Title: Daxx enhances Fas-mediated apoptosis in a murine pro-B cell line, BAF3

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Daxx has been shown to play an essential in type I interferons (IFN-α/β)-mediated suppression of B-cell development and apoptosis. Recently, we demonstrated that Tyk2 is directly involved in IFN signaling for the induction and translocation of Daxx, which may result in growth arrest and/or apoptosis of B lymphocyte progenitors. To clarify the mechanism of Daxx-mediated apoptosis signaling in B lymphocyte progenitors, here we introduced an efficient suicide switch in a murine pro-B cell line, BAF3 by expressing FK506-binding protein-fused Fas intracellular domain (FKBP-Fas) and Daxx. It arrows us to monitor Fas/Daxx-mediated signal by induction of Fas-dimerization with the dimerizer drug, AP20187. AP20187-mediated Fas dimerization induced not only apoptosis but also Jun N-terminal kinase (JNK) activation. However, AP20187 had no effect on cells expressing either Fas or Daxx only. Furthermore, expression of JNK inhibitor, JNK binding domain of JIP-1 resulted in a resistance to AP20187-mediated apoptosis in cells expressing FKBP-Fas and Daxx. These results imply that our novel suicide switch system may provide a powerful tool to delineate or identify the signaling molecules for Daxx-mediated apoptotic machinery in B lymphocyte progenitors through JNK activation.

Key words: Daxx, B lymphocyte progenitor, Fas, apoptosis, Jun N-terminal kinase (JNK)
INTRODUCTION

Sequential rearrangements of immunoglobulin heavy and light chain genes occur during B-cell development, and the defective or dangerous lymphocytes are eliminated by negative selection (1). Several cytokines and chemokines in bone marrow microenvironment deliver inhibitory or stimulatory signals to B lymphocyte progenitors. For example, type I interferons, IFN-α/β that are produced by resident bone marrow macrophages can inhibit the IL-7-induced growth and survival of B lymphocyte progenitors (2, 3). Cross-linking of Fas, a member of the tumor necrosis-nerve growth factor receptor superfamily, can induce apoptosis in B lymphocyte progenitors (4). It is widely believed that dysregulation or absence of these regulatory factors could contribute autoimmune and/or malignant diseases. Indeed, mice carrying the lymphoproliferation (lpr) mutation have defects in the Fas antigen gene. The lpr mice develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune diseases (5).

Our experiments using Tyk2-deficient mice revealed that Tyk2 is essential for the IFN-α/β-induced inhibition of colony formation of B lymphocyte progenitors in response to interleukin (IL)-7 as well as the up-regulation and nuclear translocation of Daxx (6). Because Daxx plays crucial roles in IFN-α-induced growth suppression of B lymphocyte progenitors (7), it is very informative to analyze how Daxx is involved in the growth arrest and/or apoptosis in early B-cell development.

Daxx was first identified as a Fas-binding protein by yeast two-hybrid screening and was known as a pro-apoptotic protein that can enhance Fas-mediated apoptosis through Jun N-terminal kinase (JNK) activation (8). However, disruption of Daxx in mice increased
apoptosis during the embryonic development, suggesting that Daxx acts as an anti-apoptotic protein in the embryo (9). Because of the diverse effects of Daxx between in vitro and in vivo experimental systems, roles of Daxx in apoptotic signals still remain unclear. While the interaction between Daxx and Fas indicated the importance of Daxx in cytoplasm, nuclear localization of Daxx was observed in various cell lines and the interactions of Daxx with several nuclear proteins such as the centromeric protein CENP-C, DNA methyltransferase-I, Pax-3, Pax-5 and PML were reported (9-15). Thus, Daxx is likely to play alternative roles in shuttling between nucleus and cytoplasm.

In this study, we established BAF3 cells expressing both Daxx and a fusion protein composed of FK506-binding protein (FKBP) and membrane-anchored intracellular domain of Fas, or each of them. A bivalent FKBP ligand could be a trigger for apoptosis only when Daxx was overexpressed. Using these series of transfected BAF3 cells, we analyzed and discussed roles of Daxx in Fas-mediated apoptotic signals during B-cell development.
MATERIALS AND METHODS

