrophages, key cytokines, and glutathione (GSH) levels. The numbers of macrophage (F4/80), CD4, CD8-positive T cells, and B220 positive cells were significantly reduced in the glomeruli and interstitium of *nrf2−/− lpr/lpr* kidneys compared with *nrf2+/+ lpr/lpr* kidneys in immunohistochemical examination (*A* and *B*). Colony stimulating factor 1 (CSF-1), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), and interferon γ (IFN-γ) levels were reduced in *nrf2−/− lpr/lpr* kidneys compared to *nrf2+/+ lpr/lpr* kidneys [reverse transcription-polymerase chain reaction (RT-PCR) method] (*C*). Renal GSH was significantly reduced in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice (*D*). Each bar represents the mean ± SEM. $^*P < 0.05$.

at 100 units, the titer of anti-double-stranded DNA autoantibody in *nrf2+/+ lpr/lpr* female mice was equal to MRL/*lpr* female mice.

Lymphadenopathy scoring

MRL/*lpr* mice develop massive lymphadenopathy due to a defect in Fas [16]. Therefore, lymphadenopathy in *nrf2−/− lpr/lpr* mice was evaluated. The scoring of lymphadenopathy was assigned by an observer, as described in the **Methods** section. The average score value was decreased in *nrf2−/− lpr/lpr* mice (Table 1).

FACS analysis of lymph nodes

We found lymphadenopathy was improved in *nrf2−/− lpr/lpr* mice. Next, we performed FACS analysis to examine the subset of lymphocytes and macrophages in lymph nodes. The numbers of CD4, CD8-positive, double-negative T cells and macrophage (F4/80) was significantly reduced in *nrf2−/− lpr/lpr* lymph nodes compared with *nrf2+/+ lpr/lpr* lymph nodes (Fig. 4).

TUNEL assay of spleen and kidney

Since Fas-mediated apoptosis is defective in *lpr* mice, the immunologic improvements seen in *nrf2−/− lpr/lpr* mice suggested that the activity of Nrf2 might somehow be linked to Fas-mediated apoptosis. Therefore, apoptosis in spleen and kidney was examined. The indices of TUNEL-positive cells were significantly increased in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice (Fig. 5). We observed apoptosis was increased in *nrf2−/− lpr/lpr* spleens and kidneys.

Induction of TNF-α–mediated apoptosis in splenocytes

We examined the effect of apoptosis triggered by anti-Fas antibody in splenocytes obtained from *nrf2−/−*

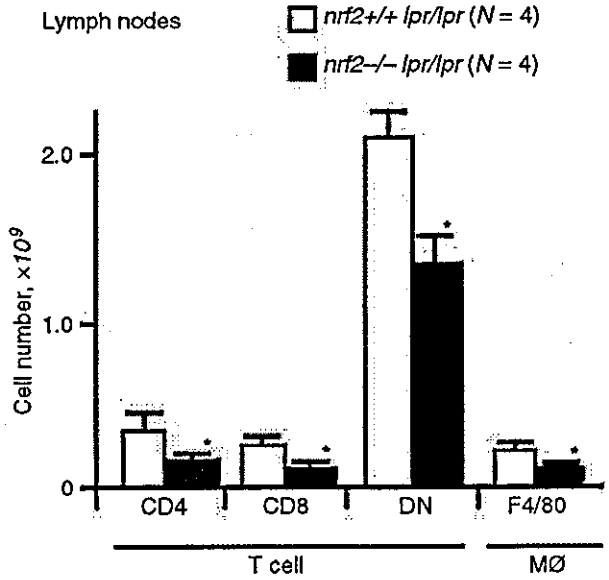

Fig. 4. Lymphocytes and macrophages in *nrf2−/− lpr/lpr* and *nrf2+/+ lpr/lpr* mice lymphnodes. Twenty-week-old *nrf2−/− lpr/lpr* ($N = 4$) and *nrf2+/+ lpr/lpr* ($N = 4$) female mice were used for fluorescence-activated cell sorter (FACS) analysis. Each bar represents the mean ± SEM. $^*P < 0.05$.

lpr/lpr mice. However, there were no difference in apoptotic splenocytes between from *nrf2−/− lpr/lpr* mice and *nrf2+/+ lpr/lpr* mice (data not shown). This result suggested that the immunologic improvements in *nrf2−/− lpr/lpr* mice were not due to an enhancement of Fas-mediated apoptosis. Fas and TNF receptor I (TNF-RI) share homology in their cytoplasmic death domain, a region important for apoptotic signaling [33]. It has been reported that, in *lpr* mice lacking TNF-RI, autoimmunity is greatly accelerated [34]. These facts indicate that TNF-RI might play a compensatory role in Fas-mediated apoptosis in *lpr* mice. Therefore, we examined the effect

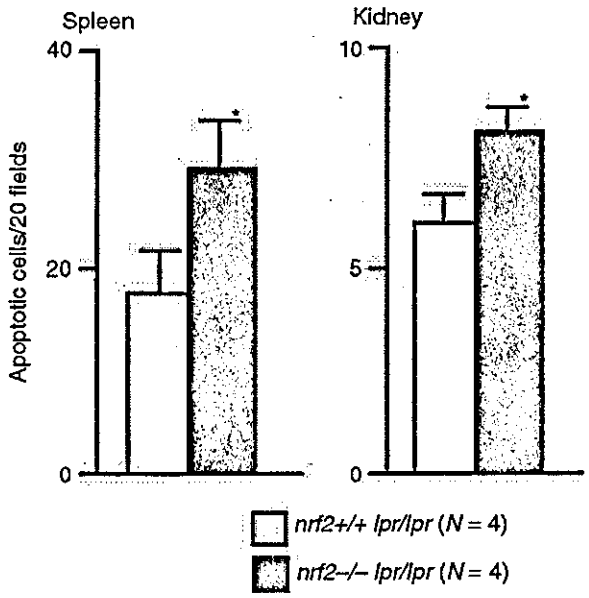

Fig. 5. Detection of apoptotic cells in the spleen of *nrf2−/− lpr/lpr* mice by terminal deoxynucleotidyl transferase (TdT) nick end-labeling (TUNEL) method. Using the TUNEL method, apoptosis was estimated in the spleens and kidneys of 20-week-old *lpr/lpr* female mice. Each bar represents the mean ± SEM. $^*P < 0.05$.

of TNF-α on cultured splenocytes from *nrf2−/− lpr/lpr* mice. We showed that the survival rate of splenocytes from *nrf2−/− lpr/lpr* mice was decreased compared to *nrf2+/+ lpr/lpr* mice ($P < 0.05$) (Fig. 6A). It has been reported that prolonged GSH depletion enhances death receptor (Fas, TNF-RI)–mediated apoptosis [35–37]. We and others reported that intracellular GSH levels were reduced in Nrf2-deficient cells [8, 9]. Moreover, we have reported Nrf2 deficiency increases the sensitivity to Fas and TNF-RI through intracellular glutathione depletion [38]. Therefore, we suspected that the observed sensitivity of TNF-α–mediated apoptosis in *nrf2−/− lpr/lpr* mice could be due to a depletion of cellular GSH levels resulting from a deficiency in Nrf2. GSH-OEt is a compound capable of passing the cell membrane and up-regulating the intracellular levels of GSH [39]. To prove the enhancement of TNF-α–mediated apoptosis in splenocytes from *nrf2 −/− lpr/lpr* mice was due to a decrease in intracellular GSH levels, GSH-OEt was administrated before TNF-α administration. The administration of GSH-OEt increased the survival rate in *nrf2−/− lpr/lpr* mice (Fig. 6B). These results indicate that Nrf2 deficiency can enhance the sensitivity of TNF-α–mediated apoptosis through GSH depletion in vitro.

Hepatocellular apoptosis induction by TNF-α

It is well known that the administration of TNF-α to GalN-sensitized mice can induce fulminant hepatitis as a result of TNF-α–mediated apoptosis [40]. GalN increases the susceptibility of mice to hepatotoxicity and the lethal effects of TNF-α [40]. The effect of hepatocellular apoptosis in vivo triggered by TNF-α in *nrf2−/− lpr/lpr* mice was examined. Serum ALT activity was monitored as indices of hepatotoxicity. We showed that serum ALT activity after TNF-α administration was significantly increased ($P < 0.05$) in *nrf2−/− lpr/lpr* mice compared to *nrf2+/+ lpr/lpr* mice (Table 2).

