

## Original article

## TRAIL mediated signaling in human mast cells: the influence of IgE-dependent activation

**Background:** Mast cells activation through FcεRI cross-linking has a pivotal role in the initiation of allergic reactions. The influence of this activation on programmed cell death of human mast cells has not yet been clarified. This study evaluates the influence of IgE-dependent activation alone and in synergy with TRAIL on the expression of molecules involved in the apoptotic signal transduction.

**Methods:** Human cord blood derived mast cells (CBMC) were cultured with myeloma IgE followed by activation with anti-human IgE. The expression of proteins involved in apoptotic signal transduction was assessed by immunoblot analysis. To test the effect of activation on a pro-apoptotic stimulus, activated, IgE-treated and resting CBMC were incubated with TRAIL, or in a medium with suboptimal concentrations of stem cell factor (SCF).

**Results:** In accordance with a previous study of ours, it was found that IgE-dependent activation increased TRAIL-induced caspase-8 and caspase-3 cleavage. However, it did not have a significant influence on CBMC death induced by SCF withdrawal. IgE-dependent activation increased the expression of FLIP and myeloid cell leukemia 1 (MCL-1) anti-apoptotic molecules as well as the pro-apoptotic one, BIM. In addition, a decrease in BID expression was observed. TRAIL could reverse the increase in FLIP but did not influence the upregulation of MCL-1 and of BIM.

**Conclusions:** These findings suggest that IgE-dependent activation of human mast cells induces an increase in both pro-survival and pro-apoptotic molecules. We therefore hypothesized that IgE-dependent activation may regulate human mast cell apoptosis by fine-tuning anti-apoptotic and pro-apoptotic factors.

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Mast cells are tissue dwelling cells whose long life span was previously described (1). Mast cells play a pivotal role in allergic reactions through high affinity IgE-receptor (FcεRI) mediated responses. Cross-linking of FcεRI on mast cells activates multiple signaling pathways, which finally result in the secretion of pre-formed mediators that are stored in cytoplasmic granules as well as in *de novo* synthesis of pro-inflammatory lipid mediators, chemokines and cytokines. Although in normal conditions the number of mast cells in the tissue is relatively constant, in allergy as well as in other inflammatory processes (e.g. psoriasis, rheumatoid arthritis, parasite infections, etc.), a mast cell hyperplasia can be observed (2). The main factor that regulates human mast cell viability is stem cell factor (SCF) (3). The *in vitro* treatment of human mast cells with SCF increases the levels of pro-survival Bcl-2 and Bcl-X<sub>L</sub> factors (3, 4).

However, other events such as IgE-dependent activation as well as monomeric IgE were also found to modulate murine mast cell viability (5–7). The enhancement of murine mast cell viability by monomeric IgE, which maintains Bcl-xL levels and causes pro-survival cytokine production [i.e. interleukin (IL)-4, IL-6, IL-13 and TNF-α], is now well established (6). However, the influence of IgE-dependent activation on mast cell viability is still controversial. Some studies revealed that an increase in mast cell viability is observed when cells are activated with high antigen concentrations (8). Other studies found no influence of activation on mast cell viability (5). All these works were carried out on murine mast cells. We previously showed that TNF-related apoptosis-inducing ligand (TRAIL) death receptors are expressed on human lung derived mast cells, human cord blood derived mast cells (CBMC) and human mast cells line (HMC-1). We also demonstrated

that TRAIL receptors are functional and their activation causes apoptosis in CBMC and that TRAIL-induced apoptosis can be upregulated by IgE-dependent activation (9).

TRAIL binds the specific cell surface death receptors DR4 and DR5 and initiates the extrinsic apoptotic cascade via activation of pro-caspase-8. Activated caspase-8 can either directly cleave downstream effector caspases without involving mitochondria activation (type I cells) or can require the engagement of the mitochondria-mediated pathway (intrinsic pathway) through cleavage of the pro-apoptotic Bcl-2 family protein BID (type II cells) (10).

TRAIL-induced apoptosis is regulated by anti-apoptotic molecules that block the apoptotic signals or divert them into alternative responses. The protein that directly inhibits apoptosis induced by TRAIL is Fas-associated death domain (FADD)-like IL-1  $\beta$ -converting enzyme-like inhibitory protease (FLIP) (11, 12). In type II cells, anti-apoptotic proteins from bcl-2 family [i.e. myeloid cell leukemia 1 (MCL-1), Bcl-2 and Bcl-xL] were found to be involved in TRAIL-induced apoptosis resistance (13–15).

In this study, we examined the change that TRAIL induces in the apoptotic signal transduction on CBMC in the context of IgE-dependent activation. We have demonstrated that IgE-dependent activation of human mast cells upregulates both the pro-apoptotic FLIP and MCL-1 and the anti-apoptotic BIM. We therefore hypothesize that IgE-dependent activation by a fine tuning of anti-apoptotic and pro-apoptotic factors may regulate human mast cell apoptosis.

## Methods

### Cells

Human cord blood derived mast cells were obtained and cultured as described (16). For the experiments, CBMC were resuspended in supplemented MEM- $\alpha$  (10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 10  $\mu$ g/ml ribonucleases; all obtained from Biological Industries, Kibbutz Beit Haemek, Israel) containing SCF at a concentration of 100 ng/ml (a generous gift from Amgen, Thousand Oaks, CA, USA).

