

Involvement of Promyelocytic Leukemia Protein in the Ethanol-induced Apoptosis in Mouse Embryo Fibroblasts

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The promyelocytic leukemia (PML) gene is a tumor suppressor gene associated with cell apoptosis, cell proliferation, and senescence. However, the role of PML in the ethanol-induced apoptosis is not fully-known. In this study, using wild-type mouse embryo fibroblasts (MEF) and PML null MEF cells, we found that (1) ethanol (100 mM and 200 mM) could obviously induce apoptosis of wild-type MEF cells, whereas, in PML null MEF cells, the pro-apoptotic function of ethanol was partially blocked; (2) the expression levels of phosphorylated p53 and two of its target genes, p21 and Bax, could be significantly up-regulated by ethanol (200 mM) in wild-type MEF cells in a time-dependent manner, but not in PML null MEF cells. These results indicate that PML plays an important role in ethanol-induced apoptosis, and p53-dependent apoptotic pathway may be involved in this process.

Key words—promyelocytic leukemia protein; apoptosis; ethanol

INTRODUCTION

Ethanol has been implicated as a potential messenger involving programmed cell death.^{1–3} Increased levels of ethanol and repeated exposures trigger apoptosis in hepatocytes,¹ astrocytes,⁴ neurons,⁵ T lymphocytes,⁶ macrophages,⁷ gastric mucosas,⁸ and mouse embryo fibroblasts (MEF),⁹ leading to liver injury, neuro-behavioral problems, modulation of immune responses, and enhanced risk for cancer. Although the cellular spectrum of apoptosis induced by ethanol is well known, the signal transduction molecules involved in this process remain poorly defined.

The PML gene, involved in the t(15;17) chromosomal translocation of acute promyelocytic leukemia (APL), encodes a protein implicated in apoptosis, cell proliferation, and senescence.¹⁰ PML epitomizes a multiprotein nuclear structure, the PML-nuclear body (PML-NB), whose proper formation and function depend on PML. Studies in knockout mice and cells unraveled an essential pleiotropic role for PML in multiple p53-dependent and -independent apoptotic pathways.¹¹ As a result, PML^{-/-} mice and cells are protected from apoptosis

triggered by a number of stimuli such as ionizing radiation, interferon, ceramide, Fas, and tumor necrosis factor (TNF).¹² It is becoming apparent that PML and the PML-NB act as molecular hubs for the induction and/or reinforcement of programmed cell death through selective and dynamic regulation of pro-apoptotic transcriptional factors, and then by affecting related target genes expression, such as Bax and p21.¹¹ However, whether PML is involved in apoptosis induced by ethanol, a well-known stimulus to cytotoxicity, is unclear.

In the present study, we investigated the role of PML in ethanol-induced apoptosis and its possible mechanism using wild-type MEF and PML^{-/-} MEF cells.

MATERIALS AND METHODS

Cells Culture and Ethanol Treatment Immortalized PML^{-/-} mouse embryo fibroblasts (MEF) and normal MEF (kindly provided by Dr. P.P. Pandolfi at Memorial Sloan-Kettering Cancer Center, NY) were cultured in Dulbecco's minimum essential medium (DMEM, Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco BRL), 100 µg/ml streptomycin sulfate, and 100 units/ml penicillin G. Control cells (untreated cells) were exposed only to plain essential medium (DMEM),

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while the treated cells were incubated with ethanol (100 mM and 200 mM).

DNA Fragmentation Assay DNA fragmentation was done with slight modification as previously described.¹³⁾ After various treatments for 48 h, cells were collected by centrifugation and lysed with lysis buffer [0.5% Triton X-100, 5 mmol/l Tris Buffer (pH 7.4), 20 mmol/l ethylenediamine tetraacetic acid (EDTA)], and RNA was removed by incubation with RNase A (0.8 mg/ml) at 37°C for 30 min. DNA was extracted with phenol/chloroform and precipitated with 1/10 volume of 3 mol/l sodium acetate (pH 5.2) and two volumes of 100% ethanol. DNA pellets were obtained by centrifugation, dried, and resuspended in 1×TAE (40 mmol/l Tris-acetate and 1 mmol/l EDTA). The samples were separated on 2% agarose gels stained with ethidium bromide and the appearance of DNA laddering was visualized under an UV transilluminator. The DNA marker was also run to determine the approximate size of fragmented DNA.