Histopathologic examination also revealed that apoptotic hepatocytes were increased in *nrf2−/− lpr/lpr* mice (Fig. 6D) compared to *nrf2+/+ lpr/lpr* mice (Fig. 6C). To confirm the enhancement of hepatitis in *nrf2−/− lpr/lpr* mice was due to a decrease in intracellular GSH levels, GSH-OEt was administrated before TNF-α injection. The administration of GSH-OEt decreased the ALT activity in *nrf2−/− lpr/lpr* mice (Table 2). Histopathologic findings also demonstrated GSH-OEt addition rescued *nrf2−/− lpr/lpr* mice from TNF-α–mediated hepatocellular apoptosis (Fig. 6E). Moreover, we performed a quantitative analysis of TUNEL-positive apoptotic cells induced by TNF-α. The number of apoptotic hepatocytes after TNF-α treatment was significantly increased in *nrf2/−/−lpr/lpr* mice ($N = 4$) compared to *nrf2+/+ lpr/lpr* mice ($N = 4$) (Fig. 6F). These results indicate that Nrf2 deficiency can enhance the sensitivity of TNF-α–mediated apoptosis through GSH depletion and that the enhanced sensitivity of TNF-α–mediated apoptosis can improve the immunologic abnormality seen in *lpr* mice.

DISCUSSION

We previously reported that *nrf2* might be one of the candidate genes in determining the susceptibility to autoimmune diseases [12]. MRL/*lpr* mice, having a defect in the Fas molecule, develop spontaneous autoimmune nephritis with generalized peripheral lymphadenopathy [16]. We speculated that *lpr* could stimulate autoimmune disease under conditions where the *nrf2* gene had been deleted. Unexpectedly, this study showed autoimmune nephritis was improved in *nrf2−/− lpr/lpr* mice. Interestingly, we observed that ablation of the *nrf2* gene in *lpr* mice restimulated apoptosis, a response normally blocked as a result of a mutation in *fas*. Fas is expressed at high levels in activated T cells and thought to be critically involved in activation-induced apoptosis in T cells [17, 41–45]. The lymphadenopathy and autoimmune disease seen in MRL/*lpr* mice has been attributed to a defect in Fas-mediated apoptosis in which activated T cells do not undergo activation-induced cell death. In this paper, we clearly demonstrate that apoptotic cells were increased in spleen and kidney in *nrf2−/− lpr/lpr* mice compared with *nrf2+/+ lpr/lpr* mice (Fig. 5). The *lpr* gene consists of a transposon insertion into the *fas* gene resulting in very few Fas molecules being present on the cell surface [16].

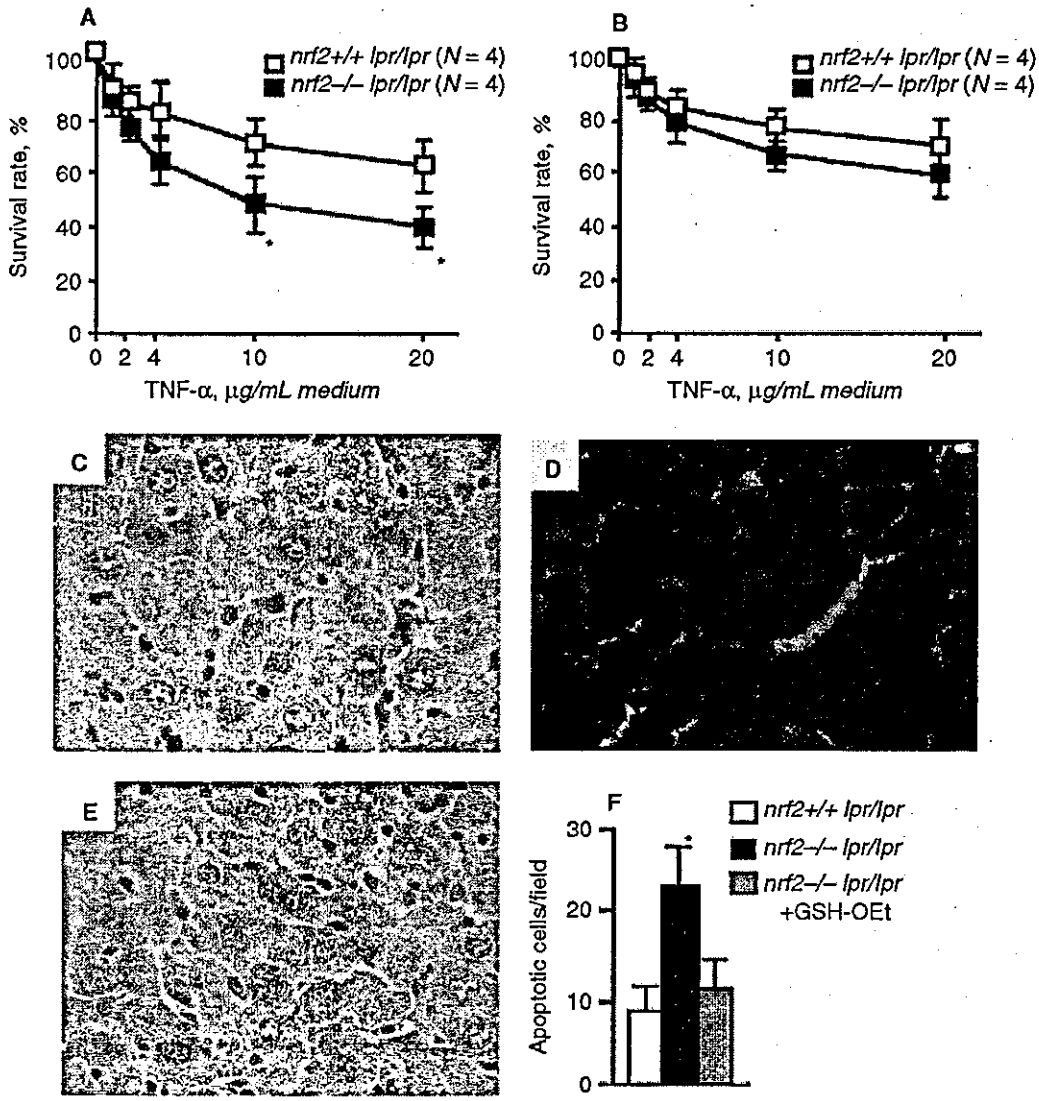

Fig. 6. *nrf2−/− lpr/lpr* mice enhanced tumor necrosis factor-α (TNF-α)–mediated apoptosis whereas administration of glutathione ethyl monoester (GSH-OEt) rescued *nrf2−/− lpr/lpr* mice from TNF-α–mediated apoptosis. Effects of Nrf2 deficiency on cultured splenocytes treated with TNF-α. (*A*) Survival rate of *nrf2−/− lpr/lpr* splenocytes after TNF-α stimulation. Each bar represents the mean ± SEM. $^*P < 0.05$. (*B*) GSH-OEt protected *nrf2−/− lpr/lpr* splenocytes against TNF-α–mediated apoptosis. Splenocytes were pre-incubated with 2mM GSH-OEt. (*C*) Liver section from *nrf +/+ lpr/lpr* mouse (×400, hematoxylin and eosin stain). (*D*) *nrf2 −/− lpr/lpr* mouse. Arrows indicate apoptotic nuclei (×400). (*E*) *nrf2−/− lpr/lpr* mouse administered GSH-OEt. The number of apoptotic cells was decreased with GSH-OEt administration (×400). (*F*) Quantitative analysis of terminal deoxynucleotidyl transferase (TdT) nick end-labeling (TUNEL)-positive apoptotic hepatocytes by TNF-α. Each bar represents the mean ± SEM. $^*P < 0.05$.

