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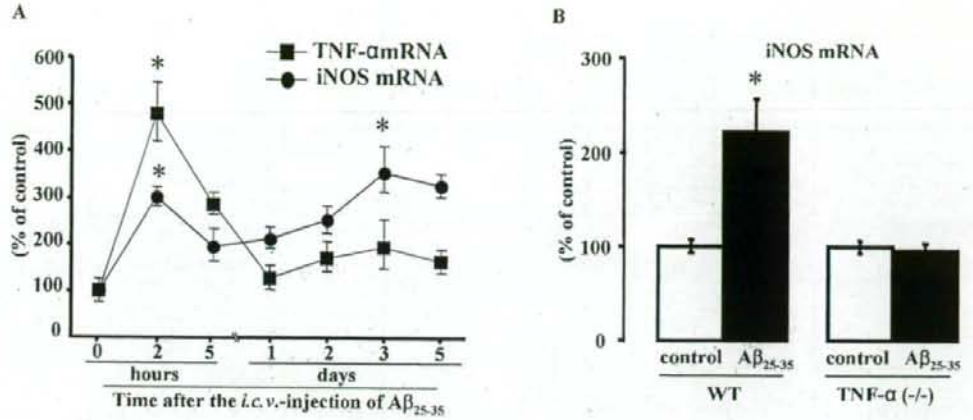

Fig. 2-1

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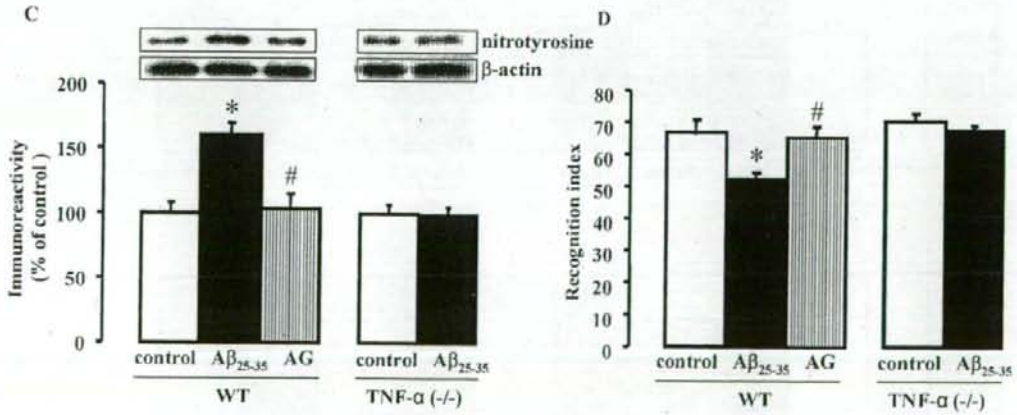

Fig. 2-2

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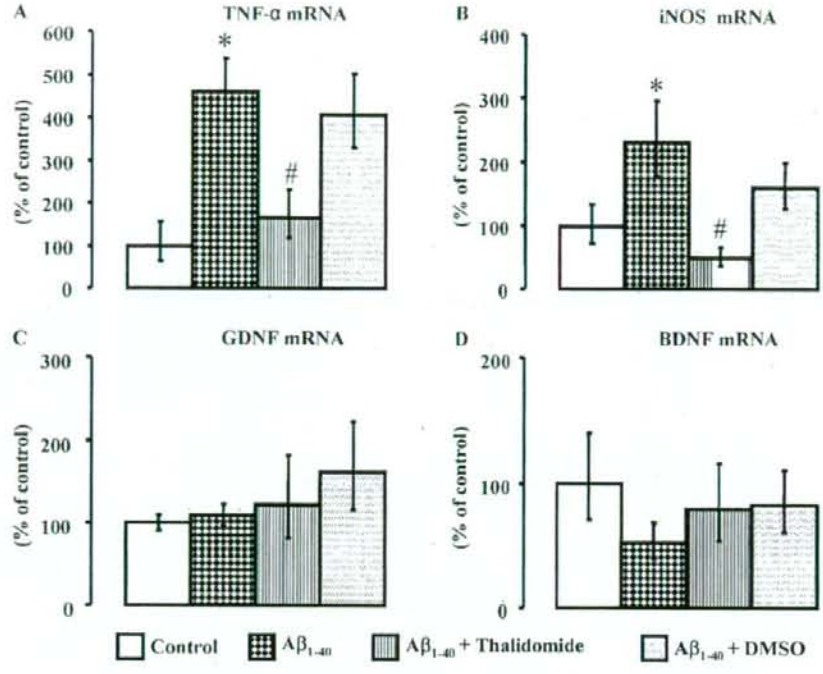

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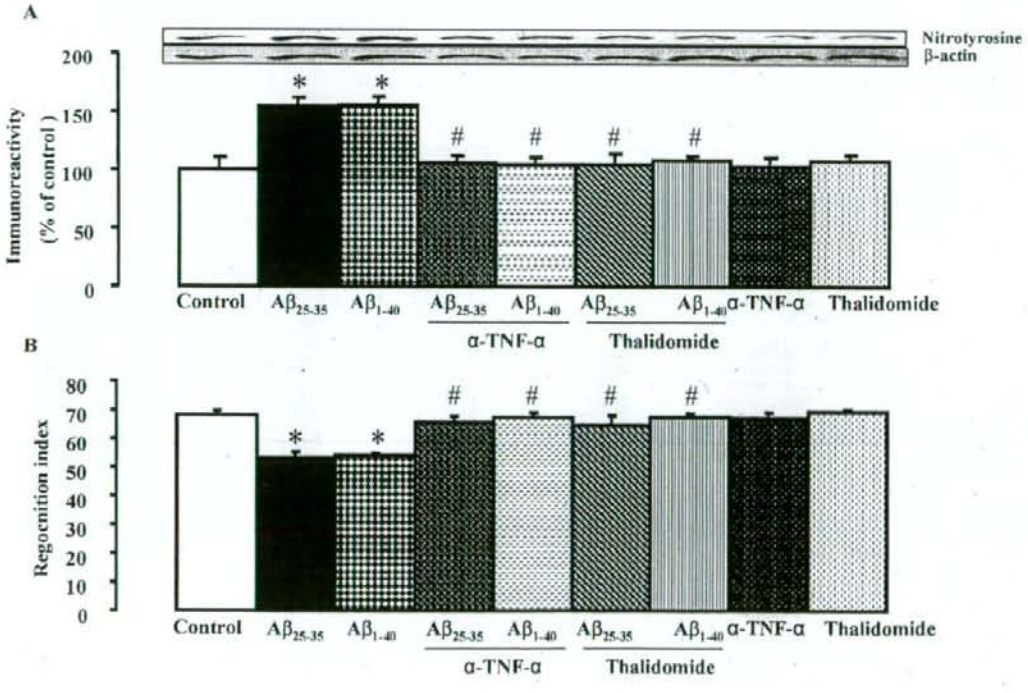

Fig. 4

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Production and functions of IL-17 in microglia

Jun Kawanokuchi [a], Kouki Shimizu [a], Atsumi Nitta [b], Kiyofumi Yamada [b], Tetsuya Mizuno [a], Hideyuki Takeuchi [a], Akio Suzumura [a,*]

[a] Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusaku, Nagoya 464-8601, Japan
[b] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine Showa-ku, Nagoya 466-8560, Japan

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Abstract

Interleukin (IL)-17-producing helper T cells may play a pivotal role in the pathogenesis of multiple sclerosis. Here, we examined the effects of IL-17 on microglia, which are known to be critically involved in multiple sclerosis. Treatment with IL-17 upregulated the microglial production of IL-6, macrophage inflammatory protein-2, nitric oxide, adhesion molecules, and neurotrophic factors. We also found that IL-17 was produced by microglia in response to IL-23 or IL-1β. Because microglia produce IL-1β and IL-23, these cytokines may act in an autocrine manner to induce IL-17 expression in microglia, and thereby contribute to autoimmune diseases, such as MS, in the central nervous system.
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Keywords: Cytokine; IL-17; EAE; MS; Microglia