Apoptosis Assay The cells were treated with different concentrations of ethanol as described above. After 48 h, cells were harvested by centrifugation and washed twice in PBS. Apoptosis was measured by flow cytometry (BD FACSCalibur) in the FL1-H and FL2-H channels after staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V (AV, 5:100, V:V) and propidium iodide (PI, 5 µg/ml) as described by the manufacturer.

Western Blot Analysis For immunoblot studies cells were lysed in lysis buffer (1% NP40, 20 mM Tris-HCl, pH 8, 130 mM NaCl, 10 mM NaF, 10 µg/ml aprotinin, 40 µM leupeptin, 1 mM DTT, 1 mM Na₃VO₄ and 1 mM PMSF) by incubating on ice for

30 min. Then, denatured proteins were electrophoresed on SDS-PAGE, transferred to PVDF membranes, and incubated at room temperature for 1 h or at 4°C overnight with mouse monoclonal anti-p53 antibody (1: 400; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-phospho-p53 (Ser15) antibody (Cell Signaling Technology, Beverly, MA), mouse monoclonal anti-p21 antibody (1:500; Santa Cruz Biotechnology), and mouse monoclonal anti-Bax antibody (1:500; Santa Cruz Biotechnology). After washing in Tris buffered saline with 0.05% Tween 20 (TBS-Tween), blots were incubated with horseradish peroxidase-conjugated antibodies: goat-anti-mouse IgG (1: 5000; Santa Cruz Biotechnology). Finally, blots were developed using the enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech). Results were normalized to the internal control β-actin.

RNA Extraction and Reverse Transcription-PCR After treated MEFs with ethanol (200 mM) for 12, 24, and 48 h, total RNA was extracted from cells with the TRIzol Reagent (Invitrogen-Life Technologies, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. RNA was reverse-transcribed with Superscript II Transcriptase (Invitrogen-Life Technologies, Inc.) in the presence of oligo-dT and random primers. Primer sequences and PCR condition are shown in Table 1. After PCR, products were analyzed by 1% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

So as to investigate the role of PML in ethanol-induced apoptosis, DNA fragmentation assay, reflecting the endonuclease activity characteristic of apopto-

Table 1. Primers, Amplicon Size, and Protocol Details for PCR

PCR	Forward (F) and reverse (R) primers (5' to 3')	Size, bp	T (°C)	Extension Time (s)	No. of cycles
p53	F: ATGACTGCCATGGAGGAGTC R: CTAGCAGTTTGGGCTTCC	1146	56	60	35
p21	F: GTGATTGCGATGCGCTCATG R: TCTCTTGCAAGACCAATC	378	58	30	30
Bax	F: GCAGAGGATGATTGCTGACG R: CTCAGCCCATCTTCTCCAG	353	55	30	35
β-actin	F: GTATGGAATCCTGTGGCATC R: AAGCACTTGCGGTGCACGAT	322	58	30	30

T (°C): Annealing temperature.

sis, was performed in wild-type MEF (Wt MEF) and PML^{-/-} MEF cells. As shown in Fig. 1, ethanol treatment (200 mM) induced the formation of definite fragments in wild-type MEF cells, which could be seen *via* electrophoresis as a characteristic ladder pattern. In contrast, no apoptosis was induced by ethanol (100 and 200 mM) in the PML^{-/-} MEF cells (Fig. 1).

Further to ascertain the role of PML in ethanol-induced apoptosis, we detected apoptosis by Annexin V

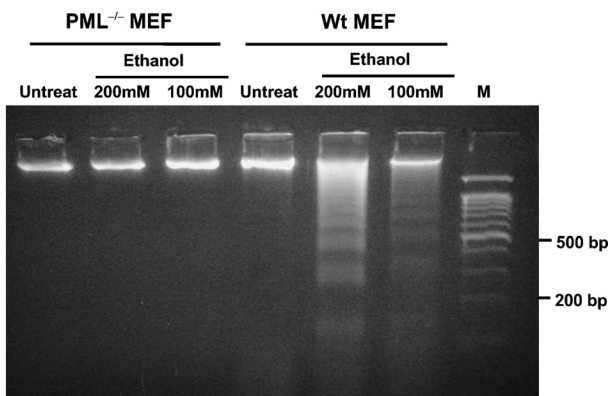


Fig. 1. Ethanol-induced Apoptosis
 Representative data are shown for the wild-type (Wt) mouse embryonic fibroblast (MEF) cells and PML^{-/-} MEF cells. Cells were either left untreated or treated with 100 and 200 mM ethanol. Total cells were harvested at 48-h intervals, and the recovered DNA was electrophoresed on 2% agarose gels with a standard marker (M).