Therefore, it was possible that the observed improvement of apoptosis in *nrf2−/− lpr/lpr* mice could be due to enhanced sensitivity of Fas-mediated apoptosis. However, the administration of anti-Fas antibody to *nrf2−/− lpr/lpr* splenocytes had no stimulatory effect on apoptosis (data not shown). A further reason for the immunologic improvement seen in the *nrf2−/− lpr/lpr* mice may be a modulation in TNF receptor signaling. Accumulating evidence indicates that TNF signaling is also important for apoptosis. Fas and TNF-RI share a region of homology within their cytoplasmic portions called the death domain, which is responsible for the delivery of intercellular death inducing signals [33]. It has been reported that, in *lpr* mice, substantial numbers of T cells still undergo apoptosis, even though Fas-mediated apoptosis is defective [46]. Further studies have shown that in *lpr* mice lacking TNF-RI, the development of lymphadenopathy and autoantibody production was greatly accelerated compared with TNF-RI wild-type *lpr* mice [34]. These mice also exhibited high mortality and early onset autoimmune disease characterized by massive mononuclear cell infiltration in liver, kidney, lung, and knee joints [34]. These

Table 2. Hepatotoxicity and lethal effect in *nrf2 −/− lpr/lpr* mice induced by tumor necrosis factor-α (TNF-α)[a]

Treatment		Time *hours*	ALT(IU/L)		Survival rate[b]	
			nrf2 −/− lpr/lpr	*nrf2 +/+ lpr/lpr*	*nrf2 −/− lpr/lpr*	*nrf2 +/+ lpr/lpr*
GSH-OEt(−)	TNF-α	0	26.8 ± 3.3	25.2 ± 3.9	5/5	5/5
GSH-OEt(−)	TNF-α	4	761.4 ± 140.0[c]	192.0 ± 45.5	5/5	5/5
GSH-OEt(−)	TNF-α	8	7110.3 ± 882.9[c]	1560.3 ± 145.4	4/5	5/5
GSH-OEt(−)	TNF-α	12	9767.8 ± 867.3[c]	2850.1 ± 348.2	3/5	4/5
GSH-OEt(+)	TNF-α	0	34.2 ± 4.2	28.8 ± 3.7	5/5	5/5
GSH-OEt(+)	TNF-α	4	261.1 ± 55.7	143.8 ± 32.2	5/5	5/5
GSH-OEt(+)	TNF-α	8	1230.3 ± 42.0	925.1 ± 199.6	5/5	5/5
GSH-OEt(+)	TNF-α	12	2565.3 ± 234.8	1235.7 ± 129.8	5/5	5/5

Abbreviations are: ALT, alanine aminotransferase; GSH-OEt, glutathione ethyl monoester; TNF-α, tumor necrosis factor-α.
[a]Results are shown as mean ± SEM for three to five mice; [b]Survival rate indicates the number of survived/all mice; [c]$P < 0.05$.

facts indicate that in *lpr* mice the defects in Fas-mediated apoptosis are exacerbated in the absence of TNF-RI and that normal expression of TNF-RI might partially compensate for the Fas-mediated apoptosis defect of lymphocytes in *lpr* mice. In this study, we demonstrate that Nrf2 deficiency enhanced the sensitivity of TNF-α–mediated apoptosis. Accordingly, an enhanced sensitivity in TNF-α–mediated apoptosis resulting from a deficiency in Nrf2 may have suppressed the accelerating effect of *lpr* in *nrf2−/− lpr/lpr* mice, thereby alleviating the autoimmune disease.

Our study has shown that a deficiency in Nrf2 enhances the sensitivity of TNF-α–mediated apoptosis through a decrease in GSH levels. GSH is an important nonprotein thiol molecule that protects cells against oxidative damage [47, 48]. Nrf2 activates the transcription of the heavy and light chain γ-glutamylcysteine synthetase [GCS (H) and GCS (L)] [8], and cystine membrane transporter (system Xc^-) genes [9] through an ARE/EpRE response element [3, 4]. These molecules are necessary for the synthesis of GSH [8, 9]. An inability to induce the expression of these molecules in Nrf2-deficient mice resulted in increased oxidative stress and the protective response to electrophilic and ROS-producing agents was profoundly impaired [9–11]. Therefore, Nrf2 deficiency resulted in minimum levels of intracellular GSH [9].

Previous reports have shown that a preexisting reduction in GSH levels could significantly increase cell death from TNF-α [49, 50], although the detailed molecular mechanism remains unknown. More recently, we reported that Nrf2 deficiency enhances death-inducing signals through GSH depletion [38]. In our previous study, we found that aged Nrf2-deficient female mice developed autoimmune glomerulonephritis probably due to an increase in oxidative stress [12]. Nephritis in Nrf2-deficient mice develops only in aged females. Therefore, it appears that the *nrf2* gene contributes to the susceptibility to autoimmune disease, rather than acting as an accelerating gene for the disease. In this study, we found that Nrf2 deficiency suppresses the stimulatory effects of the *lpr* gene, which is well known as an accelerating gene for autoimmune diseases. Since this is an unexpected interaction, it is still unclear whether Nrf2 is one of the susceptibility genes for autoimmune diseases. To elucidate the roles of Nrf2 in the development of autoimmune diseases, we will introduce the *nrf2* mutation into BXSB/*Yaa* mice, a disease model not defective for the *fas* gene. This should uncover any synergistic effects that exist between the *Yaa* and *nrf2* mutation.

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A Constitutively Active Arylhydrocarbon Receptor Induces Growth Inhibition of Jurkat T Cells through Changes in the Expression of Genes Related to Apoptosis and Cell Cycle Arrest*

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is known to suppress T cell-dependent immune reactions through the activation of the arylhydrocarbon receptor (AhR). Our previous findings suggest that TCDD inhibits the activation and subsequent expansion of T cells following antigen stimulation in mice, leading to a decreased level of T cell-derived cytokines involved in antibody production. In the present study, we investigated the effects of activated AhR on T cells by transiently expressing a constitutively active AhR (CA-AhR) mutant in AhR-null Jurkat T cells. In agreement with our previous findings, CA-AhR markedly inhibited the growth of Jurkat T cells. The inhibited cell growth was found to be concomitant with both an increase in the annexin V-positive apoptotic cells and the accumulation of cells in the G_1 phase. The growth inhibition was also shown to be mediated by both xenobiotic response element (XRE)-dependent and -independent mechanisms, because an A78D mutant of the CA-AhR, which lacks the ability of XRE-dependent transcription, partially inhibited the growth of Jurkat T cells. Furthermore, we demonstrated that CA-AhR induces expression changes in genes related to apoptosis and cell cycle arrest. These expression changes were shown to be solely mediated in an XRE-dependent manner, because the A78D mutant of the CA-AhR did not induce them. To summarize, these results suggest that AhR activation causes apoptosis and cell cycle arrest, especially through expression changes in genes related to apoptosis and cell cycle arrest by the XRE-dependent mechanism, leading to the inhibition of T cell growth.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)[1] is known to exert a variety of toxicities such as reproductive toxicity, immunotoxicity, teratogenicity, and neurotoxicity (1, 2). Previous studies using arylhydrocarbon receptor (AhR) knock-out mice indicate that most, if not all, of the TCDD-induced toxicity is mediated by the AhR, a basic helix-loop-helix periodicity/ARNT/single-minded (PAS) transcription factor (3, 4). In the absence of a ligand, the AhR is located in the cytoplasm in association with heat shock protein 90, X-associated protein 2, and heat shock protein 90 co-chaperone p23 (5). Once a ligand, such as TCDD, binds to the AhR, the complex is translocated into the nucleus where it forms a heterodimer with an AhR nuclear translocator (ARNT). The AhR/ARNT heterodimer binds to specific DNA sequences termed xenobiotic response elements (XREs), and it enhances the expression of genes such as cytochrome P-450 1A1 (*CYP1A1*) (4, 6). On the other hand, it has also been shown that the ligand-activated AhR directly interacts with retinoblastoma protein (RB) (7, 8) and NF-κB (RelA) (9), and their direct interactions modulate the signaling pathways involved in many physiological functions. Although many studies have been conducted, the precise mechanism for individual toxicities of TCDD remains to be clarified.