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder that affects the central nervous system (CNS). Although the etiology of MS is not fully understood, T helper 1 (Th1) cells and the cytokines that they produce are thought to play a role in the development of MS. Recently, interleukin (IL)-17 producing helper T (Th17) cells play important roles in the induction of autoimmune diseases including MS and the corresponding animal model—experimental autoimmune encephalomyelitis (EAE) (Hofstetter et al., 2005, Ishizu et al., 2005, Iwakura and Ishigame, 2006). It has been shown that IL-17 mRNA levels are high in both the cerebrospinal fluid and plaques of MS patients (Matusevicius et al., 1999, Lock et al., 2003). IL-17 is a T cell-derived proinflammatory molecule that stimulates epithelial, endothelial, and fibroblastic cells to produce other inflammatory cytokines and chemokines, including IL-6, macrophage inflammatory protein (MIP)-2, granulocyte-colony stimulating factor (G-CSF), and monocyte chemoattractant protein (MCP)-1 (Aggarwal and Gurney, 2002; Yao et al., 1995; Kennedy et al., 1996; Fossiez et al., 1996; Linden et al., 2000; Cai et al., 1998; Jovanovic et al., 1998; Laan et al., 1999). IL-17 also synergizes with other cytokines such as tumor necrosis factor (TNF)α and IL-1β to further induce chemokine expression (Jovanovic et al., 1998; Chabaud et al., 1998). Although the precise mechanisms that control Th17 cell development have yet to be elucidated, Th17 cells are thought to develop from naïve T helper (Th0) cells via a pathway that is different than the pathways that lead to the development of Th1 and Th2 cells. In the absence of interferon (IFN)γ and IL-4, IL-23 has been shown to maintaining Th17 phenotype in a manner that is not dependent on the transcription factors STAT1, T-bet, STAT4, and STAT6 (Aggarwal et al., 2003; Harrington et al., 2005; Park et al., 2005; Bettelli et al., 2006). Interestingly, a recent study revealed that IL-27 is a critical regulator of IL-17 production. IL-27 receptor-deficient mice were found to generate more IL-17-producing T helper cells and were hypersusceptible to EAE, suggesting that IL-27 negatively regulates the development of Th17 cells (Batten et al., 2006).

* Corresponding author. Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusaku, Nagoya 464-8601, Japan. Tel.: +81 52 789 3881; fax: +81 52 789 3885.
E-mail address: suzumura@riem.nagoya-u.ac.jp (A. Suzumura).

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IL-17 levels have been shown to be significantly higher in the cerebrospinal fluid of patients with active optico-spinal MS (Ishizu et al., 2005) and in the CNS of EAE mice (Hofstetter et al., 2005). The effects of IL-17 on CNS cells, however, are unclear. In order to uncover the contribution of IL-17 to inflammatory demyelination in the CNS, we have examined the effects of IL-17 on microglia, which function as antigen-presenting cells and effector cells in the CNS during inflammatory demyelination.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS), human recombinant transforming growth factor (TGF)-β, and mouse recombinant IL-17 and IL-23 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse recombinant IL-1β, TNFα, and IFNγ were purchased from Techne (Minneapolis, MN, USA). Sulfonylamide, *N*-(1-naphthyl)ethylenediamine, and phosphate for Griess reagent (Ignarro et al., 1987) were also purchased from Sigma-Aldrich.

2.2. Cell culture

The protocols for the animal experiments were approved by the Animal Experiment Committee of Nagoya University. All primary cultures were prepared from C57BL/6J mice (Japan SLC, Hamamatsu, Shizuoka, Japan). Microglia were isolated from primary mixed glial cell cultures prepared from newborn mice on day 14 using the "shaking off" method as previously described (Suzumura et al., 1987); the purity of the cultures was almost 100%, as determined by immunostaining with anti-CD11b antibodies. The cultures were maintained in Dulbecco's modified Eagle's minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA), 5 μg/ml bovine insulin (Sigma), and 0.2% glucose.

Astrocyte-enriched cultures were prepared as described previously (Kuno et al., 2006). Briefly, the mixed glial cell cultures were trypsinized after the microglia were collected, and replated in Petri dishes. After this procedure was repeated three times, the cultures that had undergone four passages were used as the astrocyte-enriched cultures. The purity of the cultures were more than 80% as determined by immunostaining with anti-glial fibrillary acidic protein (GFAP). Peritoneal macrophages were collected from mice intraperitoneally injected with thioglycolate 48 h prior to collection. T cell-rich lymphocytes were separated from mouse spleens. Neuronal cultures were prepared from mice at embryonic day 17 as described previously (Takeuchi et al., 2005). Briefly, cortices were dissected and freed of meninges. Cortical fragments were dissociated into single cells using dissociation solution, and they were resuspended in Nerve-Cell Culture Medium (serum-free conditioned medium from 48-h rat astrocyte confluent cultures based on Dulbecco's modified Eagle's minimum essential medium/F-12 with N2 supplement, Sumitomo Bakelite, Akita, Japan). The purity of the cultures was more than 95% as determined by NeuN-specific immunostaining.

2.3. Expression of IL-17 receptors

The mRNA expression of the IL-17 receptor was examined using reverse transcription-polymerase chain reactions (RT-PCRs). Microglia, astrocytes, or neurons were cultured for 3 days before total cellular RNA was extracted using an RNase Mini Kit (Qiagen). cDNA encoding the IL-17 receptor was examined by RT-PCR analysis using SuperScript II (Invitrogen), AmpliTaq DNA polymerase (Applied Biosystems), and the specific primers shown in Table 1. Amplification within the linear range using 5 μl of each cDNA sample was achieved following 30 cycles in a DNA thermal cycler under conditions that were optimized for each set of primers.

The protein level of IL-17 receptor expression was examined using Western blot analysis. Samples (20 μg/well) were electrophoresed on 7.5% SDS-polyacrylamide gels (Invitrogen) according to the Laemmli method (Laemmli and Favre, 1973). After electrophoresis, proteins were transferred from the gels to nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK) using standard procedures (Towbin et al., 1979). Nonspecific binding was blocked with 5% nonfat dry milk in TBST buffer (5 mM Tris–HCl, pH 7.6, 136 mM NaCl, 0.05% Tween 20) for 1 h. Blots were incubated for 12 h at 4 °C with rat anti-mouse IL-17 receptor antibody (R&D Systems) (1:1000 dilution). Blots were washed four times in TBST: the first time for 20 min and 10 min each time thereafter. We then incubated the washed blots for 1 h at room temperature with a

Table 1
Primer sequences used for RT-PCR analysis

GAPDH sense, 5′-ACTCACGGGAAATTCAACG
GAPDH antisense, 5′-CCCTGTTGCTGTAGCCGTA
IL-17R sense, 5′-CTAAACTGCACGGTCAAGAAT
IL-17R antisense, 5′-ATGAACCAGTACACCCAC
TNFα sense 5′-ATGAGCACAGAAAGCATGATCCGC
TNFα antisense 5′-CCAAAGTAGACCTGCCCGGACTC
IL-1β sense, 5′-ATGGCAACTGTTCCTGAACTCAACT
IL-1β antisense, 5′-CAGGACAGGTATAGATTCTTTCCTTT
IL-6 sense, 5′-ATGAAGTTCCTCTCTGCAAGAGACT
IL-6 antisense, 5′-CACTAGGTTTGCCGAGTAGGATCTC
MIP-2 sense, 5′-CCGGCTCCTCAGTGCTG
MIP-2 antisense, 5′-GGTCAGTTAGCCTTGCCTTT
IL-17 sense, 5′-CAGGACGCGCAAAACATGA
IL-17 antisense, 5′-GCAACAGCATCAGAGAGACACAGAT
iNOS sense, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC
iNOS antisense, 5′-GGCTGTCAGAGCCTCGTGGCTTTGG
NGF sense, 5′-CATAGCGTAATGTCCATGTTGTTCT
NGF antisense, 5′-CTTCTCATCTGTTGTCAACGC
BDNF sense, 5′-AGCCTCCTCTGCTCTTTCTG
BDNF antisense, 5′-TTGTCTATGCCCCTGCAGCC
GDNF sense, 5′-ATTTTATTCAAGGCCACCATTA
GDNF antisense, 5′-GATACATCCACACCGTTTTAGC
MHC class II antigen sense, 5′-AAGAAGGAGACTGTCTGGATGC
MHC class II antigen antisense, 5′-TGAATGATGAAGATGGTGCCC
ICAM-1 sense, 5′-TTCACACTGAATGCCAGCTC
ICAM-1 antisense, 5′-GTCTGCTGAGACCCCTCTTG
VCAM-1 sense, 5′-ATTTTCTGGGGCAGGAAGTT
VCAM-1 antisense, 5′-ACGTCAGAACAACCGAATCC