(AV, an indicator of early apoptosis) and propidium iodide (PI, an indicator of cell necrosis) double-staining method. The data showed that when wild-type MEF cells were exposed to ethanol for 48 h, the proportions of AV⁺/PI⁺ (necrotic cells and late apoptosis cells) were increased from 3.21% of control cells to 6.86% and 7.64% of the 100 and 200 mM ethanol groups, respectively (Fig. 2). In addition, the number of AV⁺/PI⁻ (early apoptotic cells) increased from 5.52% of control cells to 20.95% and 25.47% of the 100 and 200 mM ethanol groups, respectively (Fig. 2). These results suggest that both necrosis and apoptosis contribute to the ethanol-induced death of MEF cells and ethanol-induced apoptosis is the main cause. Compared with the obvious increasing of ethanol-induced apoptosis in wild-type MEF cells, ethanol exposure (100 and 200 mM) just slightly elevated the proportions of necrotic cells and apoptosis cells in PML^{-/-} MEF cells (Fig. 2). These findings indicate that PML, a mediator of programmed cell death, may be involved in ethanol-induced apoptosis. Furthermore, our results showed that ethanol-induced apoptosis was decreased but not completely blocked in PML^{-/-} MEF cells. One possible reason for this could be that, besides a PML-dependent mechanism, a PML-independent mechanism is also involved in ethanol-induced apoptosis. To

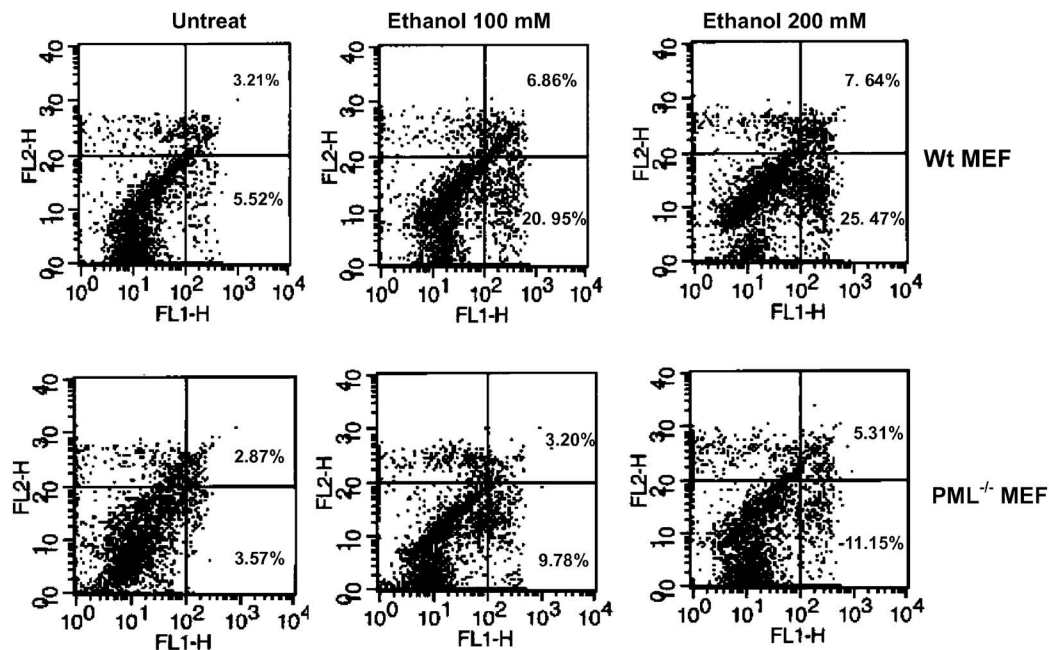


Fig. 2. Apoptotic Cells Detected by Flow Cytometry with Annexin V Conjugated with Propidium Iodide Staining
 Wild-type (Wt) MEF and PML^{-/-} MEF cells were treated without or with ethanol at 100 and 200 mM for 48-h. The assay was performed according to the materials and methods. FL1-H (Annexin V); FL2-H (propidium iodide).