As regards immunotoxicity, TCDD induces thymus atrophy and suppresses both humoral and cellular immunity in an AhR-dependent manner (10, 11). Recent studies using chimeric mice with the AhR in either hemopoietic or stromal tissues showed that TCDD induces thymus atrophy by directly affecting thymocytes (immature T cells) and not dendritic or stromal cells (12, 13). Additionally, it has been suggested that TCDD-induced thymus atrophy can be attributed to the inhibition of G_1/S cell cycle progression in thymocytes at the double negative stages of T cell development (12). In terms of cellular immunity, it has been reported that full suppression of the cytotoxic T lymphocyte response by TCDD required AhR expression in both $CD4^+$ and $CD8^+$ T cells; this indicates that T cells are direct targets of TCDD (14). With regard to the suppressive effect of TCDD on humoral immunity, primary effects on both B cells and T cells have been reported by several groups (11, 15). Recently, we showed that TCDD considerably reduces the production of the T cell growth factor IL-2 and $CD4^+$ type 2 helper T cell-derived cytokines prior to the inhibition of antibody suppression in mice immunized with ovalbumin (16–18). Furthermore, TCDD suppressed the increase in the number of T cells in the spleen, following immunization. This suggests

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[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, arylhydrocarbon receptor; ARNT, arylhydrocarbon receptor nuclear translocator; XRE, xenobiotic response element; CA-AhR, constitutively active arylhydrocarbon receptor; PAS, periodicity/ARNT/single-minded; RB, retinoblastoma protein; IL-2, interleukin-2; GFP, green fluorescent protein; PI, propidium iodide; RT, reverse transcriptase; CYP1A1, cytochrome P-450 1A1; FACS, fluorescence-activate cell sorting; TGF, transforming growth factor; CDA1, cell division autoantigen-1; GADD45A, growth arrest and DNA-damage-inducible, alpha.

that TCDD inhibits the activation of antigen-specific T cells and their subsequent expansion, which probably leads to deteriorated antibody production (16). All these findings strongly suggest that T cells are a vulnerable target of TCDD toxicity, with regard to not only thymus atrophy and inhibition of cellular immunity but also the suppression of humoral immunity. Our recent finding that primary T cells have functional AhR also supports this mechanism (19).

In the present study, to investigate the effects of activated AhR on T cells and their underlying mechanism, we transiently expressed a constitutively active AhR (CA-AhR) mutant in human leukemic Jurkat T cells, because all the T cell lines examined thus far, including Jurkat T cells, do not have functional AhR (20, 21). We used a CA-AhR mutant lacking the minimal PAS B motif, which is constitutively localized in the nucleus and activates AhR-dependent transcription independent of the ligand (22, 23). We also generated an A78D mutant of the CA-AhR, which lacks the ability of XRE-dependent transcription (24), and examined the involvement of XRE-dependent transcription in the effects of CA-AhR on Jurkat T cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pEB6CAGFP (an Epstein-Barr virus-based expression vector for green fluorescent protein (GFP) driven by a CAG promoter) (25) and pEB6CAGMCS (containing multicloning sites) were kindly provided by Dr. Yoshihiro Miwa (University of Tsukuba, Tsukuba, Japan). A CA-AhR cDNA[2] was subcloned into a KpnI-HindIII site of pEB6CAGMCS, and pEB6CAG-CA-AhR-GFP, encoding a CA-AhR fused to GFP, was generated by inserting the SalI-AflII fragment of pEGFP-N3 (Clontech Laboratories, Inc., Palo Alto, CA). To examine whether the effects of CA-AhR on Jurkat T cells are mediated by XRE-dependent transcription, the mutation changing the alanine at position 78 to aspartic acid (A78D) (24) in the CA-AhR was introduced by the use of a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A primer with the sequence 5′-GTCAGCTACCTGAGGGACAAGAGCTTCTTTGATG-3′ and its complementary equivalent were employed.

Cell Line, Transient Transfection, and Sorting—Jurkat T cells were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 10 mM HEPES (pH 7.1), 1 mM pyruvate, and 50 μM 2-mercaptoethanol at 37 °C in 5% CO_2. Jurkat T cells (2×10^6 cells) were transiently transfected with 6 μg of pEB6CAGFP, pEB6CAG-CA-AhR-GFP, or pEB6CAG-A78D-GFP using DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions. After 2 days, GFP-positive cells from each transfectant were sorted using a FACSVantage SE (BD Biosciences). The efficiency of the sorting was confirmed using a FACSCalibur (BD Biosciences), and 98–99% of the sorted cells were GFP-positive. The sorted cells were cultured at 1×10^5 cells/ml, and then growth rate, apoptosis, and cell cycle distribution were examined as described below. The results obtained in each experiment were confirmed in another independent experiment, and a set of representative results has been shown under "Results."

Detection of Apoptosis—For the detection of apoptotic cells by annexin V binding and propidium iodide (PI) staining, we used an Annexin V-biotin apoptosis detection kit (BioVision, Palo Alto, CA) according to the manufacturer's instructions, with minor modifications. At 0, 2, and 4 days after sorting, the cells were incubated with biotin-labeled annexin V for 5 min at room temperature. After washing, the cells were incubated with streptavidin-labeled allophycocyanin (SA-APC, BD Biosciences) for 15 min at room temperature. After washing, PI was added, and the cells were analyzed using a FACSCalibur.

Furthermore, the induction of apoptosis was confirmed by apoptotic morphological changes. The sorted cells were cultured for 2 days and then stained with 4 μM bisbenzimide (Hoechst 33342, ICN Biomedicals Inc., Aurora, OH) for 15 min. The apoptotic cells were examined by the changes in their nuclear morphology, *i.e.* nuclear fragmentation, under a UV-visible fluorescence microscope. Approximately over 100 cells were counted in four microscopic fields, and the percentage of apoptotic cells was estimated.

Cell Cycle Analysis—At 0, 2, and 4 days after sorting, the cells were stained with PI using a CycleTEST Plus DNA reagent kit (BD Biosciences) according to the manufacturer's instructions, and their DNA content was measured using a FACSCalibur. The percentages of cells in the G_1, S, and G_2/M phases were analyzed using ModFit software (BD Biosciences).

Affymetrix GeneChip Analysis—Affymetrix GeneChip analysis was performed according to the Affymetrix expression analysis technical manual (Affymetrix, Santa Clara, CA), with some modifications. After sorting, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Chatsworth, CA). Double-stranded cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and T7 oligo$(dT)_{24}$ primer (Affymetrix). The double-stranded cDNA was purified by the phenol/chloroform extraction method, followed by ethanol precipitation. The *in vitro* transcription reaction was performed using a BioArray high yield RNA transcript-labeling kit (Enzo Diagnostics, Farmingdale, NY). 15 μg of the biotin-labeled cRNA was fragmented and hybridized to a Human Genome U133A array (Affymetrix). The hybridized probe array was washed, stained, and scanned. The data were analyzed using Affymetrix Microarray Suite 5.0 software. A comparison analysis was performed to obtain genes with at least 2-fold changes in Jurkat T cells expressing CA-AhR-GFP as compared with cells expressing GFP alone.

Semiquantitative RT-PCR—To confirm the gene expression changes observed by the Affymetrix GeneChip analysis, semiquantitative RT-PCR was performed on the double strand cDNA prepared for Affymetrix GeneChip analysis. Primers used in the present study were designed using PRIMER3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and NCBI UniSTS (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists), based on human sequences published in the NCBI data base. Primer sequences, PCR cycle numbers, and the annealing temperatures of each gene are shown in Table I. Each double-stranded cDNA was amplified in the exponential phase of PCR using TaKaRa *Taq* (TaKaRa Shuzo Co., Ltd., Tokyo, Japan). The amplification was carried out by an initial incubation at 94 °C for 2 min, followed by 19–30 cycles of 94 °C for 30 s, 55 or 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR products were separated in a 1.2% Synergel (Diversified Biotech, Boston, MA) containing 0.5 μg/ml ethidium bromide. The net intensity of the bands was quantified using Kodak EDAS 290. The expression level of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase or β-actin.

RESULTS

CA-AhR Inhibits Growth of Jurkat T Cells—To examine the effect of AhR activation on T cells, we used a CA-AhR mutant lacking the minimal PAS B motif (Fig. 1*A*). The CA-AhR mutant has been reported to form a heterodimer with ARNT and induce XRE-dependent gene expression in Chinese hamster ovary cells and MCF-7 cells in the absence of a ligand (22, 23). First, we examined CYP1A1 expression to confirm that CA-AhR induces XRE-dependent gene expression in Jurkat T cells. Jurkat T cells, which expressed ARNT but not AhR (data not shown), were transiently transfected with an expression vector for either CA-AhR-GFP or GFP alone. Two days after the transfection, GFP-positive cells were sorted and RT-PCR for CYP1A1 mRNA was performed. As shown in Fig. 1*B*, CA-AhR-GFP, but not GFP alone, markedly induced CYP1A1 mRNA expression, indicating that the CA-AhR is functional in Jurkat T cells. In addition, the green fluorescence emitted from CA-AhR-GFP was mainly found in the nuclear compartment (data not shown).

To examine the effect of CA-AhR on the growth rate of Jurkat T cells, the sorted cells were cultured for up to 4 days, and the cell numbers were counted at the indicated times. As shown in Fig. 2, the cells expressing GFP alone increased 10-fold, 4 days after sorting. In contrast, the expression of CA-AhR-GFP completely inhibited the increase in cell numbers up to 4 days after sorting, indicating that the activation of AhR greatly inhibits the growth of Jurkat T cells.