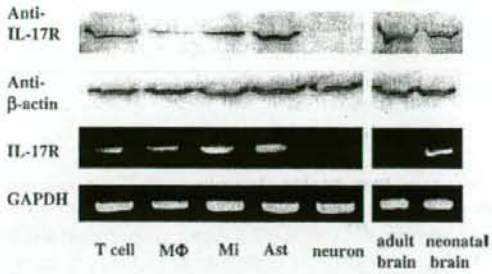

Fig. 1. Expression of IL-17 receptor mRNA and protein in neural cells. Microglia and astrocytes along with splenic T cells and macrophages express IL-17 receptor mRNA and protein, whereas neurons do not. MΦ, macrophages; Mi, microglia; Ast, astrocytes (left panel). The mRNA expression of IL-17 receptor in adult brain was lower than that of neonatal, but protein level of IL-17 receptor was the same as neonatal (right panel).

1:5000 dilution of peroxidase-conjugated, anti-rat IgG secondary antibody (Amersham Bioscience) followed by an additional rinse. IL-17 receptor was detected by ECL (Amersham Bioscience). The molecular weight of IL-17 receptor was determined by running molecular weight markers (Invitrogen) in an adjacent lane. Spleen cells served as a positive control.

2.4. Effects of IL-17 on the production of cytokines, neurotrophic factors and NO by microglia

Microglia and astrocytes were cultured in 24-well plates at a concentration of 1×10^6 cells/ml with or without 1 μg/ml LPS for 24 to 72 h in presence of various doses of IL-17 (1–100 ng/ml). The supernatants were then collected and stored at −80 °C until they were assessed. Total cellular RNA was extracted from remaining cells using an RNase Mini Kit (Qiagen). cDNAs encoding mouse TNFα, IL-1β, IL-6, iNOS, MIP-2 (the functional analogue of human IL-8), Nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and IL-17 were generated and amplified in RT-PCRs as described above using the specific primers shown in Table 1.

Cytokine production was measured using ELISA kits specific for TNFα, IL-6 (Techne), MIP-2, and IL-17 (R&D).

Cellular levels of NGF and BDNF in the microglia were also assessed as follows: stimulated microglial cultures were washed four times in cold PBS, the cells were lysed using sonication in ice-cold PBS containing protease inhibitors (complete mini EDTA-free; Roche, Mannheim, Germany), and the lysates were assayed for cellular NGF and BDNF using ELISA kits specific for NGF and BDNF (Promega, WI, USA). Cytoplasmic GDNF content was measured by an enzyme immunoassay (EIA) as described (Nitta et al., 1999). The EIA system for GDNF was based on the method originally developed for the EIA of NGF, BDNF, and NT-3 (Furukawa et al., 1983; Kaechi et al., 1993; Nitta et al., 1999; Nitta et al., 2004). Antibodies against GDNF were produced by immunizing rabbits with purified human recombinant GDNF. GDNF protein (0.5 mg) in phosphate-buffered saline (PBS; 5 ml) was emulsified with an equal volume of Freund's adjuvant and injected intradermally into rabbits four times at 2-week intervals. Animals were exsanguinated 1 week after the final injection. To affinity purify the antibody, antiserum (1 ml) first was loaded onto a GDNF-linked column (1-ml bed volume; Affi-Gel 10; Bio-Rad, Hercules, CA). After extensive sequential washing with three buffers, 0.1 M Tris–HCl (pH 7.4) containing 0.9% NaCl, 0.05 M borate buffer (pH 8.0), and 0.05 mM sodium acetate buffer (pH 5.0), the bound antibodies were eluted with a 0.1 M glycine–HCl buffer (pH 2.0). A part of the purified anti-GDNF antibody preparation was eluted and biotinylated. The detection limit of the EIAs was as low as 1 pg/ml.

NO production was determined using the Griess reaction as described (Pollock et al., 1991). Briefly, 50-μl aliquots of the supernatants were mixed with an equal volume of Griess reagent (0.1% *N*-ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) and incubated for 5 min at room temperature. The absorbance at 540 nm was measured

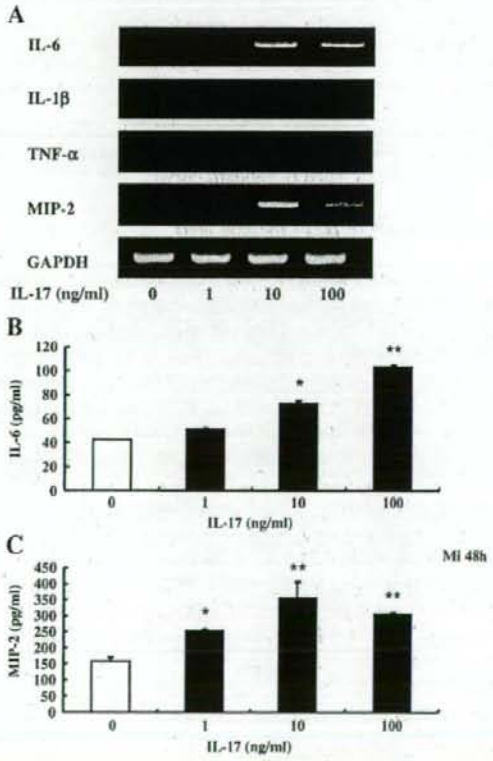

Fig. 2. The effects of IL-17 on cytokine production by microglia. Microglia were treated with IL-17 for 48 h. (A) At concentrations greater than 1 ng/ml, IL-17 induced the expression of IL-6 and MIP-2 mRNA. Similar results were obtained for IL-6 (B) and MIP-2 (C) protein using specific ELISAs. The values shown are the means±S.D. $^*P<0.05$ and $^{**}P<0.01$ compared with untreated microglia. The data represent typical samples performed in triplicate in three independent experiments.

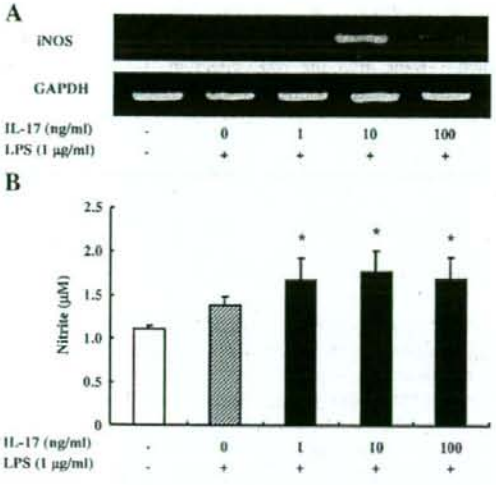

Fig. 3. The effects of IL-17 on NO production and iNOS expression in LPS-stimulated microglia. Microglia were stimulated with LPS for 48 h in the presence of various concentrations of IL-17. IL-17 enhanced iNOS mRNA expression (A) and NO production (B). The values shown are the means±S.D. *$P<0.05$ compared with LPS-stimulated microglia in the absence of IL-17. The data represent typical samples performed in triplicate in three independent experiments.

using a microtiter plate reader. Nitrite concentrations were calculated from a $NaNO_2$ standard curve.

2.5. Production of IL-17 by glial cells

Microglia and astrocytes were cultured for 72 h in 6-well plates at a concentration of 1×10^6 cells/ml with various doses of IL-23 (1–100 ng/ml). The culture supernatants were then collected and stored at −80°C until they were assessed. IL-17 production was measured using an ELISA kit specific for murine IL-17 (R&D). Total cellular RNA was extracted from the remaining cells using an RNase Mini Kit (Qiagen). cDNAs encoding mouse IL-17 were amplified using RT-PCRs as described above and the specific primers shown in Table 1. In some experiments, cytoplasmic levels of IL-17 in the microglia were also assessed as follows: stimulated microglial cultures were washed four times in cold PBS, the cells were lysed using sonication in ice-cold PBS containing protease inhibitors (complete mini EDTA-free; Roche, Mannheim, Germany), and the lysates were assayed for cellular IL-17 using ELISAs.