Reagents and antibodies

A synthetic dimerizer, AP20187 and an expression vector, pC4M-Fv2E containing an N-terminal myristoylation signal, two copies of FKBP, followed by a C-terminal HA-tag were kindly provided by ARIAD Gene Therapeutics (Cambridge, MA). Expression vectors, FLAG-tagged Daxx, JNK binding domain (JBD) of JIP-1(16) and murine Fas cDNA(17) were kindly provided by Dr. H. Ariga and Dr. T. Taira (Hokkaido Univ., Sapporo, Japan), Dr. R. J. Davis (Univ. of Massachusetts Med. Sch., MA) and Dr. S. Nagata (Osaka Univ., Osaka, Japan), respectively. FKBP-Fas fusion was constructed by introducing the murine Fas cytoplasmic region into pC4M-Fv2E vector as described previously (18-21). Anti-HA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). JNK activation was determined by PhosphoPlus JNK antibody Kit (Cell Signaling Technology, MA) according to the manufacturer’s instructions. Z-VAD-fmk was purchased from Peptide Institute, INC (Osaka, Japan).

Cell culture, transfections, RT-PCR and cell viability assays

An IL-3-dependent murine pro-B cell line, BAF3 was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 10% conditioned medium from WEHI-3B cells as a source of IL-3 (22). Stable transformants expressing either FKBP-Fas, Daxx, or both of them were established as described previously (22) and maintained in the above medium in the presence of G418 (1mg/ml). AP20187 stimulation was also performed in the presence of IL-3. RT-PCR was performed using RT-PCR high-Plus- Kit (TOYOBO,
Tokyo, Japan) (Primer sequences are available upon request). Cell viability was determined by Cell Counting Kit-8 (Wako Chemicals, Tokyo, Japan) according to the manufacturer’s instructions.

Caspase assays, and cell death assays
Caspase activity was determined by Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega Corporation, WI) according to the manufacturer’s instructions. Annexin V assay was performed by Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. TUNEL (TdT-mediated dUTP nick end labeling) assay was performed by In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

Immunoprecipitation, immunoblotting and DNA fragmentation
The immunoprecipitation and Western blotting were performed as described previously (23). Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to Immobilon filter (Millipore, Bedford, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). DNA fragmentation assay was performed as described previously (24).
Development of a conditional Fas/Daxx suicide switch model in a pro-B cell line, BAF3

An IL-3-dependent murine pro-B cell line, BAF3 was employed to analyze mechanisms of growth arrest or apoptosis via Daxx, because IFN-α inhibited their growth and up-regulated their Daxx expression (data not shown). To simplify the assay system, we engineered a novel suicide switch model based on the murine Fas receptor to trigger cell death in BAF3 cells. We established three kinds of the stably transfected BAF3 cells. BAF/F2 and BAF/F3 expressed the HA-tagged fusion protein composed of FKBP and membrane-anchored intracellular domain of Fas (FKBP-Fas), and BAF/D9 expressed FLAG-tagged Daxx. BAF/FD7 and BAF/FD10 expressed both FKBP-Fas and Daxx. Protein expression of the exogenous FKBP-Fas and Daxx was confirmed with immunoblot analysis using anti-HA or anti-FLAG antibodies (Fig. 1A).

In general, cross-linking of Fas results in the recruitment of a death-inducing signaling complex, the activation of a proteolytic cascade of caspases, and the induction of cell death by apoptosis. Thus, aggregation of FKBP-Fas proteins by the addition of a bivalent FKBP ligand, AP20187 expected to trigger the apoptotic death signals in BAF3 cells. However, original BAF3 as well as BAF/F2 and BAF/F3 cells expressing FKBP-Fas did not die after the treatment with AP20187 (Fig.1B). Overexpression of Daxx in BAF/D9 also had no effects on the proliferation or survival. It is worthy that BAF/FD7 and BAF/FD10 expressing both FKBP-Fas and Daxx strongly underwent cell death with the addition of AP20187 as shown in Fig. 1B. Furthermore, aggregation of FKBP-Fas on BAF/FD7 cells induced the association of FKBP-Fas with Daxx (Fig. 1C). Therefore, Daxx directly interacts with the
intracellular domain of Fas and overexpression of Daxx enhances apoptotic signals mediated by Fas in a murine pro-B cell line, BAF3.

**AP20187-induced apoptosis in BAF/FD7 cells**

Fas-induced apoptosis is known to be a rapid event that occurs within a few hours. Some of the earliest detectable events in the apoptotic pathway are loss of the cytoplasmic polarization of plasma membrane phosphatidylserine and the fragmentation of nuclear DNA. To determine the kinetics of AP20187-induced cell death in BAF/FD7 cells, we treated BAF/FD7 cells with 1nM AP20187 and stained with annexin V at different times (Fig. 2A). Annexin V-positive cells were clearly detectable within 3 hrs, and the percentage of annexin V-positive cells continued to increase over time. After 24 hrs of treatment with AP20187, 90% of the cells were annexin V-positive. TUNEL staining showed a similar kinetics with that of annexin V (Fig. 2B). To confirm apoptosis, the fragmentation of nuclear DNA in AP20187-untreated or -treated BAF-FD7 cells was analyzed. As shown in Fig. 2C, after 12 hrs of treatment with AP20187, BAF/FD7 cells clearly showed the degradation of chromosomal DNA. Therefore, a dimerizer of FKBP-Fas, AP20187 effectively induces apoptosis in BAF/FD7 cells.

