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Enhanced Expression of Fas-Associated Death Domain-Like IL-1-Converting Enzyme (FLICE)-Inhibitory Protein Induces Resistance to Fas-Mediated Apoptosis in Activated Mast Cells

Hideshi Yoshikawa, Yasuo Nakajima, and Kachio Tasaka

Mast cells play a critical role in host immune responses and are implicated in the pathogenesis of allergic inflammation. Though mouse mast cell line MC/9 expresses cell surface Fas Ag and is sensitive to Fas-induced apoptosis, activated MC/9 cells are resistant to Fas-induced cell death by cross-linking of FcεRI or FcγRI. Fas-associated death domain-like IL-1-converting enzyme (FLICE)-inhibitory protein (FLIP), a caspase-8 inhibitor that lacks the cisteine domain, is one of the negative regulators of receptor-mediated apoptosis. In this report, we show that activation of mast cells by cross-linking of FcεRI or FcγRI can induce enhanced expression of FLIP and consequently a resistance to Fas-induced apoptosis, although the expression level of Fas Ag is not changed. Addition of antisense oligonucleotide for FLIP prevents resistance to Fas-induced apoptosis of activated mast cells, suggesting that endogenous FLIP inhibits Fas-mediated apoptosis in activated mast cells. Thus, the enhanced expression of FLIP in activated mast cells contributes to the resistance to Fas-induced apoptosis, which may result in the development and prolongation of allergic inflammation. The Journal of Immunology, 2000, 165: 6262–6269.

Materials and Methods

**Mast cells and cross-linking of FcεRI or FcγRI**

MC/9 is an IL-3-dependent murine mast cell line derived from fetal liver cells of a (B6 × A/J)F1, mouse and kindly provided by Y. Kitamura (Osaka University, Osaka, Japan). MC/9 cells were cultured in RPMI 1640 with 10% FCS, 1 × 10^−5 M 2-ME, and antibiotics, supplemented with 10% (v/v) WEHI-3-conditioned medium as a source of IL-3 and maintained at 37°C in 5% CO2 atmosphere. BMMC were obtained from bone marrow of BALB/c mice and cultured for 4–6 wk in RPMI 1640 with 10% FCS supplemented with 20% (v/v) WEHI-3-conditioned medium as a source of IL-3 as described previously (13). For the cross-linking of FcεRI, cells were incubated with 10 μg/ml of anti-DNP IgE (Sigma, St. Louis, MO) for 12 h at 37°C. Then, these cells were washed twice with PBS and resuspended in the above medium without IL-3. Finally, IgE-sensitized cells were added with 500 ng/ml DNP-albumin (Sigma), incubated for the appropriate times and used for assays. For the cross-linking of FcγRI, mouse IgG (10 mg/ml) was heated at 63°C for 20 min and used as heat-aggregated IgG without further purification as described previously (10).
CellQuest software (Becton Dickinson). calibur flow cytometer (Becton Dickinson, Mountain View, CA) using the secondary Ab. Flow cytometric analyses were performed by a FACS

Measurement of Fas expression by flow cytometry

MC/9 cells were cultured under each condition for 24 h, and cell surface Fas Ag was stained by single-color indirect immunofluorescence. The first Ab was a rat anti-mouse Fas mAb (MBL, Nagoya, Japan) or an isotype control of rat IgG (thin line) for 1 h. After washing, FITC-conjugated goat F(ab')2 of anti-rat IgG was added and incubated another 1 h. Then flow cytometric analyses were performed by a FACScalibur flow cytometer. A representative of three experiments is shown.

Antibodies

Rabbit anti-Bcl-2, anti-Bax, and anti-Bad polyclonal Abs were purchased from StressGen (Victoria, Canada). Rabbit anti-Bcl-xL polyclonal Ab was purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-mouse FLIP polyclonal Ab was purchased from Millennium (Ramona, CA).