3. Results

3.1. Expression of the IL-17 receptor

Increased levels of IL-17 have been observed in the cerebrospinal fluid from patients with active MS as well as in the CNS of EAE mice. Thus, we assessed the expression of IL-17 receptor in CNS cells. RT-PCR demonstrated that neonatal microglia and astrocytes along with splenic T cells and peripheral macrophages expressed IL-17 receptor mRNA, whereas embryonic neurons did not (Fig. 1). Western blot analysis demonstrated that neonatal microglia and astrocytes along with splenic T cells and peripheral macrophages expressed IL-17 receptor protein, whereas embryonic neurons did not (Fig. 1).

We next compared the expression of IL-17 receptor mRNA and protein in adult brain with that of neonatal brain. The expression of mRNA for IL-17 receptor in adult brain was lower than that of neonatal, but protein level of IL-17 receptor was almost identical in these 2 samples (Fig. 1, left panel).

3.2. Effects of IL-17 on microglia

We then assessed the effects of IL-17 on microglia. IL-17 induced the mRNA expression of the inflammatory cytokines IL-6 and MIP-2 with maximum induction observed at 10 ng/ml (Fig. 2A). IL-17, however, did not significantly induce the expression of mRNA encoding IL-1β or TNFα. Upregulation of the expression of IL-6 and MIP-2 protein by IL-17 was confirmed with ELISAs; IL-17 at a concentration of 10 ng/ml or greater significantly increased the production of IL-6 and MIP-2 by microglia (Fig. 2B), whereas IL-1β and TNFα were not detected in the supernatants (data not shown). Although IL-17 by itself did not induce the expression of iNOS mRNA and NO production in unstimulated microglia, it enhanced iNOS mRNA expression and NO production in LPS-stimulated microglia; the maximum increase was observed with 10 ng/ml IL-17 (Fig. 3). In addition, IL-17 dose-dependently upregulated the expression of neurotrophic factors NGF, BDNF, and GDNF (Figs. 4 and 5). IL-17 itself did not induce the expression of mRNA encoding class II major histocompatibility complex (MHC) antigen or cell adhesion molecules (data not shown). On the other hand, the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 mRNA was upregulated in INFγ-stimulated microglia following treatment with IL-17, whereas it did not affect the expression of class II MHC antigen in these cells (Fig. 6). In contrast to the proinflammatory effects, IL-17 increased the expression of neurotrophic factors in microglia, which may contribute to anti-inflammatory defense mechanisms in the CNS and implies that microglia have multiple functions.

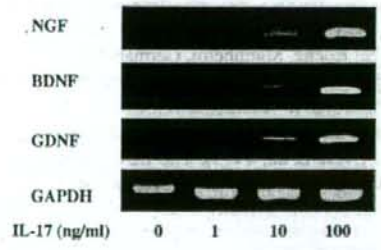

Fig. 4. The effects of IL-17 on the expression of mRNA coding for neurotrophic factors in microglia. Microglia were treated with IL-17 for 72 h. IL-17 dose-dependently induced the expression of NGF, BDNF, and GDNF mRNA.

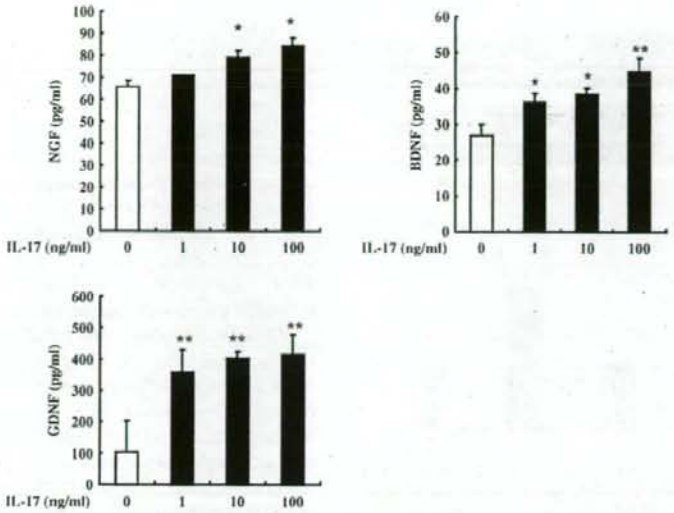

Fig. 5. The effects of IL-17 on the expression of neurotrophic factors in microglia. Microglia were treated with IL-17 for 72 h. IL-17 dose-dependently induced the expression of NGF, BDNF, and GDNF. The values shown are the means ± S.D. *$P<0.05$ and **$P<0.01$ compared with untreated microglia. The data represent typical samples performed in triplicate in three independent experiments.

3.3. Production of IL-17 by microglia

IL-17 is reported to be a T cell-specific cytokine (Yao et al., 1995). A study that analyzed human astrocytes using cDNA microarrays, however, suggested that CNS cells produce IL-17 (Meeuwsen et al., 2003). Thus, we assessed IL-17 production in glial cells. Although unstimulated microglia did not express mRNA coding for IL-17, IL-23 induced IL-17 mRNA expression in microglia in a dose-dependent manner. Because ELISAs failed to detect IL-17 in the supernatant of IL-23-stimulated microglia, we measured the cytoplasmic levels of IL-17 in IL-23-stimulated microglia. IL-23 (≥1 ng/ml) significantly increased the cytoplasmic level of IL-17 in a dose-dependent manner (Fig. 7). IL-1β (≥1 ng/ml) also induced the expression of IL-17 mRNA and increased the cytoplasmic level of IL-17 in microglia; maximum induction was observed at 10 ng/ml (Fig. 8). On the contrary, stimulation with IL-23 or IL-23 and IL-1β did not induce IL-17 mRNA expression in astrocytes (data not shown). In the presence of IL-6, TGF-β derived from regulatory T cells induces upregulation of IL-23 receptor expression in Th17 cells (Ivanov et al., 2006). Neither IL-6 nor TGF-β, however, enhanced IL-17

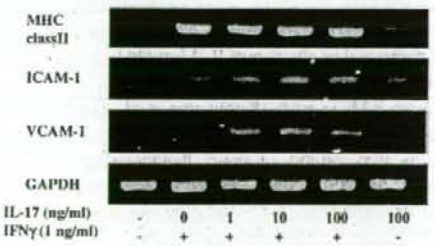

Fig. 6. The effects of IL-17 on the expression of mRNA encoding MHC antigen and adhesion molecules. Microglia were stimulated with IFNγ for 48 h in the presence of various concentrations of IL-17. IL-17 enhanced ICAM-1 and VCAM-1 mRNA expression in INFγ-stimulated microglia, whereas it did not affect MHC class II antigen mRNA levels in these cells.

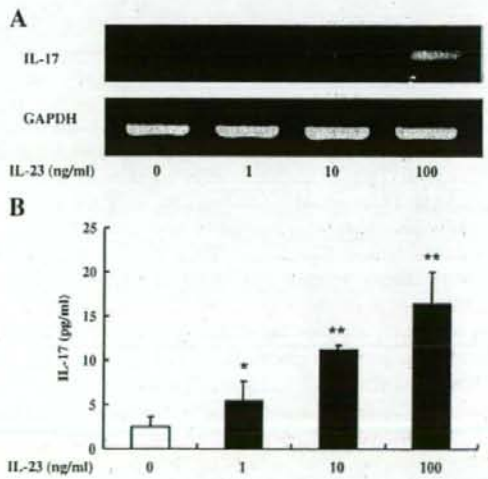

Fig. 7. IL-23 induces IL-17 production by microglia. Microglia were stimulated with various concentrations of IL-23 for 72 h. IL-23 dose-dependently induced IL-17 mRNA expression (A) and increased cytoplasmic IL-17 levels in microglia (B). The values shown are the means ± S.D. *$P<0.05$ and **$P<0.01$ compared with untreated microglia. The data represent typical samples performed in triplicate in three independent experiments.