**Caspase activation and the enhanced JNK activation by AP20187 in BAF/FD7 cells**

To delineate the molecular mechanisms in AP20187-induced apoptosis of murine B lymphocyte progenitors, AP20187-induced caspase activation in BAF/FD7 cells was analyzed. As shown in Fig. 3A, the caspase-3/7 activity increased after 3hrs with AP20187
in BAF/FD7 but not BAF/F2 cells. We then examined whether the activation of caspase cascade is related to AP20187-induced cell death. As shown in Fig. 3A, Z-VAD-fmk, a broad caspase inhibitor inhibited AP20187-induced cell death in BAF/FD7 cells. Addition of Z-VAD-fmk also inhibited AP20187-induced apoptosis monitored by annexin V and TUNEL staining (data not shown). Therefore, caspase activation is necessary for AP20187-induced apoptosis in BAF/FD7 cells.

Previous studies demonstrated that Fas/Daxx also activates JNK pathway (8). Recently, Daxx is also shown to physically interact with the cytoplasmic domain of the type II TGF-β receptor and mediate TGF-β-induced apoptosis and activation of JNK (25). To assess the possibility whether AP20187 induces JNK activation in BAF/FD7 cells, the kinetics of AP20187-induced JNK activation in BAF/FD7 cells were analyzed. JNK activation was monitored by immunoblotting using an antibody directed to phospho-JNK Thr183/Tyr185. As shown in Fig. 3B, upon AP-20187 stimulation, the strong sustained phosphorylation of JNK was induced in BAF/FD7 cells, compared with that of BAF/F2 cells. We next examined whether the sustained activation of JNK is critical in AP20187-mediated apoptosis in BAF/FD7 cells or not. For this purpose, we expressed the JNK binding domain (JBD) from JIP-1 in BAF/FD7 cells, which is known as a potent JNK inhibitor. As shown in Fig. 3C, each established BAF/FD7 clone expressing JBD showed a resistance to AP20187-induced apoptosis. Therefore, JNK activation by AP20187 is also essential in the apoptotic signals in BAF/FD7 cells.
Conclusions

We have here shown the development of a conditional Fas/Daxx suicide switch for use in murine B lymphocyte progenitors. We introduced an efficient suicide switch in a murine pro-B cell line, BAF3 by expressing Daxx and FKBP-Fas. Using this cell line, we succeeded in monitoring Fas/Daxx-mediated apoptotic signals by induction of Fas-dimerization with the dimerizer drug, AP20187. AP20187-mediated Fas dimerization induced not only caspase activation but also JNK activation. Furthermore, expression of a JNK inhibitor resulted in a resistance to AP20187-mediated apoptosis in BAF3 cells expressing FKBP-Fas and Daxx. These results demonstrated that our novel suicide switch system in B lymphocyte progenitors is dependent on Fas and its enhancer, Daxx.

Daxx was reported to be involved in the Fas- and TGF-β-mediated apoptotic signaling pathway (8, 25). Daxx was originally cloned as a Fas-associated protein and binds specifically to the death domain of Fas, although Daxx itself lacks a death domain (8). There are two independent signaling pathways downstream of Fas, involving the adapter protein Fas-associated death domain (FADD) and Daxx (5, 8). The activation of FADD induces a protease cascade (26), while that of Daxx enhances JNK activation, leading to apoptosis (8). Overexpression of Daxx enhances Fas-induced apoptosis (8, 13), and the targeted disruption of the Daxx gene in mice results in embryonic lethality (9). Daxx is also involved in coupling of the type II TGF-β receptor signalings with components of the apoptotic machinery (25). TGF-β induces apoptosis in primary and cultured lymphocytes (27). Daxx associates with the cytoplasmic domain of the type II TGF-β receptor and transduces apoptotic signals by TGF-β (25).
Recently, Daxx was also reported to be essential for IFN-induced suppression of B-cell development (7). IFN-α enhances Daxx expression, with concomitant increases in Daxx protein levels and nuclear body translocation. Moreover, Daxx antisense oligonucleotides rescue IFN-α-treated pro-B cells from growth arrest and apoptosis. We also demonstrated that Tyk2 is essential for the transduction of IFN-α-induced suppression of B-cell development through the activation of some signaling molecules other than STAT1, followed by the up-regulation and nuclear translocation of Daxx (6). The apoptotic signaling pathway downstream of Daxx still remains unknown. One candidate of Daxx targets is a JNK signaling pathway. Alternatively, the localization of Daxx is also important, since the localization of Daxx in either the cytoplasmic or nuclear compartment was reported to be dependent upon the cell type and/or its functional status (28).