CA-AhR Induces Apoptosis in Jurkat T Cells—Because CA-AhR was shown to induce growth inhibition, we examined whether the expression of CA-AhR induces apoptosis in Jurkat T cells. Apoptotic cells were detected with annexin V, which monitors the appearance of phosphatidylserine on the cell sur-

[2] Y. Fujii-Kuriyama and J. Mimura, unpublished data.

TABLE I
List of primers used for semiquantitative RT-PCR

Description	GenBank™ accession number	Primer sequence (5′–3′)	PCR cycle number	Annealing temperature	Product size
				°C	*bp*
AhR	L19872	TTACCTGGGCTTTCAGCAGT AACTGGGGTGGAAAGAATCC	19	60	506 (CA-AhR)
β-Actin	X00351	GAGGCCCAGAGCAAGAGAG GGCTGGGGTGTTGAAGGT	19	60	225
Caspase 8	U58143	CTTGGATGCAGGGGCTTTGACC GTTCACTTCAGTCAGGATGG	23	55	550
CDA1	AF254794	TGAGGAGGAAGGAAGTGAAGA TAGTGGGTGGGGGATACAGA	26	60	171
c-Jun	BC002646	GGTATCCTGCCCAGTGTTGT CGCACTAGCCTTTGGTAAGC	25	60	382
c-Myc	V00568	TCGGATTCTCTGCTCTCCTC CGCCTCTTGACATTCTCCTC	24	60	413
Cyclin G2	U47414	TGGACAGGTTCTTGGCTCTT AATACAGATGGTTTTGCTTTTGA	28	55	367
CYP1A1	X02612	CTTGGACCTCTTTGGAGCTG CGAAGGAAGAGTGTCGGAAG	30	60	212
DUSP6	AB013382	CGATGAACGATGCCTATGAC TGCCAAGAGAAACTGCTGAA	29	60	262
Fas	M67454	CTGCCATAAGCCCTGTCCT CAAAGCCACCCCAAGTTAGA	27	60	360
GADD34	U83981	CCTCCTCTGTCCCTTCGTC AGTTGGTCTCAGCCACGC	26	60	127
GADD45A	M60974	CTGGAGGAAGTGCTCAGCAAAG TTGATCCATGTAGCGACTTTCC	27	60	399
GAPDH	M33197	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	19	60	452
IL-9 receptor	M84747	TTCACCATCACTTTCCACCA AACGCTCCTCCTCTACCACA	26	60	370
$p21^{waf1}$	U03106	GGGAAGGGACACACAAGAAG GGGAGCCGAGAGAAAACAG	25	60	478
TGF-β receptor II	D50683	CGGCTCCCTAAACACTACCA GGTCCCTTCCTTCTCTGCTT	27	60	478

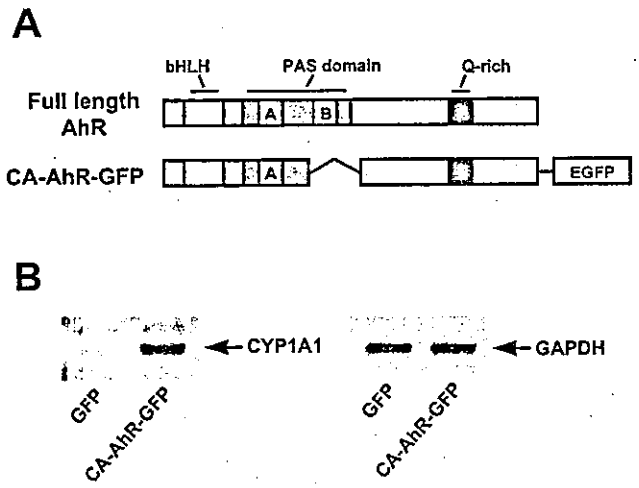

FIG. 1. **Structure of a constitutively active mutant of an aryl-hydrocarbon receptor (CA-AhR) and CYP1A1 induction in Jurkat T cells expressing CA-AhR.** *A*, the structures of wild-type AhR and a CA-AhR fused to GFP (CA-AhR-GFP) are shown. The CA-AhR lacks the minimal PAS B motif. *B*, the expression vector for either CA-AhR-GFP or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. Two days after transfection, GFP-positive cells were sorted using a FACSVantage SE. Total RNA was isolated from the cells, and mRNA expression levels of CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase were examined by semiquantitative RT-PCR.

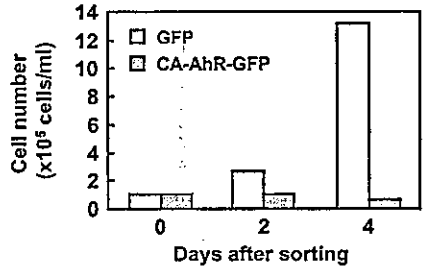

FIG. 2. **CA-AhR inhibits growth of Jurkat T cells.** The expression vector for either CA-AhR-GFP or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. After 2 days, GFP-positive cells from each transfected cells were sorted using a FACSVantage SE and then cultured at 1×10^5 cells/ml. The cell numbers at the indicated times were determined by trypan blue exclusion.

face during apoptosis. In addition, PI was used to distinguish between early and late apoptosis, because PI is excluded only by live and early apoptotic cells. As shown in Fig. 3, where annexin V-positive, PI-negative cells (*upper left quadrant*) represent early apoptotic cells and annexin V, PI-double positive cells (*upper right quadrant*) represent late apoptotic/necrotic cells, GFP-alone-transfected cells did not show remarkable changes in the ratio of dead cells. On the other hand, CA-AhR-GFP increased the percentage of apoptotic cells, especially 2 and 4 days after sorting. Two days after sorting, the percentage of early apoptotic cells was 3-fold higher in cells expressing CA-AhR-GFP than in those expressing GFP alone. Moreover, 4 days after sorting, CA-AhR-GFP augmented the percentages of early apoptotic cells and late apoptotic/necrotic cells by approximately 8- and 6-fold, respectively, as compared with GFP alone.

Furthermore, the induction of apoptosis was confirmed by nuclear morphological changes. Each group of sorted cells was cultured for 2 days and stained with Hoechst 33342 dye. Fragmented nuclei were observed in a number of cells expressing CA-AhR-GFP (Fig. 4*A*, *arrowheads*), and apoptotic cells reached about 30%; however, the apoptotic cells only reached 10% in cells expressing GFP alone (Fig. 4*B*).

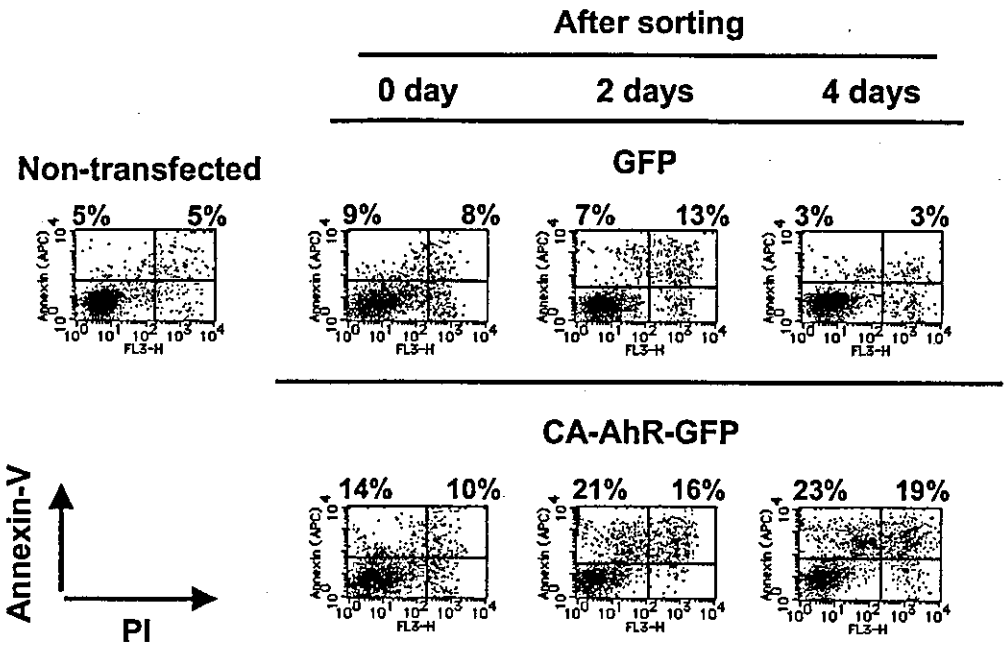

FIG. 3. **CA-AhR induces apoptosis in Jurkat T cells.** The expression vector for either CA-AhR-GFP or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. After 2 days, GFP-positive cells were sorted from the transfected cells using a FACSVantage SE and then cultured for the indicated time periods. The cells were incubated with biotin-labeled annexin V, followed by staining with SA-APC and PI, and analyzed using a FACSCalibur. The *upper left quadrant* (annexin V-positive, PI-negative) represents early apoptotic cells, whereas the *upper right quadrant* (annexin V, PI-double positive) represents late apoptotic/necrotic cells.