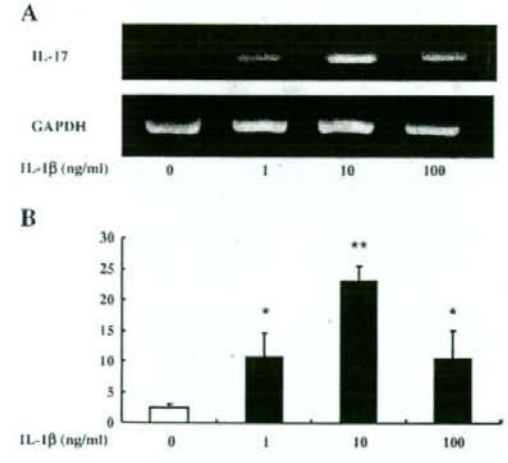

Fig. 8. IL-1β induces IL-17 production by microglia. Microglia were stimulated with various concentrations of IL-1β for 72 h. IL-1β induced IL-17 mRNA expression (A) and increased cytoplasmic IL-17 levels in microglia (B). The values shown are the means±S.D. $^{*}P<0.05$ and $^{**}P<0.01$ compared with untreated microglia. The data represent typical samples performed in triplicate in three independent experiments.

production in unstimulated microglia or in IL-23-stimulated microglia (data not shown). Stimulation with both IL-6 (100 ng/ml) and TGF-β (10 ng/ml) also failed to enhance the level of IL-17 produced by IL-23-stimulated microglia (data not shown).

4. Discussion

IL-17 has been associated with various autoimmune diseases, although its regulation and functional roles remain to be clarified. Antibodies specific for IL-17 reportedly inhibit chemokine expression in the brain during EAE, whereas overexpression of IL-17 in lung epithelia results in chemokine production and leukocyte infiltration. Thus, IL-17 expression characterizes a unique T helper lineage that regulates tissue inflammation (Park et al., 2005). Here we have evaluated the effects of IL-17 on neural cells *in vitro*. In the CNS, microglia and astrocytes express IL-17 receptors, whereas neurons do not. We then examined the effects of IL-17 on microglia—the antigen-presenting effector cells that can induce autoimmune inflammatory processes in the CNS. Because both IL-4 and IFNγ negatively regulate the production of IL-17 by T helper cells during the effector phase, Th17 cells may have roles that are distinct from those of Th1 and Th2 cells. The effects of IL-17 on microglia, however, are similar to those of Th1 cytokines; IL-17 enhanced inflammatory cytokine and chemokine production by microglia. IL-17 did not affect IFNγ-induced MHC class II antigen expression by microglia, whereas it increased the IFNγ-induced expression of adhesion molecules by these cells. IL-17 by itself did not induce iNOS expression or NO production, although it enhanced both of these phenomena in LPS-stimulated microglia. In rodent astrocytes, it has been shown that IL-17 enhances IFNγ-induced iNOS expression, which is suppressed by inhibitors of NF-κB or p38 MAP kinase (Trajkovic et al., 2001). Thus, IL-17 functions as a proinflammatory cytokine that works synergistically with other inflammatory stimuli in the CNS.

In addition to the proinflammatory effects on microglia, IL-17 also enhanced the expression of neurotrophic factors by microglia. We and other groups have previously shown that proinflammatory cytokines or inflammatory stimuli induce the expression of neurotrophic factors in microglia (Suzumura et al., 2006; Bessis et al., 2007). This may contribute to anti-inflammatory defense mechanisms in the CNS and implies that microglia may have multiple functions.

Previous cDNA microarray and immunohistochemical studies have suggested that astrocytes produce IL-17 (Meeuwsen et al., 2003; Li et al., 2005). In this study, we showed for the first time that microglia produce IL-17 in response to IL-23 or IL-1β. Although IL-1β and IL-23 induced IL-17 mRNA expression in microglia, we did not detect IL-17 in the supernatant of these cells. It is possible that IL-1β and IL-23 stimulate microglia to produce very low amount of IL-17 that cannot be detected with a commercially available ELISA kit, or that microglia may require another stimulatory signal before they release IL-17.

The same stimulus, however, did not induce the expression of IL-17 mRNA and protein in astrocytes. Thus, other stimuli may induce astrocytes to produce IL-17. Alternatively, this result may be due to differences between the species. As we and other groups have shown, IL-1β and IL-23 are produced by microglia in the CNS (Sonobe et al., 2005; Suzumura et al., 2006; Li et al., 2007). Therefore, it is possible that IL-1β and IL-23 may function as autocrine mediators that induce IL-17 expression by microglia.

Both Th1 and Th2 cytokines negatively regulate the differentiation of IL-17-producing T cells (Iwakura and Ishigame, 2006). In contrast, recent studies suggest that TGF-β derived from regulatory T cells induces an upregulation of IL-23 receptor expression and differentiation of Th17 cells in the presence of IL-6 (Ivanov et al., 2006; Valdhoen et al., 2006). Neither TGF-β nor IL-6, however, affects IL-17 production in microglia. Treatment of IL-23-stimulated microglia with both TGF-β and IL-6 also failed to enhance IL-17 production. These results suggest that different regulatory mechanisms control IL-17 production in microglia and Th17 cells.

Microglia play a pivotal role in the pathogenesis of inflammatory autoimmune diseases in the CNS. Thus, therapeutic targeting of the microglial production of IL-17 might be a useful strategy to treat MS. Because IL-17 deficiency has been demonstrated to ameliorate EAE in mice (Komiyama et al., 2006), induction of neutralizing antibodies that target the IL-23/IL-17 immune axis in microglia may provide a novel therapeutic approach for the treatment of MS. Indeed, targeting IL-23 with neutralizing antibodies ameliorates EAE and reduces serum levels of IL-17 (Chen et al., 2006). Moreover, a previous study demonstrated that blocking IL-17 with neutralizing antibodies induced by an active vaccination efficiently delays the onset of disease and reduces the severity of EAE (Röhn et al., 2006). Inflammatory autoimmune diseases such as MS, however, are chronic in nature, and treatment of these diseases with autoantibodies is extremely costly. Active

vaccination against IL-17 may therefore be an attractive therapeutic alternative, which could allow more patients access to an effective therapy that acts at an earlier stage of disease.

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Role of the mitochondrial membrane permeability transition in cell death

Yoshihide Tsujimoto · Shigeomi Shimizu

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

Y. Tsujimoto (✉) · S. Shimizu
Osaka University Medical School, Department of Medical Genetics, SORST of the Japan Science and Technology Agency, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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

Introduction

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

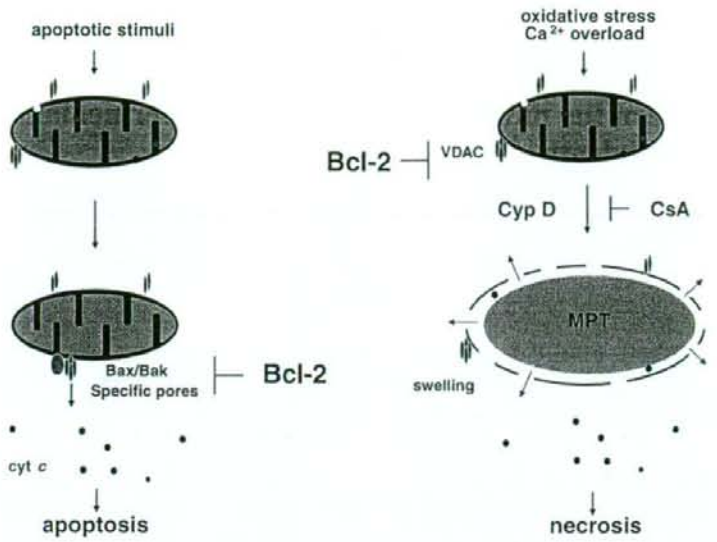

Fig. 1 Role of the mitochondria in apoptosis and necrosis. An increase in the permeability of the outer mitochondrial membrane is crucial for apoptosis to occur and is regulated by multidomain pro-apoptotic members of the Bcl-2 family (Bax and Bak), resulting in the release of several apoptogenic factors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