The present paper describes an establishment of an IL-3-dependent pro-B cell line, BAF/FD7, which has an efficient suicide switch dependent of Fas/Daxx. This cell line is useful to delineate the apoptotic signaling pathway downstream of Daxx through JNK signaling pathway and is also a powerful tool to identify novel molecules involved in Daxx-mediated apoptosis in B lymphocyte progenitors.
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interferon-like cytokine that preferentially influences B-lymphocyte precursors.


FIGURE LEGENDS

Fig. 1. Development of a conditional Fas/Daxx suicide switch for use in BAF3 cells. (A) Expression of FKBP-Fas and/or Daxx proteins in each transformants as indicated. The immunoprecipitate with anti-HA antibody (upper) and total extracts (lower) were examined by Western bolt analysis using anti-HA or anti-FLAG antibodies as indicated. (B) Each transformants were treated with the indicated concentrations of AP20187 for 24h. Cell viability was determined by Cell Counting Kit-8 as described in MATERIALS AND METHODS. The results are presented from three independent experiments, and the error bars represent the standard deviations. (C) AP20187-dependent association of FKBP-Fas with Daxx in BAF-FD7 cells. BAF/FD7 cells (2x10^7) were treated with or without AP20187 (10nM) for 30 min. Cell lysates were then immunoprecipitated and immunoblotted with anti-FLAG (upper) or anti-HA (middle) antibodies as indicated. Total extracts (20μg) were examined by Western bolt analysis using anti-FLAG antibody (lower).

Fig. 2. AP20187-induced apoptosis in BAF/FD7 cells. (A) BAF/FD7 cells (2x10^5) were treated with AP20187 (1nM) for the indicated time and stained with Annexin-V-FLUOS Staining Kit as described in MATERIALS AND METHODS. (B) BAF/FD7 cells (5x10^5) were treated with AP20187 (1nM) for the indicated time and stained with TUNEL (TdT-mediated dUTP nick end labeling) using In Situ Cell Death Detection Kit as described in MATERIALS AND METHODS.
(C) BAF/FD7 cells (1x10^7) were treated without or with AP20187 (1nM) for 12 h. Total DNA was extracted from cells and electrophoresed on a 1% agarose gel in the presence of 1μg/ml ethidium bromide.

Fig. 3. **Caspase activation and the enhanced JNK activation by AP20187 in BAF/FD7 cells.**

(A) Caspase activation by AP20187 and effect of caspase inhibitor on AP20187-induced cell death in BAF/FD7 cells. BAF/F2 or BAF/FD7 cells (2x10^4) was treated with AP20187 (1nM) for the indicated time and the caspase activity was determined by Apo-ONE Homogeneous Caspase-3/7 Assay Kit as described in *MATERIALS AND METHODS*. BAF/FD7 cells (2x10^4) were treated without or with AP20187 (1nM) in the absence or presence of the caspase inhibitor Z-VAD-fmk (100nM) for 24h. Cell viability was determined by Cell Counting Kit-8 as described in *MATERIALS AND METHODS*. The results are presented from three independent experiments, and the error bars represent the standard deviations.

(B) JNK activation by AP20187 in BAF/FD7 cells. BAF/F2 or BAF/FD7 cells (2x10^6) was treated with DMSO or AP20187 (1nM) for the indicated time and lysed, and immunoblotted with an anti-phospho JNK antibody (upper panel) or an anti-JNK antibody (lower panel).

(C) Effect of JBD expression on AP20187-induced cell death in BAF/FD7 cells. JBD and G3PDH transcripts were analyzed by RT-PCR in each transformants as indicated. Each cells (2x10^4) were treated with the indicated concentrations of AP20187. 24 h after transfection, cell viability was determined by Cell Counting Kit-8 as described in *MATERIALS AND METHODS*. The results are presented from three independent experiments, and the error bars
represent the standard deviations.
Fig. 3