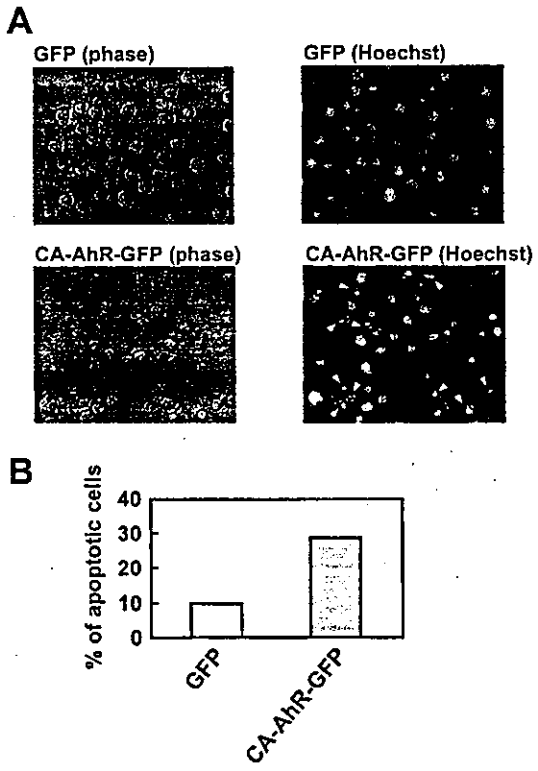

FIG. 4. **CA-AhR induces apoptotic morphological changes in Jurkat T cells.** *A*, the expression vector for either CA-AhR-GFP or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. After 2 days, GFP-positive cells were sorted from the transfected cells using a FACSVantage SE. The sorted cells were cultured for 2 days and then stained with 4 μM Hoechst 33342 for 15 min. The apoptotic cells were examined by the changes in their nuclear morphology, *i.e.* with fragmentation under a phase-contrast and a UV-visible fluorescence microscope. The *arrowheads* indicate the fragmented nuclei of the apoptotic cells. *B*, approximately over 100 cells were counted in four microscopic fields under a UV-visible fluorescence microscope, and the percentage of the apoptotic cells was estimated.

CA-AhR Induces the Accumulation of Cells in the G_1 Phase—We also investigated the possibility that CA-AhR induces cell cycle arrest. As shown in Fig. 5, in non-transfected Jurkat T cells, 48% of the cells were in the G_1 phase, 37% were in the S phase, and 15% were in the G_2/M phase. Immediately after sorting (0 day), no difference was observed in the DNA profile among non-transfected cells, cells expressing GFP alone, and those expressing CA-AhR-GFP. Two days after sorting, the percentage of cells expressing CA-AhR-GFP rose to 61% in the G_1 phase and correspondingly decreased to 24% in the S phase, whereas no change in cell cycle distribution was observed in cells expressing GFP alone. Four days after sorting, the percentages in the individual phases were not significantly changed from those obtained 2 days after sorting. These results suggest that CA-AhR affects cell cycle progression, especially in the G_1 phase.

The Inhibition of Growth by CA-AhR Is Mediated by Both XRE-dependent and -independent Mechanisms—To elucidate whether the inhibitory effect of CA-AhR on the growth of Jurkat T cells is mediated by an XRE-dependent or -independent mechanism, we generated an A78D mutant of the CA-AhR. The A78D mutant of wild-type AhR is translocated into the nucleus in the presence of TCDD and forms a heterodimer with ARNT. However, it lacks the potential for XRE-driven gene expression due to impaired XRE binding (24). To confirm that the disruption of transcription by A78D mutation is available for the CA-AhR, we examined the effects of A78D mutation introduced into the CA-AhR on localization and gene expression. When either CA-AhR-GFP or A78D mutant of CA-AhR-GFP (A78D-GFP) was transiently expressed in Jurkat T cells, a FACS analysis showed the same GFP expression patterns between both types of transfected cells, before and after sorting (Fig. 6*A*). Furthermore, the mRNA expression of CA-AhR-GFP or A78D-GFP was detected at the same level in both transfected cells by semiquantitative RT-PCR (Fig. 6*B*). The green fluorescence emitted by the GFP was mainly observed in the nuclear compartment in cells expressing A78D-GFP as well as CA-AhR-GFP (data not shown). However, as shown in Fig. 6*B*, only

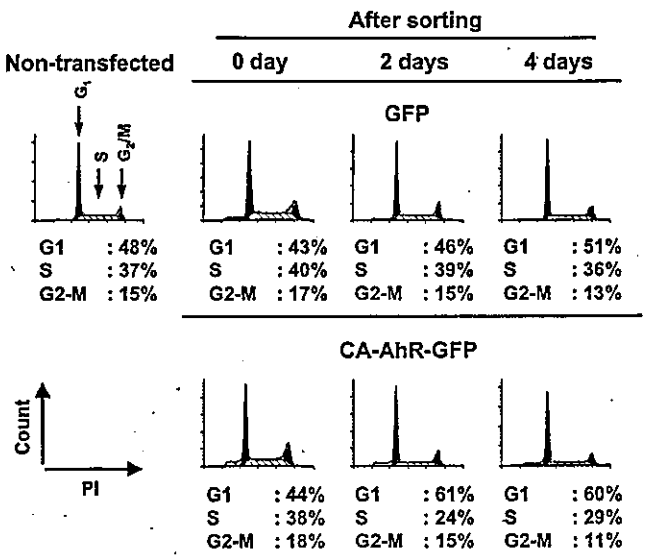

FIG. 5. **CA-AhR increases the percentage of cells in the G_1 phase.** The expression vector for either CA-AhR-GFP or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. After 2 days, GFP-positive cells were sorted from the transfected cells using a FACSVantage SE and then cultured for the indicated time periods. The cells were stained with PI using a CycleTEST Plus DNA reagent kit, and DNA content was measured using a FACSCalibur. The percentages of cells in the G_1, S, and G_2/M phases were analyzed using ModFit software.

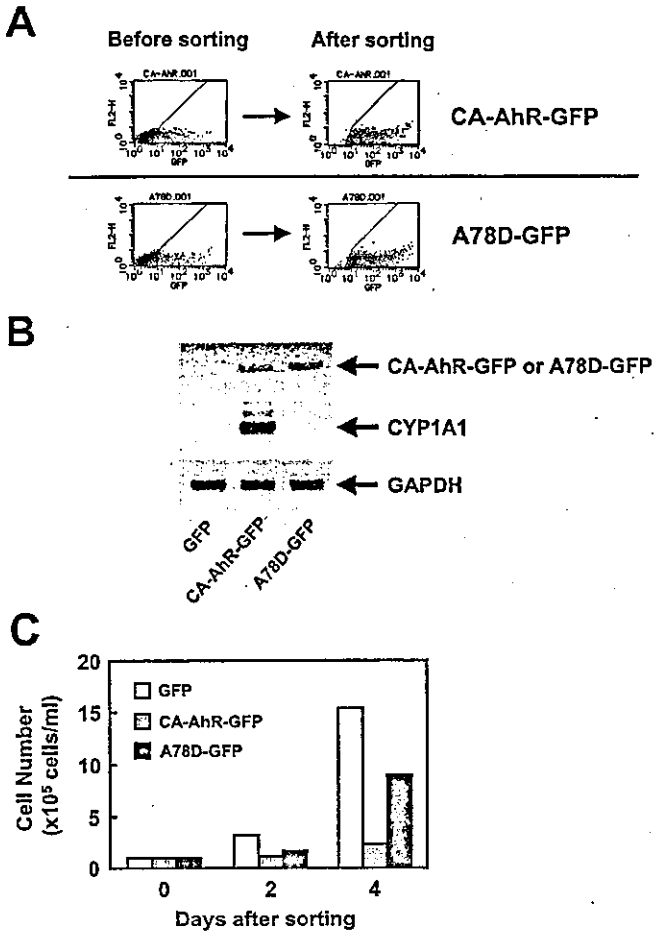

FIG. 6. **CA-AhR inhibits the growth of Jurkat T cells by the XRE-dependent and -independent mechanisms.** *A*, the expression vector for either CA-AhR-GFP, A78D-GFP, or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. Two days after the transfection, GFP-positive cells were sorted using a FACSVantage SE. The GFP expression of the cells was analyzed before and after sorting, using a FACSCalibur. *B*, total RNA was isolated from the cells and mRNA expression levels of CYP1A1, CA-AhR-GFP, and glyceraldehyde-3-phosphate dehydrogenase were examined by semiquantitative RT-PCR. *C*, the sorted cells were cultured at 1×10^5 cells/ml, and the cell numbers at the indicated times were determined by trypan blue exclusion.