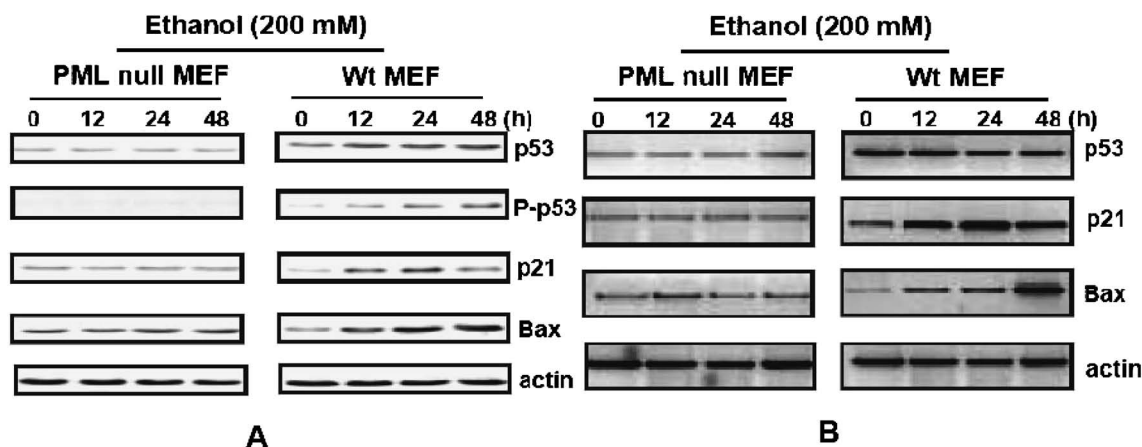


Fig. 3. Effects of Ethanol on Expressions of p53, Phosphorylated p53, p21, and Bax in Wild-type (Wt) MEF and PML^{-/-} MEF Cells

A, immunoblot analysis of apoptotic-related proteins. B, RT-PCR analysis of apoptotic-related genes. β -Actin was used to normalize protein and mRNA levels.

our knowledge, this is the first report of the relationship between PML and ethanol-induced apoptosis.

It is reported that PML regulates both p53-dependent and -independent apoptosis.¹¹⁾ To explore whether the silenced PML inhibiting ethanol-induced apoptosis is mediated through a p53-dependent pathway, we investigated the effect of ethanol on p53, phosphorylated p53, and its target genes, p21 and Bax, using Western blot and RT-PCR methods in wild-type MEF and PML^{-/-} MEF cells. Our data indicate that the protein levels of phosphorylated p53 and Bax increased significantly in a time-dependent manner in wild-type MEF cells after treatment with ethanol at a concentration of 200 mM (Fig. 3A). In addition, we found there was no change in total p53 protein after ethanol treatment. Notably, p21 protein was expressed at the highest level at 24 h, but slightly decreased at 48 h (Fig. 3A), suggesting that the overexpression of p21 by ethanol is not solely *via* induction of p53 activation, and other transcription factors, for example Smads and Sp1,¹⁴⁾ might also contribute to the overexpression of p21 by ethanol. In contrast, PML^{-/-} MEF cells were almost entirely refractory to the same treatment. Further to ascertain the up-regulation of p21 and Bax transcription induced by ethanol, we performed RT-PCR. The results showed a similar pattern to that of immunoblot data (Fig. 3B). Taken together, our results demonstrate that not only PML but also p53 is involved in ethanol-induced apoptosis. It is well known that tumor suppressor genes of PML and p53 are frequently subject to inactivation in malignant tumors,

which promote the progression of cancer.¹⁵⁻¹⁷⁾ Therefore, it is possible that ethanol could induce apoptosis in the presence of these tumor suppressors, whereas in the absence of these tumor suppressors, ethanol exposure might induce carcinogenesis due to cell survival and biochemical molecular alterations. Recently, Koch *et al.*¹⁸⁾ reported that, in the hepatocytes of mutant mice (p53^{-/-}) exposed to ethanol, widespread signs of dysplasia were observed, whereas in ethanol-fed normal mice these features were absent. This verifies our viewpoint. However, the detailed roles of PML and p53 in ethanol-induced cell apoptosis and carcinogenesis merit further investigation.

In conclusion, the present study demonstrates for the first time that the tumor suppressor gene PML might be involved in ethanol-induced apoptosis through a p53-dependent pathway.

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