Measurement of apoptosis

We studied apoptosis by flow cytometry. Briefly, we incubated cells under each condition for 24 h, then added 1 μg/ml of apoptosis-inducing anti-Fas Ab (PharMingen, San Diego, CA) and cultured the cells another 6 h. Cells (5 × 10^5) were washed with PBS, and 500 μl of FITC-conjugated annexin V (Caltag, Burlingame, CA) and propidium iodide (PI) (5 μg/ml) in a calcium-containing buffer was added. After incubation for 10 min at room temperature, the samples were immediately analyzed on a FACScalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

Measurement of caspase activities

Quantitative measurement of caspase activity was performed using a caspase colorimetric protease assay kit including caspase-3 and -8, using specific substrates, DEVD-pNA and IETD-pNA, respectively. Briefly, MC/9 cells were cultured under each condition for 24 h and incubated another 6 h with or without anti-Fas Ab. Then cytosolic protein was extracted and diluted to 200 μg in a volume of 50 μl, and incubated with corresponding substrate at 37°C for 2 h. The OD at 405 nm was measured using a TiterTak Multiskan Plus microplate reader (Flow Laboratories, McLean, VA).

Measurement of mitochondrial transmembrane potential (ΔΨm)

Perturbation in ΔΨm was monitored by flow cytometry using a modified method described previously (30). Briefly, MC/9 cells were cultured under each condition for 24 h and incubated another 6 h with or without anti-Fas Ab. After staining with 5 μg/ml of JC-1 (Molecular Probes, Eugene, OR) for 20 min at 37°C, fluorescence intensity was estimated by exciting the probes with a laser at 480 nm, and emission was measured through 575/26 nm (aggregated) and 530/30 nm (monomer) bandpass filters. Logarithmic amplification was used to detect fluorescence intensity.

RT-PCR

MC/9 cells were cultured under each condition for 4 h and total RNA was isolated using the guanidium isothiocyanate method. Five micrograms of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase. The products obtained by reverse transcription were PCR amplified using sets of primers on a thermal cycler (Atto, Tokyo, Japan). Amplification was done at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Each cDNA was amplified for 30 cycles. The following sense and antisense primer sets were synthesized with a model 381A DNA synthesizer (Applied Biosystems, Foster City, CA): bc1-2, 5'-TGACCTGAGCGCCCCTTCAC-3' and 5'-TAGCTGTATCCACATTTGCTGTA-3'; bc1-xL, 5'-TG GTGCATTTTCCTCTCTCTAC-3' and 5'-GAGATCAAAAGAAMTGC-3'; bax, 5'-ACAGATCATGACACAGCCGG-3' and 5'-CAAAGTGAAGGAGGCAACC-3'; bad, 5'-CAGATATGTTCCAGATCC-3' and 5'-AGACTGTTAATGCGGTC-3'; and flip, 5'-GTTAGGTAGCGACGTTGG-3' and 5'-CCTGCTTGTGCTCAG-3'.

Western blot analysis

Protein extracts (50 μg) obtained by SDS lysis were separated on a 10% polyacrylamide gel. After transfer to a polyvinylidene difluoride membrane and blocking overnight at 4°C with 1% BSA in PBS to block nonspecific Igs, the membrane was incubated for 1 h at room temperature with each Ab. After washing, the membrane was incubated with an HRP-conjugated secondary Ab for 1 h at room temperature, and specific bands were detected using enhanced chemiluminescence according to the manufacturer’s protocols.

Antisense oligonucleotide treatment

MC/9 cells were incubated with either stimulation of Fce receptor or Fcy receptor polymerization in the presence of the indicated concentration of a morpholino-oligonucleotide (5'-GCTCTGAGAACACCAAGCAAC-3'), complementary to the 25 bp of mouse flip mRNA sequence (antisense oligonucleotide), or standard control (5'-CCTCTTACCTCTACTAACAATATATCTACCTACAATT-3') corresponding to β-globin pre-mRNA of thalassemia (control oligonucleotide).
Statistics

The statistical significance was analyzed using Student’s t test. Data were presented as means ± SD. Differences were considered to be significant at \( p < 0.05 \).