CA-AhR-GFP, but not A78D-GFP, induced CYP1A1 mRNA expression. These observations show that A78D-GFP is constitutively localized in the nucleus in the absence of TCDD, but it cannot induce gene expression by binding to the XRE.

Using these AhR mutants, the XRE dependence of the inhibitory effect of activated AhR was examined. As shown in Fig. 6*C*, CA-AhR markedly inhibited the increase in cell number (in agreement with the data shown in Fig. 2). On the other hand, the A78D mutant only partially inhibited the increase. This result indicates that both XRE-dependent and -independent mechanisms are involved in the CA-AhR-induced growth inhibition.

CA-AhR Induces Expression Changes of Genes Related to Apoptosis and Cell Cycle Arrest by an XRE-dependent Mechanism—Because CA-AhR induced apoptosis and the accumulation in the G_1 phase in Jurkat T cells, we examined whether CA-AhR changes the expression of genes related to apoptosis and cell cycle arrest and whether the regulations of these genes are mediated by XRE-dependent transcription. Two days after transfection, total RNA was isolated from the GFP-positive cells, and the gene expression was analyzed using an Affymetrix GeneChip. Genes related to apoptosis and cell cycle arrest were selected from the genes that showed at least a 2-fold change in gene expression in the cells expressing CA-AhR-GFP, as compared with cells expressing GFP alone, and their expression changes were confirmed by semiquantitative RT-PCR. Furthermore, we determined the relative -fold induction of each of the confirmed genes in cells expressing CA-AhR-GFP and in cells expressing A78D-GFP by a comparison with the cells expressing GFP alone (Fig. 7 and Table II). We found that CA-AhR up-regulates genes related to apoptosis (caspase 8, c-Jun, and Fas) (26, 27) and cell cycle arrest (cyclin G2, growth arrest and DNA-damage-inducible, alpha (GADD45A), $p21^{waf1}$, cell division autoantigen-1 (CDA1), and IL-9 receptor) (28–32). CA-AhR also up-regulated the genes involved in both apoptosis and cell cycle arrest (dual specificity phosphatase 6, GADD34, and TGF-β receptor II) (33–36). On the other hand, c-Myc, which plays an important role in the G_1/S transition (37), was down-regulated in cells expressing CA-AhR-GFP. Furthermore, our results clarified that all the changes in these genes were dependent on transcription through the XRE, because only CA-AhR, but not A78D, induced expression changes of these genes (Fig. 7 and Table II).

DISCUSSION

In the present study, $CD4^+$ T cell line Jurkat T cells were transiently expressed with a CA-AhR, a model of ligand-activated AhR. We demonstrated that CA-AhR remarkably inhibits the growth of Jurkat T cells. We also clarified that CA-AhR induces both apoptosis and accumulation in the G_1 phase, which strongly suggests that these effects induce growth inhibition in Jurkat T cells. Furthermore, we showed that CA-AhR-induced growth inhibition is mediated by both XRE-dependent and -independent mechanisms, using an A78D mutant of the CA-AhR. With regard to the XRE-dependent mechanism, our results demonstrate that CA-AhR induces expression changes in genes related to apoptosis and cell cycle arrest. On the other hand, the XRE-independent growth inhibition, which is caused by the A78D mutant of the CA-AhR, may result from the interaction between CA-AhR and its target molecules. For in-

stance, it has been reported that RB specifically interacts with both an LXCXE motif in PAS B of the AhR and the C-terminal region of the AhR and that their direct interaction inhibits E2F-driven gene expression, leading to G_1 arrest (7, 8, 38). Although the CA-AhR protein lacks a PAS B region containing an LXCXE motif, it possesses the ability to specifically interact with RB through the C-terminal region. The semiquantitative RT-PCR data in our present and previous study (19) suggest that the CA-AhR was expressed in Jurkat T cells at a much higher level than the native AhR in mouse thymocytes and splenocytes. Therefore, we also cannot rule out the possibility that the XRE-independent growth inhibition was due to artifactual effects, such as stress response, of overexpression of the AhR protein in the nucleus. Further studies will be needed to elucidate the mechanism of XRE-independent growth inhibition.

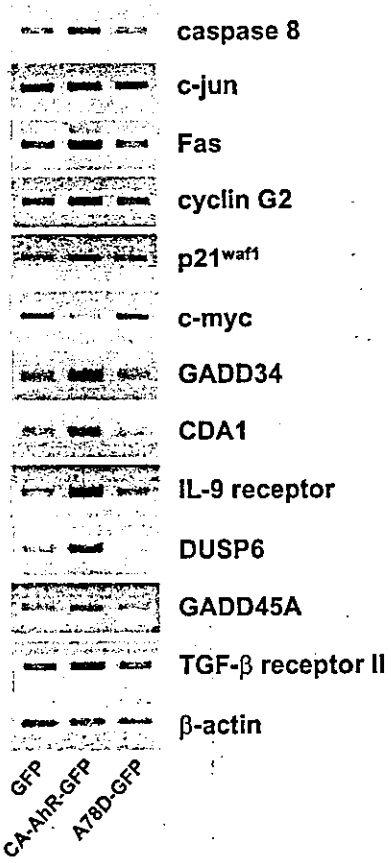

FIG. 7. **CA-AhR induces expression changes of genes related to apoptosis and cell cycle arrest by an XRE-dependent mechanism.** The expression vector for either CA-AhR-GFP, A78D-GFP, or GFP alone was transiently transfected into Jurkat T cells using DMRIE-C reagent. Two days after the transfection, GFP-positive cells were sorted using a FACSVantage SE. Total RNA was isolated from the cells, and gene expression was analyzed using an Affymetrix GeneChip. Genes related to apoptosis and cell cycle arrest were collected from the genes that showed at least a 2-fold change in gene expression in the cells expressing CA-AhR-GFP, as compared with the cells expressing GFP alone, and the expression changes of these genes were confirmed by semiquantitative RT-PCR.

The gene expression analysis suggests the involvement of several signaling pathways in the growth suppression of Jurkat T cells. The increase in Fas and caspase 8 transcripts by CA-AhR suggests that the Fas signaling pathway is involved in the CA-AhR-induced apoptosis. In agreement with our present data, previous studies using mice having a deficiency in the Fas signaling pathway have shown that TCDD decreases the cell number of anti-CD3-activated T cells through a Fas signaling pathway (39, 40). The study by Zeytun *et al.* (40) reported that Fas ligand was up-regulated in the spleen cells of mice exposed to TCDD. However, CA-AhR did not increase the expression level of Fas ligand in Jurkat T cells. This discrepancy between our study and that of Zeytun *et al.* suggests that TCDD-induced up-regulation of Fas ligand is due to the effect on non-T cells in spleen cells. As another apoptosis-related gene, we found that CA-AhR increases TGF-β receptor II transcript. The TGF-β signaling pathway has been reported to induce apoptosis and cell cycle arrest in T cells (36). Although CA-AhR induces no changes in the expression level of the TGF-β family (TGF-β1, β2, and β3) in Jurkat T cells (data not shown), the up-regulation of TGF-β receptor II by AhR activation in T cells may cause an increase in their susceptibility to TGF-β. CA-AhR also regulated a number of genes related to cell cycle arrest. We found that CA-AhR induces expression changes of genes involved not only in G_1 arrest (cyclin G2 and c-Myc) (28, 37) but also in G_2 arrest (GADD45A) (29), in both G_1 and G_2 arrest ($p21^{waf1}$) (30), and in multiphase cell cycle arrest (CDA1) (31). Through a cell cycle analysis, we showed that CA-AhR induced the accumulation in the G_1 phase by culturing for 2 days after sorting. However, further accumulation in the G_1 phase was not found by culturing for 4 days after sorting. These results may suggest

TABLE II

Relative-fold induction of CA-AhR-regulated genes in the XRE-dependent and -independent fashion

Gene expression changes by CA-AhR were examined by Affymetrix GeneChip analysis, and genes related to apoptosis and cell cycle arrest with at least two-fold changes in CA-AhR-GFP-transfected cells, as compared with GFP-alone transfected cells, were selected. Relative expression levels of the selected genes in cells expressing either CA-AhR-GFP or A78D-GFP were determined by semiquantitative RT-PCR.