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

MPT

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

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

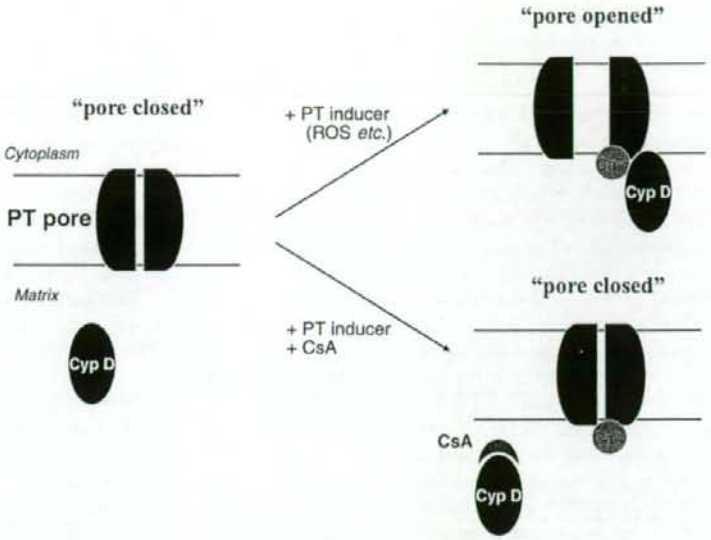

Fig. 2 Model of the MPT pore Under normal conditions, Cyp D is localized to the mitochondrial matrix, and the MPT pore is closed. In the presence of permeability transition inducers, Cyp D is considered to bind to and induce a conformational change of a channel in the inner membrane, resulting in opening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

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

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

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

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

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

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

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

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

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

Role of the MPT

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

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

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

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

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

Regulation of the MPT by Bcl-2

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

Role of the Cyp D-dependent MPT in disease

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

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

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A Bax/Bak-independent Mechanism of Cytochrome *c* Release*

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Takeshi Mizuta, Shigeomi Shimizu, Yousuke Matsuoka, Takashi Nakagawa, and Yoshihide Tsujimoto[1]

From the Laboratory of Molecular Genetics, Department of Medical Genetics, Osaka University Medical School, and Solution Oriented Research for Science and Technology (SORST) of the Japan Science and Technology Agency (JST), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Bax and Bak are multidomain pro-apoptotic members of the Bcl-2 family of proteins that regulate mitochondria-mediated apoptosis by direct modulation of mitochondrial membrane permeability. Since double-knock-out mouse embryonic fibroblasts with deficiency of Bax and Bak are resistant to multiple apoptotic stimuli, Bax and Bak are considered to be an essential gateway for various apoptotic signals. Here we showed that the combination of calcium ionophore A23187 and arachidonic acid induced cytochrome *c* release and caspase-dependent death of double-knock-out mouse embryonic fibroblasts, indicating that other mechanisms of cytochrome *c* release exist. Furthermore, A23187/arachidonic acid (ArA)-induced caspase-dependent death was significantly suppressed by the treatment of several serine protease inhibitors including 4-(2-aminoethyl)benzenesulfonylfluoride and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone but not the overexpression of anti-apoptotic Bcl-2 family of proteins or the inhibition of mitochondrial membrane permeability transition. These results indicate that there are at least two mechanisms of cytochrome *c* release leading to caspase activation, a Bax/Bak-dependent mechanism and a Bax/Bak-independent, but serine protease(s)-dependent, mechanism.

Apoptosis plays a critical role in the regulation of development processes, tissue homeostasis, and elimination of damaged cells. It has been shown that the mitochondria play a crucial role in apoptosis by releasing several apoptogenic molecules, such as cytochrome *c*, Smac/Diablo, and HtrA2/Omi (1, 2). After release into the cytosol, cytochrome *c* binds to Apaf-1 to cause recruitment of caspase-9, which leads to the initiation of a caspase cascade that includes caspase-3 and results in the occurrence of apoptotic cell death. Smac/Diablo and HtrA2/Omi bind directly with members of the inhibitor of apoptosis protein family, which are endogenous caspase inhibitory proteins and thus contribute to caspase activation (1, 2).

The best characterized regulators of apoptosis are the Bcl-2 family of proteins, which directly modulate outer mitochondrial membrane permeability during apoptosis. This family of proteins can be categorized into anti-apoptotic members (such as Bcl-2, Bcl-x_L, and Mcl-1) and pro-apoptotic members, which consist of multidomain proteins (such as Bax and Bak) and BH3-only proteins (including Bid, Bim, Bik, Bad, Noxa, and Puma) (3). The multidomain pro-apoptotic proteins Bax/Bak are essential and redundant regulators of a diverse intrinsic mitochondrial cell death pathway; Bax/Bak double-deficient murine embryonic fibroblasts (MEFs)[2] are resistant to multiple apoptotic stimuli that increase outer mitochondrial membrane permeability, including staurosporine, ultraviolet radiation, growth factor deprivation, and etoposide (4).

It was recently reported that double-knock-out (DKO) MEFs undergo death in a caspase-dependent manner, although at a lower rate when compared with WT MEFs, after exposure to agents such as arachidonic acid and Ca^{2+} ionophore (5), raising the possibility that there is a Bax/Bak-independent mechanism that regulates mitochondrial membrane permeability. In this present study, we found that the treatment of the combination of arachidonic acid and A23187 caused cytochrome *c* release and subsequently caspase activation succeeding the cell death in DKO MEFs. These events were inhibited by the treatment of 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), a serine protease inhibitor, but not the treatment of mitochondrial permeability transition (mPT) inhibitors, deletion of cyclophilin D (mPT component), and overexpression Bcl-2 or Bcl-x_L.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—The following antibodies were used. Numbers in parentheses indicate dilutions used. Anti-caspase-3 (1:250) and anti-caspase-9 (1:1000) monoclonal antibodies were purchased from BD Transduction Laboratories and MBL (Nagoya, Japan), respectively. Anti-lamin B1 (1:1000) and anti-GAPDH (1:1000) monoclonal antibodies were obtained from Zymed Laboratories Inc. Inc. (South San Francisco, CA) and BD Biosciences, respectively. Anti-cytochrome *c* monoclonal antibodies for Western blotting (clone 7H8.2C12, 1:1000) and immunostaining (clone 6H2.B4, 1:500) were purchased from Pharmingen. A23187 and arachidonic acid were obtained from Calbiochem and Sigma, respectively. AEBSF, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), and L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone (TLCK)

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[1] To whom correspondence should be addressed. Tel.: 81-6-6879-3363; Fax: 81-6-6879-3369; E-mail: tsujimot@gene.med.osaka-u.ac.jp.

[2] The abbreviations used are: MEF, murine embryonic fibroblast; DKO, double-knock-out; TKO, triple-knock-out; ArA, arachidonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride; AEBSA, 4-(2-aminoethyl)benzenesulfonamide; TPCK, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone; TLCK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone; WT, wild type; mPT, mitochondrial permeability transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DFP, diisopropylfluorophosphate; GFP, green fluorescent protein; siRNA, small interfering RNA; PBS, phosphate-buffered saline; CsA, cyclosporin A; Cyp D, cyclophilin D; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; Ac, acetyl; MCA, methylcoumarylamide.