Results

Expression of Fas Ag and effect of anti-Fas mAb on the viability of MC/9 cells

Flow cytometry was used to determine cell surface expression of Fas Ag on MC/9 cells cultured with or without IL-3, or with cross-linking of FcεRI or FcγR for 24 h. As shown in Fig. 1, MC/9 cells constitutively express Fas Ag. In addition, depletion of IL-3 and cross-linking of FcεRI or FcγR resulted in no change on the expression of Fas Ag. We next evaluated the function of Fas Ag expressed on MC/9 cells. After incubation under each condition, MC/9 cells were cultured with or without 1 μg/ml of anti-Fas mAb for another 6 h, and the percentage of apoptotic cells was determined by flow cytometry of double-staining with annexin V and PI. Early apoptotic events (annexin V+ , PI-) were shown in the upper left quadrants of each panel. As shown in Fig. 2, 1 μg/ml of anti-Fas mAb effectively induced apoptosis even in the presence of IL-3. However, Fas-induced apoptosis was significantly reduced by cross-linking of FcεRI or FcγR. On the contrary, depletion of IL-3 for 30 h without anti-Fas Ab induced apoptosis and was also inhibited by cross-linking of FcεRI or FcγR. We previously reported the mechanism by which IL-3 depletion-induced apoptosis was prevented by cross-linking of FcεRI or FcγR, and it was mediated by a paracrine of IL-3. In this study, Fas-induced apoptosis was not inhibited in the presence of IL-3, suggesting that cross-linking of FcεRI or FcγR prevents Fas-induced apoptosis by other mechanisms but not through paracrine of IL-3.

Fas-induced apoptosis of MC/9 cells was mediated by the activation of caspases, but not the decrease in DCm

To evaluate the mechanisms by which cross-linking of FcεRI or FcγR prevents Fas-induced apoptosis of MC/9 cells, we determined the signaling pathway of Fas-induced apoptosis in MC/9 cells. The signaling pathway of Fas-mediated apoptosis was divided into two major routes by the dependence on mitochondria.
and the differential modulation of apoptosis sensitivity was cell-type specific. So we first evaluated the caspase activity. After incubation with or without IL-3, or cross-linking of FcRI or FcγR for 24 h, MC/9 cells were each cultured with or without 1 μg/ml of anti-Fas mAb for another 6 h, and the activity of caspase-3 and -8 were measured. As shown in Fig. 3, the activity of both caspase-3 and -8 was increased by the addition of anti-Fas Ab regardless of the presence of IL-3. However, they were significantly reduced by cross-linking of FcRI or FcγR as well as by Fas-induced apoptosis. At the same time, we evaluated the change in ΔΨm using a specific probe (JC-1) by flow cytometry. As shown in Fig. 4, although a slight decrease in ΔΨm was observed with the addition of anti-Fas Ab in the presence of IL-3, no significant decrease in ΔΨm was observed under other conditions.

By contrast, depletion of IL-3 itself induced a significant decrease in ΔΨm, suggesting a difference in dependence on mitochondria between Fas-mediated and IL-3 depletion-induced apoptosis of MC/9 cells.

Cross-linking of FcRI or FcγR induces enhanced expression of FLIP, but not Bcl-2, Bcl-xL, Bax, or Bad

To evaluate the mechanisms by which cross-linking of FcRI or FcγR prevents Fas-induced apoptosis of MC/9 cells, we evaluated the expression of apoptosis-related proteins. Based on the result that a slight decrease in ΔΨm was observed with the addition of anti-Fas Ab in the presence of IL-3 (Fig. 4), we first examined the expression of mitochondria-related Bcl family proteins such as Bcl-2, Bcl-xL, Bax, and Bad using RT-PCR and Western blotting. As shown in Fig. 5, no significant change in the expression of Bcl-2, Bcl-xL, Bax, or Bad was observed by cross-linking of FcRI or FcγR at both mRNA and protein levels. However, a decrease only in Bcl-2 expression was observed with the depletion of IL-3, suggesting a relationship between Bcl-2 expression and a decrease in ΔΨm in IL-3 depletion-induced apoptosis. We next evaluated the expression of FLIP, which had been thought to inhibit caspase-8 activation, because the decreased activity of caspase-8 was observed in Fig. 3. In addition, caspase-8 had been reported to work at the beginning of the caspase activation cascade of receptor-mediated apoptosis. RT-PCR analysis indicated that expression of flip mRNA was detectable at low levels in MC/9 cells cultured with or without IL-3, but was significantly enhanced by cross-linking of FcRI or FcγR (Fig. 6, upper panel). Western blot confirmed the enhanced expression of FLIP protein by cross-linking of FcRI or FcγR (Fig. 6, lower panel), suggesting the possibility that enhanced expression of FLIP functions as an inhibitor of Fas-mediated apoptosis in activated MC/9 cells by cross-linking of FcRI or FcγR.