Description	Change[a]	Functions	CA-AhR, change[b]	A78D, change[c]
			-fold	
Caspase 8	Up	Apoptosis	1.72	0.76
c-Jun	Up	Apoptosis	1.42	0.85
Fas	Up	Apoptosis	2.66	0.62
Cyclin G2	Up	G_1 arrest	2.20	0.78
c-Myc	Down	G_1/S transition	0.29	0.75
GADD45A	Up	G_2 arrest	1.30	0.71
$p21^{waf1}$	Up	G_1 arrest and G_2 arrest	1.98	1.19
CDA1	Up	Growth arrest	2.36	0.73
IL-9 receptor	Up	Growth arrest	4.79	1.33
DUSP6	Up	Apoptosis and growth arrest	3.00	0.57
GADD34	Up	Apoptosis and growth arrest	2.18	0.69
TGF-β receptor II	Up	Apoptosis and growth arrest	1.79	0.89

[a] Gene expression changes were determined by Affymetrix GeneChip analysis.

[b] Relative gene expression level was determined in cells expressing CA-AhR-GFP, as compared with cells expressing GFP alone, by semiquantitative RT-PCR.

[c] Relative gene expression level was determined in cells expressing A78D-GFP, as compared with cells expressing GFP alone, by semiquantitative RT-PCR.

that CA-AhR causes alteration of the G_1/S transition at an early stage and then inhibits cell cycle progression at various stages of the cell cycle.

With regard to how CA-AhR regulates the transcription of these genes, a search for the human genome sequences of the NCBI demonstrated that the 5′-flanking regions of GADD34, IL-9 receptor, CDA1, and c-Jun genes contain the core consensus sequence of XRE (5′-TNGCGTG-3′ or 5′-CACGCNA-3′), suggesting that these genes are directly regulated by activated AhR through the XRE, whereas other genes seem to be regulated by indirect mechanisms. The expression of Fas, GADD45A, $p21^{waf1}$, and caspase 8 are known to be up-regulated by the activation of p53 (41, 42). Recently, it has been reported that GADD34 induces phosphorylation of p53 and enhances $p21^{waf1}$ expression (35). Likewise, CA-AhR may up-regulate genes such as Fas, GADD45A, and caspase 8 through induction of GADD34 and following p53 activation. In addition, it has been reported that the induction of CYP1A1 causes DNA damage (43), probably leading to the activation of p53. Therefore, this p53-dependent pathway may be involved in CA-AhR-induced apoptosis and cell cycle arrest.

Previous studies have reported that TCDD induces apoptosis in AhR-null T cell clones, including Jurkat T cells, in an AhR-independent manner (20, 44). However, Jurkat T cells used in this study were not susceptible to apoptosis even in the presence of 10 nM TCDD (data not shown). Although the reason for the discrepancy is unclear, our results well indicate that activated AhR is essential for the inhibition of T cell growth by TCDD, in agreement with the findings that AhR expression is indispensable for TCDD-induced immunosuppression *in vivo* (10, 11).

In summary, we demonstrated that CA-AhR induces the growth inhibition of Jurkat T cells, with an increase in apoptosis and the accumulation of the cells in the G_1 phase. Furthermore, we showed that both XRE-dependent and -independent mechanisms are involved in CA-AhR-induced growth inhibition and that CA-AhR regulates the expression of several genes related to apoptosis and cell cycle arrest in an XRE-dependent manner. Further studies will aim to identify target gene(s) and protein(s) mainly responsible for the inhibition of T cell growth by the XRE-dependent and -independent mechanisms. $CD4^+$ helper T cells play an important role in both humoral and cellular immunity, where TCDD inhibited the increase in the number of $CD4^+$ T cells, following immunization (16, 45). The present data may provide a mechanism for the suppression of both humoral and cellular immunity.

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Activation of Hepatic Nrf2 *In Vivo* by Acetaminophen in CD-1 Mice

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The transcription factor NF-E2-related factor 2 (Nrf2) plays an essential role in the mammalian response to chemical and oxidative stress through induction of hepatic phase II detoxification enzymes and regulation of glutathione (GSH). Enhanced liver damage in Nrf2-deficient mice treated with acetaminophen suggests a critical role for Nrf2; however, direct evidence for Nrf2 activation following acetaminophen exposure was previously lacking. We show that acetaminophen can initiate nuclear translocation of Nrf2 *in vivo*, with maximum levels reached after 1 hour, in a dose dependent manner, at doses below those causing overt liver damage. Furthermore, Nrf2 was shown to be functionally active, as assessed by the induction of epoxide hydrolase, heme oxygenase-1, and glutamate cysteine ligase gene expression. Increased nuclear Nrf2 was found to be associated with depletion of hepatic GSH. Activation of Nrf2 is considered to involve dissociation from a cytoplasmic inhibitor, Kelch-like ECH-associated protein 1 (Keap1), through a redox-sensitive mechanism involving either GSH depletion or direct chemical interaction through Michael addition. To investigate acetaminophen-induced Nrf2 activation we compared the actions of 2 other GSH depleters, diethyl maleate (DEM) and buthionine sulphoximine (BSO), only 1 of which (DEM) can function as a Michael acceptor. For each compound, greater than 60% depletion of GSH was achieved; however, in the case of BSO, this depletion did not cause nuclear translocation of Nrf2. In conclusion, GSH depletion alone is insufficient for Nrf2 activation: a more direct interaction is required, possibly involving chemical modification of Nrf2 or Keap1, which is facilitated by the prior loss of GSH. (HEPATOLOGY 2004;39:1267–1276.)

The cellular defense response to chemical or oxidative stress is characterized by a coordinated induction of phase II drug-metabolizing enzymes and glutathione (GSH) synthesis, which protect the cell through the elimination of electrophiles and reactive oxygen species (ROS).[1,2] Central to this transcriptional response is a common DNA sequence found within the promoter regions of these phase II genes, which is referred to as the antioxidant (or electrophile) responsive element (ARE/EpRE).[3,4]

The ARE, first identified in the upstream regulatory region of the rat *GSTA2* gene,[5] was found to respond to oxidative stress.[6] The resemblance of the consensus ARE to the DNA *cis* element recognized by nuclear factor-erythroid 2 (NF-E2),[7] aligned with the discovery of a subset of basic leucine zipper (bZip) transcription factors known as the cap 'n' collar proteins that presently comprise the NF-E2-related factors 1, 2, and 3 (Nrf1, Nrf2, and Nrf3), and Bach1 and Bach2, revealed a family of ARE-interacting factors. Studies using forced expression of Nrf2 have demonstrated this bZip protein to be a functionally critical component for ARE activation.[8–11] Gene deletion studies have also shed light on the importance of Nrf2 in driving the antioxidant transcriptional response.[12–15]

Under normal homeostatic conditions, Nrf2 is believed to reside predominantly within the cytoplasm of the cell. Activation of Nrf2, which initially depends on its nuclear translocation, has been postulated to occur through a number of signal transduction pathways (for a

Abbreviations: GSH, reduced glutathione; ROS, reactive oxygen species; ARE/EpRE, antioxidant/electrophile response element; NF-E2, nuclear factor-erythroid 2; bZip, basic zipper protein; Nrf, NF-E2-related factor; Keap1, Kelch-like ECH-associated protein 1; NAPQI, N-acetyl-p-benzoquinoneimine; IP, intraperitoneal; DEM, diethyl maleate; BSO, buthionine sulfoximine; TBS, Tris-buffered saline; ALT, alanine transaminase; HO-1, heme oxygenase-1; GCLC, glutamate cysteine ligase catalytic subunit; mEH, microsomal epoxide hydrolase; mRNA, messenger RNA.

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