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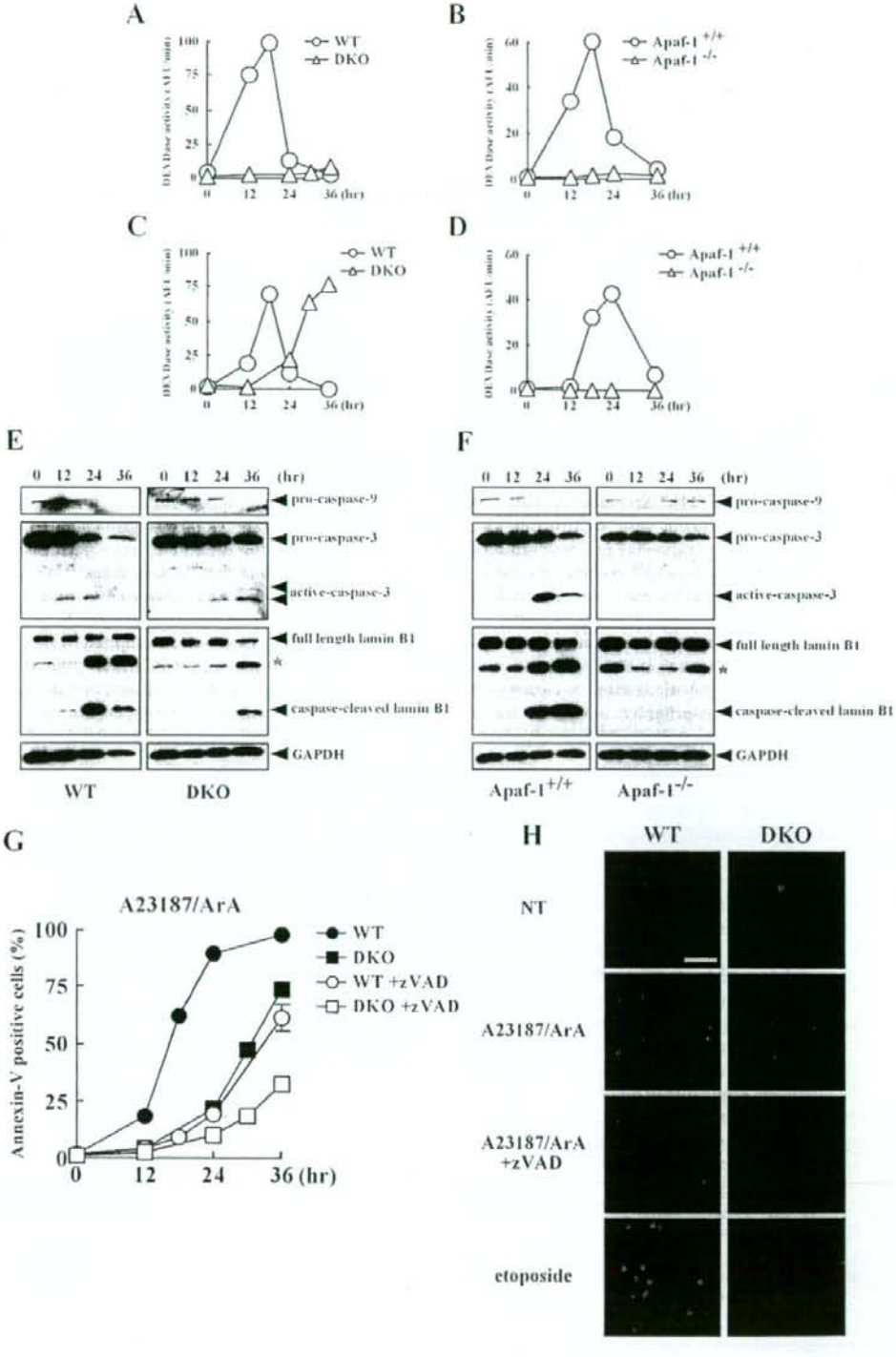

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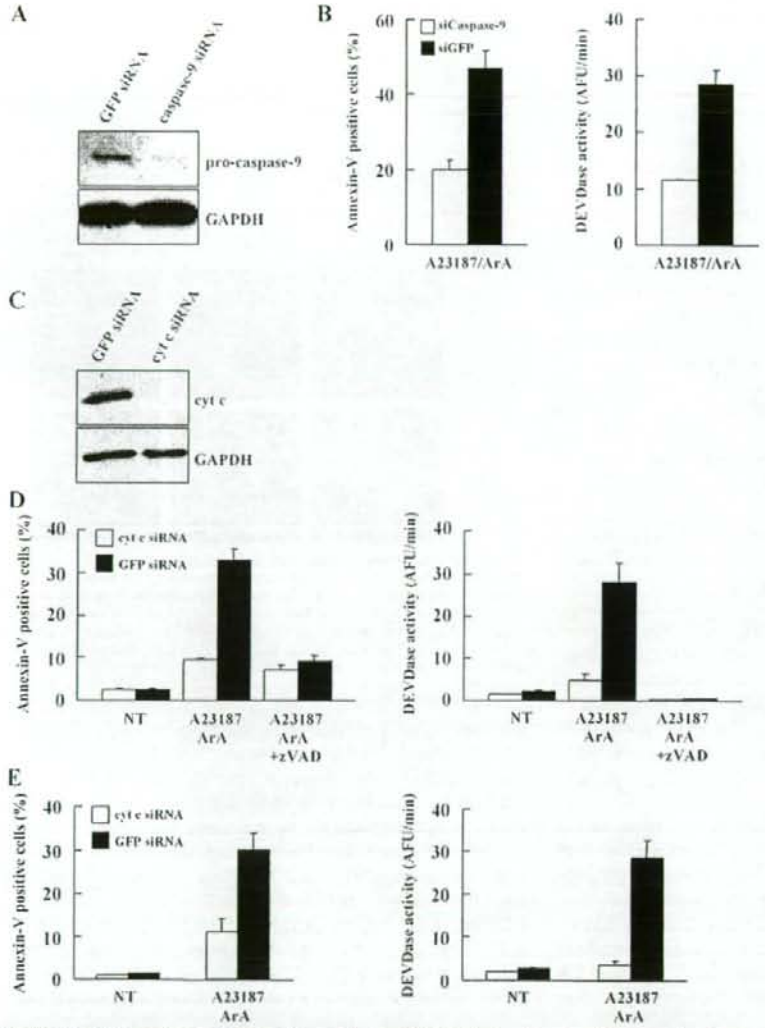

FIGURE 2. **Inhibition of A23187/ArA-induced death of DKO MEFs by silencing of caspase-9 and cytochrome *c*.** *A*, DKO MEFs were treated with 10 μg of caspase-9 siRNA as described under "Experimental Procedures," after which the expression of caspase-9 and GAPDH (loading control) was analyzed by Western blotting. *B*, DKO MEFs with silencing of caspase-9 (*siCaspase-9*) were treated with 10 μM A23187 plus 100 μM ArA for 24 h, and then the extent of cell death (defined by annexin V staining) and DEVDase activity was measured ($n = 4$). *siGFP*, silencing of GFP. *C*, DKO MEFs were treated with 5 μg of cytochrome *c* siRNA. Expression of cytochrome *c* (*cyt c*) and GAPDH was analyzed. *D* and *E*, DKO MEFs with silencing of cytochrome *c* were treated with A23187/ArA in the absence or presence of 100 μM Z-VAD-fmk for 24 h. *NT*, not treated. *E*, the same experiments as shown in *D* were performed, except in the presence of 1 μM antimycin A for 12 h. Cell death (defined by annexin-V staining) and DEVDase activity were measured ($n = 4$).

were purchased from Roche Applied Science (Penzberg, Germany). 4-(2-Aminoethyl)benzenesulfonamide (AEBSA) were purchased from Aldrich (Steinheim, Germany). Diisopropylfluorophosphate (DFP) and other chemicals were purchased from Wako Co. (Osaka, Japan).

Cell Culture and DNA Transfection—SV40 T antigen-immortalized WT MEFs, Bax/Bak DKO MEFs (kindly provided by Dr. S. J. Korsmeyer), and Bax/Bak/CypD TKO MEFs were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. Apaf-1-deficient MEFs and control MEFs were kindly provided by Dr. X. Wang and were also grown in the same medium. DNAs encoding human Bax and human Bcl-x_L were used in the pUC-CAGGS expression vector. Cells (1×10^6) were transfected with plasmid DNA using the Amaxa electroporation system according to the supplier's protocol (kit V, program U-20). The transfection efficiency was more than 75% as assessed by co-transfection with DNA expressing green fluorescence protein (GFP). All of the siRNAs were produced by Dharmacon Research. The sequences used were as follows (numbers in parentheses indicate nucleotide positions within the respective open reading frames): mouse cytochrome *c* siRNA, 5′-GGGAGAAAGGGCAGACCUA-3′ (267–285); mouse HtrA2/Omi siRNA, 5′-GGGGAGUUUGUUGUUGCCA-3′ (760–778); and GFP siRNA, 5′-GGCUACGUCCAGGAGCGCA-3′ (274–292). Mouse caspase-9 siRNA SMARTpool™ was also purchased from Dharmacon Research. Cells (1×10^6) were transfected twice on alternate days with 10 μg of siRNA using the Amaxa electroporation system.