Enhanced expression of FLIP is necessary for the inhibition of Fas-mediated apoptosis by cross-linking of FcRI or FcγR on MC/9 cells

To investigate the role of FLIP expression for the inhibition of Fas-mediated apoptosis by cross-linking of FcRI or FcγR, anti-sense or control oligonucleotide was added to MC/9 cells during

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**FIGURE 4.** ΔΨm during Fas-mediated apoptosis in MC/9 cells. MC/9 cells were cultured with or without IL-3, IgE/Ag (IgE), or aggregated IgG (IgG) for 24 h and incubated with or without anti-Fas mAb (1 μg/ml) for another 6 h. After staining of the cells using 5 μg/ml of JC-1, two-color fluorescence intensity was measured by flow cytometry. The percentage of decrease in ΔΨm is shown. Fas stimulation decreased ΔΨm compared with unstimulated cells (*, p < 0.05) in the presence of IL-3 but the decrease of ΔΨm was not significant in the cells precultured with IgE/Ag (IgE) or aggregated IgG (IgG). Data represent means ± SD of three experiments.

**FIGURE 5.** Expression of apoptosis-related proteins in MC/9 cells. A. MC/9 cells were cultured with or without IL-3, IgE/Ag (IgE), or aggregated IgG (IgG) for 4 h. Total RNA (5 μg) was reverse transcribed and PCR amplified, as described. Products using specific primers were resolved in 2% agarose gels and visualized by staining with ethidium bromide. B. MC/9 cells were cultured under each condition for 24 h, protein extracts (50 μg) were prepared, and specific bands were detected by Western blotting.

**FIGURE 6.** Expression of FLIP in MC/9 cells. MC/9 cells were cultured with or without IL-3, IgE/Ag (IgE), or aggregated IgG (IgG) for 4 h. Total RNA (5 μg) was reverse transcribed and PCR amplified, and products using specific primers for flip were resolved in 2% agarose gel and visualized by staining with ethidium bromide (upper panel). MC/9 cells were cultured under each condition for 24 h, protein extracts (50 μg) were prepared, and specific bands of FLIP protein were detected by Western blotting (lower panel).
specific bands of FLIP protein were detected by Western blotting. In contrast, no significant effect was observed by treatment with control oligonucleotide, suggesting that the antisense oligonucleotide specifically and efficiently inhibits translation and production of FLIP protein. So we next measured the activities of caspase-3 and -8 in activated MC/9 cells by cross-linking of FcεRI or FcγRI. Moreover, as shown in Fig. 9B, the inhibition of Fas-mediated apoptosis by cross-linking of FcεRI or FcγRI, as well as caspase-8 activity, was prevented in the presence of antisense oligonucleotide for flip. However, neither caspase activation (data not shown) nor apoptosis were induced either by control or antisense oligonucleotides in the absence of anti-Fas mAb (Fig. 9A). These results suggest that enhanced expression of FLIP is necessary and sufficient for the inhibition of Fas-mediated apoptosis via caspase-8 activation by cross-linking of FcεRI or FcγRI in MC/9 cells.

Expression of FLIP is enhanced in activated BMMC and contributes to resistance to Fas-mediated apoptosis

Finally, to confirm whether the expression of FLIP contributes to resistance to Fas-mediated apoptosis of mast cells, we evaluated the expression of FLIP in unstimulated and activated BMMC. As shown in Fig. 10A, BMMC constitutively expressed flip mRNA, which was enhanced by cross-linking of FcεRI or FcγRI. In addition, BMMC was relatively resistant to Fas stimulation compared with MC/9 cells. However, antisense oligonucleotide for flip sensitized BMMC to Fas-mediated apoptosis (Fig. 10B), suggesting that the expression of FLIP is essentially important in Fas-resistance in activated mast cells.

Discussion

The Fas/Fas ligand system is one of the receptor-ligand pairs involved in cell fate determination in a variety of cell types including immunocompetent cells. The receptor-proximal events have been best characterized for Fas. Fas engagement initiates an intracellular signaling through aggregation of its death domains, leading to oligomerization of caspase-8, mediated via the adaptor fas-associated death domain protein. Caspase-8 is the most proximal of a growing family of cystein proteases that are shown to be involved in many forms of apoptosis, and caspase-3 appears to play an important role in the effector pathway in apoptosis. Recently, the involvement of a loss of ΔΨm in the apoptotic pathway has been demonstrated. However, it has been reported that pro-caspase-3 is a major target of caspase-8 and of the lack of decreased ΔΨm early in the course of Fas-mediated apoptosis (31, 32). In Bid-deficient mice, hepatocytes but not other cell lineages are resistant to Fas-induced apoptosis, suggesting that the mitochondrial pathway is not necessarily required for Fas-mediated apoptosis (33). Thus, two different Fas-signaling pathways have been demonstrated by their kinetics and dependence on the mitochondrial pathway, and they are thought to be cell-type specific (34, 35). In type I cells, caspase-8 is activated within seconds, caspase-3 within 30 min of receptor engagement, and a mitochondria-independent pathway can be used, whereas in type II cells, activation of both caspases is delayed and the mitochondria-dependent pathway is important. In MC/9 cells, Fas-induced apoptosis was mediated via delayed activation of both caspase-8, and -3, and a slight decrease in ΔΨm, suggesting that MC/9 cells belong to an intermediate type of cell in Fas-induced apoptosis.

Fas signaling for cell death may be modulated by several distinct antiapoptotic proteins, which directly bind Fas, inhibit
caspase activation or activity, or modulate mitochondrial events (20–29). All mitochondrial activities in apoptosis can be blocked by overexpression of Bcl-2 or Bcl-xL, which seemed to be acting upstream of caspase-3 (20, 21, 36). Although caspase-3 is activated during Fas-mediated apoptosis, discussion regarding the ability of Bcl-2 or Bcl-xL to inhibit this kind of apoptosis has been controversial. By contrast, Bid and Bad, which have been shown to function as signal anchor segment required for targeting mitochondria, may represent death ligands (37). It has been reported that nonphosphorylated Bad heterodimerizes with Bcl-2 or Bcl-xL at membrane sites to prevent cell death (38). In addition, Bax, despite possessing a hydrophobic C terminus, has been noted in the cytosol as well as in mitochondrial membranes, and enhanced Bax expression results in a downstream program of mitochondrial dysfunction as well as caspase activation (39). Recently, mitochondrial channel VDAC has also been reported as a binding site of Bcl-2 family proteins, such as Bcl-xL and Bax (40). In MC/9 cells, expression of these proteins, such as Bcl-2, Bcl-xL, Bax, and Bad, was not changed by cross-linking of FcεRI or FcγR, suggesting minimized involvement of the mitochondrial pathway for inhibition of Fas-mediated apoptosis. Only after withdrawal of IL-3, Bcl-2 expression and ΔΨm were decreased, suggesting the importance of the mitochondrial pathway in IL-3 depletion-induced apoptosis.