FIGURE 1. **Induction of caspase-dependent death of DKO MEFs by A23187/ArA.** *A–D*, activation of DEVDase in MEFs exposed to death stimuli. WT MEFs and DKO MEFs (*A* and *C*) or Apaf-$1^{+/+}$ and Apaf-$1^{-/-}$ MEFs (*B* and *D*) were treated with 10 μM etoposide (*A* and *B*) and 10 μM A23187 plus 100 μM arachidonic acid (*C* and *D*), and DEVDase activity was measured. Representative results from three independent experiments are shown. *E* and *F*, activation of caspases in DKO MEFs, but not in Apaf-$1^{-/-}$ MEFs, by A23187/ArA. The indicated MEFs were incubated with A23187/ArA for the indicated times. Cleavage of pro-caspase-9, pro-caspase-3, and lamin B1 was analyzed by Western blotting. GAPDH was also analyzed as a loading control. *, nonspecific band. *G* and *H*, induction of death in A23187/ArA-treated DKO MEFs. WT and DKO MEFs were not treated (*NT*) or were treated with A23187/ArA in the presence or absence of 100 μM Z-VAD-fmk (*G* and *H*) or 10 μM etoposide (*H*) for 36 h. The extent of cell death was assessed by annexin V staining (*G*). Data are shown as the mean ± S.D. ($n = 4$). Representative nuclear morphology is shown (*H*). *Bars*, 50 μm.

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Twenty-four hours after the second transfection with siRNA, cells were used for experiments.

Cell Viability and DEVDase Activity Assay—Cells (2×10^5/well) were seeded into 6-well dishes. After 24 h, the cells were treated with 10 μM A23187/100 μM arachidonic acid or 10 μM etoposide in the presence or absence of 100 μM Z-VAD-fmk or 100 μg/ml AEBSF. Cells were harvested and stained with 1 μM propidium iodide, 1 μM Cy3-conjugated annexin V, or 1 μM Hoechst 33342 for 5 min at room temperature and were analyzed with a flow cytometer (BD Biosciences, FACS Caliber) or under a fluorescence microscope (Olympus, BX50). For DEVDase assay, cells were washed three times with phosphate-buffered saline (PBS) and suspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. After the addition of Triton-X to 0.1%, cells were incubated for 30 min at on ice. Lysates were clarified by centrifugation at 8000 rpm for 3 min, and cleared lysates containing 50 μg of protein were incubated with 100 μM enzyme substrate Ac-DEVD-MCA at 37 °C for 1 h. Levels of released 7-amino-4-methlycoumarin were measured using a spectrofluorometer (Hitachi F-3000) with excitation at 380 nm and emission at 460 nm.

Preparation of the Cytosolic Fraction and Total Cell Lysate—For the detection of released cytochrome *c*, the cytosolic fraction was collected from MEFs after incubation with 0.1 mg/ml digitonin for 5 min at 37 °C in isotonic buffer (20 mM potassium-Hepes (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, and 1 mM Na^{2+}-EDTA). After centrifugation at 8000 rpm for 5 min, aliquots of the supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) were analyzed by Western blotting with an anti-cytochrome *c* antibody. In some experiments, cells were lysed with radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate (sodium salt), and 150 mM NaCl).

Immunofluorescence Staining—Cells were fixed in 4% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After incubation with 2% fetal bovine serum in PBS for 1 h, the cells were incubated with anti-cytochrome *c* for 1 h. After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa Fluor 488-conjugated anti-mouse IgG, 1:1000) for 1 h. Then fluorescence was detected under a confocal microscope (Zeiss, LSM 510).

Microinjection—DKO MEFs (1.5×10^4) were plated on 35-mm dishes 1 day before use. PBS or PBS containing 400 μM AEBSF, 400 μM AEBSA, 100 μM DFP, 100 μM TLCK, or 200 μM TPCK was microinjected through a glass capillary into the cytoplasm with a Narishige micromanipulator. For identification of injected cells, samples were mixed with green fluorescent protein. Cells were then treated with A23187/ArA at 1 h. Cell morphology was examined 20 h after A23187/ArA treatment under a fluorescence microscope.

RESULTS

We added various apoptotic reagents to Bax/Bak DKO MEFs, Apaf-$1^{-/-}$ MEFs, and their control MEFs, all of which were immortalized, and then measured DEVDase activity. In both DKO MEFs (Fig. 1*A*) and Apaf-$1^{-/-}$ MEFs (Fig. 1*B*), activation of DEVDase was not observed after exposure to etoposide that induced mitochondria-mediated apoptosis, whereas in the control MEFs, activation of DEVDase was readily observed, confirming previous observations that Bax/Bak is essential for such apoptosis (4, 6). Time-dependent decrease of DEVDase activities was due to disruption of plasma membrane. Similar results were also obtained when these cells were treated with staurosporine, UV, and X-rays (data not shown). In contrast, when the cells were treated with A23187 plus ArA, activation of DEVDase was observed in DKO MEFs but not in Apaf-$1^{-/-}$ MEFs (Fig. 1, *C* and *D*). It has previously been reported that ionomycin (another Ca^{2+} ionophore) plus ArA could induce DEVDase activation in DKO MEFs (5). Consistent with the elevation of caspase activation, A23187/ArA treatment induced cleavage of caspase-9, caspase-3, and lamin B1 in WT and DKO MEFs but not in Apaf-$1^{-/-}$ MEFs (Fig. 1, *E* and *F*). Furthermore, A23187/ArA induced the death of DKO MEFs, as assessed by staining with annexin V (Fig. 1*G*) and propidium iodide (data not shown), whereas such cell death was not completely inhibited by Z-VAD-fmk, a pan-caspase inhibitor (Fig. 1*G* and data not shown), indicating that A23187/ArA induced both caspase-dependent and caspase-independent death of DKO MEFs. It is known that not a few reagents induce both apoptosis and necrosis, such as oxidative stress (7) and Ca^{2+} overload (8). Staining with Hoechst 33342 revealed that A23187/ArA induced nuclear pyknosis, which was partially inhibited by Z-VAD-fmk (Fig. 1*H*). A23187/ArA-induced caspase activation and death of DKO MEFs were slightly delayed when compared with these processes in WT MEFs (Fig. 1, *C* and *G*). Primary cultures of DKO MEFs tended to mainly

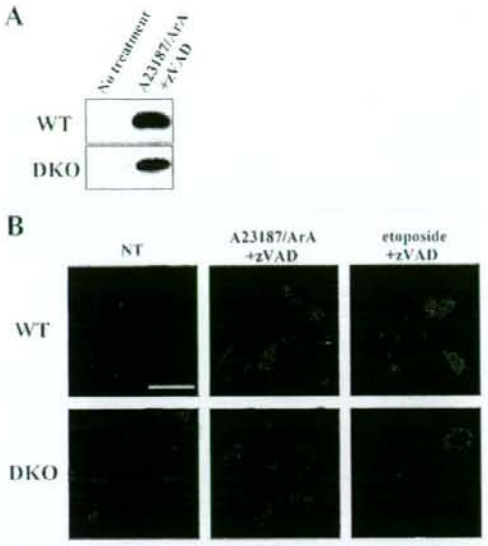

FIGURE 3. **Cytochrome *c* release in A23187/ArA-treated MEFs.** WT and DKO MEFs were not treated (*NT*) or were treated with 10 μM A23187 plus 100 μM ArA in the presence of Z-VAD-fmk (100 μM). *A*, after 18 h, the cytoplasmic fraction was recovered, and samples were subjected to Western blotting for detection of cytochrome *c*. *B*, after 12 h, MEFs were fixed and immunostained with an anti-cytochrome *c* monoclonal antibody. In *B*, MEFs were also treated with 10 μM etoposide plus 100 μM Z-VAD-fmk. *Bars*, 50 μm.

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