Aside from mitochondrial events, Fas signaling for cell death may be modulated by several distinct antiapoptotic proteins that inhibit caspase activation or activity. It has been reported that FLIP interferes with receptor-mediated apoptosis but not with the chemotherapeutic drug- or irradiation-induced apoptotic signaling pathway (41). In general, immunocompetent cells have been demonstrated to show decreased apoptosis following activation. Neutrophils activated by proinflammatory stimuli up-regulate the expression of IL-1β, resulting in resistance to the cell death program. In this case, enhanced expression of Mcl-1, which is one of the antiapoptotic proteins, has been reported (42, 43). In addition, FLIP-mediated inhibition of apoptosis has also been reported in the immune system (44). Primed/memory T cells expressing higher levels of FLIP after Ag restimulation are resistant to Fas-mediated activation-induced cell death (45, 46). IL-2 stimulated NK cells express enhanced FLIP and are resistant to Fas-mediated cell death (47). Monocytes also express FLIP and become resistant to Fas-mediated apoptosis during macrophage differentiation (48). In addition, B cells activated by cross-linking of the B cell Ag receptor (BCR) become resistant to Fas-mediated apoptosis (49).
These findings suggest a potential mechanism by which the functional lifespan of inflammatory cells and their ability to function during inflammation are regulated.

The molecular mechanisms by which FLIP is induced have not been clearly defined. In T cells, IL-2 signaling has been reported to reduce FLIP expression, in sharp contrast with the case of NK cells (50, 51). However, it has been reported that TCR engagement or Con A induces neosynthesis of FLIP, and each of them is inhibited by cyclosporin A or mitogen-activated protein kinase inhibitor, suggesting the importance of the downstream signaling pathway of TCR (52, 53). In B cells, cross-linking of BCR also induces neosynthesis of FLIP (49). In MC/9 cells, cross-linking of FcεRI or FcγRII induced enhanced expression of FLIP and resistance to Fas-mediated apoptosis. In the Fc receptor-mediated signaling pathway, the Fc receptor γ-chain is commonly used not only in FcεRI and FcγRII but also in TCR and induces common signaling, such as protein tyrosine kinase activation, calcium mobility, and activation of protein kinase C as well as BCR, suggesting the possibility of common mechanisms for FLIP induction with T or B cells.

Mast cells as well as eosinophils are major effectors of allergic inflammation and produce biologically active mediators that play pivotal roles in the pathophysiologic changes of allergic disorders. There is increasing evidence that mast cells have mechanisms that regulate the secretion of various mediators, the production of cytokines, and the number of mast cells within tissues (13). The number of mast cells in tissues is believed to be locally regulated and to depend on the balance between cell growth and death. Removal of mast cell growth factors such as IL-3 or SCF leads to mast cell apoptosis, suggesting that control of the number of mast cells depends only on the supply of growth factors (9, 12). We also reported survival of mast cells by paracrine mechanisms of growth factors and the possibility of prolongation of allergic inflammation (8). However, the identification of the dependence of mast cells on growth factor has limited applicability in developing strategies to treat diseases of mast cell proliferation. Therefore, we postulated that mast cells might be susceptible to Fas-mediated apoptosis and that mechanisms by which activated mast cells escape from cell death might exist. The expression of Fas Ag was constitutive and did not require any activation, which is in agreement with reports that murine and human mast cells are Fas positive, but down-regulation of Fas Ag by cross-linking of FcεRI was not observed in MC/9 cells as previously reported (13). In addition, Fas expression on eosinophils is differentially regulated by cytokines, which are produced by activated mast cells, suggesting the possibility that the regulation of effector cells in allergic inflammation depends on Fas-mediated signaling control (17). In vitro, we can induce Fas-mediated apoptosis in mast cells as well as in eosinophils, as previously reported using anti-Fas mAb. However, the supply of Fas ligand is not defined in vivo. Expression of Fas ligand in murine mast cells has been reported, but it is not cell lytic, owing to the intracellular localization (54). These problems have to be resolved so as to reflect those phenomena occurring in vitro as well as those in vivo. However, in fact, Fas resistance and delayed apoptosis of eosinophils in atopic dermatitis have been reported (55, 56). In that sense, our findings show the possibility that FLIP expression and resistance to Fas-mediated apoptosis of activated mast cells participate in the development and prolongation of allergic inflammation and suggest a possible therapeutic model for allergic diseases.

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