### Detection of mitochondrial membrane potential ( $\psi_m$ )

For detection of changes of  $\psi_m$ , CBMC were treated with rTRAIL (500 ng/ml; Alexis, Laufelfingen, Switzerland) for 4, 8 and 18 h. Mitochondrial injury and changes in the  $\psi_m$  were assessed by staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetrathylbenzimidazolyl carbocyanine iodide (JC-1; Alexis). This dye, existing as a monomer in solution, emitting a green fluorescence, can assume a dimeric configuration emitting red fluorescence in a reaction driven by the mitochondrial transmembrane potential. Thus, red fluorescence indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remains unprocessed because of breakdown in the mitochondrial transmembrane potential. During the last 15 min of incubation with TRAIL, CBMC were incubated with 2.5  $\mu$ g/ml

JC-1 at 37°C. Cells were washed with cold PBS twice and resuspended in PBS. Analysis was performed by FACS, and mitochondrial function was assessed as JC-1 green (uncoupled mitochondria) or red (intact mitochondria) fluorescence.

### Immunoblotting

Cells were lysed in 50  $\mu$ l 1.5 $\times$  protein sample buffer (10% glycerol, 62.4 mM Tris, pH 6.8, 3% SDS, 5%  $\beta$ -mercaptoethanol, and 1 mg/ml bromophenolblue) per  $1 \times 10^6$  cells followed by instant boiling at 95°C, for 10 min. Fifteen microliters of cell extracts were separated on SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoblot analysis was performed using the following primary antibodies: anti-caspases 3, anti-caspase-8, anti-bcl-2, anti-bcl-xL, anti-BID, anti-BIM, anti-BAD, anti-BAX (Dilution 1 : 1000; Cell Signaling Technology, Beverly, MA, USA), anti-FLIP<sub>S/L</sub>, GAPDH (1 : 1000; Calbiochem, San Diego, CA, USA) and anti-actin (Dilution 1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was either horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) and/or goat anti-rabbit IgG (Pierce, Rockford, IL, USA). Enhanced chemiluminescence reaction was performed using ECL<sup>TM</sup> Western blotting detection reagent (Amersham Biosciences, Amersham, UK).

### Mast cell activation

For IgE-dependent activation, CBMC ( $1 \times 10^6$ ) cells/ml were preincubated for 3 days with human myeloma IgE (1  $\mu$ g/ml; Calbiochem). At day 0 of the experiment, cells were resuspended in Tyrode buffer [NaCl 137 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.36 mM, KCl 2.6 mM, NaHCO<sub>3</sub> 11.9 mM, glucose 5.5 mM containing 0.1% gelatin (TG), 1.8 mM CaCl<sub>2</sub>, 0.9 mM MgCl<sub>2</sub> (TG<sup>++</sup>)] supplemented with SCF (100 ng/ml) and challenged with mouse anti-human IgE (5  $\mu$ g/ml; Southern Biotech, Birmingham, AL, USA), for the indicated time periods. MC activation was assessed by measuring  $\beta$ -hexosaminidase levels in both supernatants and cell lysates as described (17).

### Determination of CBMC viability

Resting, IgE-treated and activated CBMC ( $1 \times 10^6$ /ml) were cultured in MEM- $\alpha$  with various concentrations of SCF (0, 10, and 100 ng/ml) for 24 h. Cell death was assessed by uptake of propidium iodide (PI) (1  $\mu$ g/ml; Sigma-Aldrich, Rehovot, Israel) and FACS analysis.

### Statistical analysis

Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple-comparisons test. Differences were considered significant at a  $P$  value  $\leq 0.05$ .

## Results

### IgE-dependent activation of CBMC enhances TRAIL-induced caspase-3 and caspase-8 cleavage

We first checked whether IgE-dependent activation of CBMC influences the caspases activation induced by TRAIL. CBMC were activated for 1 h and therefore exposed to TRAIL for 4, 8 and 24 h. The treatment with

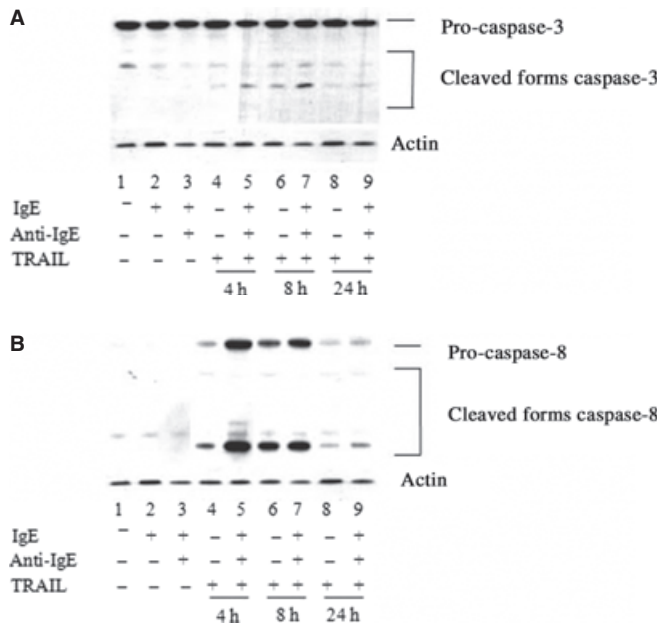


**Figure 1.** IgE-dependent activation of human mast cells enhances TRAIL-induced pro-caspase-3 and pro-caspase-8 cleavage. CBMC sensitized with IgE were incubated with anti-IgE for 30 min at 37°C. After activation CBMC were incubated with TRAIL-FLAG for 4, 8 or 24 h. The level of pro-caspase-3 and cleaved caspase-3 (A) and pro-caspase-8 and cleaved caspase-8 (B) was assessed by immunoblot analysis. Actin was used as a control for loading normalization. Data are representative of three independent experiments performed with CBMC from three different donors.

TRAIL alone caused cleavage in caspase 3 and caspase-8. IgE-dependent activation of CBMC increased TRAIL-mediated cleavage of pro-caspase-3 (Fig. 1A) and the cleavage of the executive pro-caspase-8 (Fig. 1B) after 4 h. A similar cleavage was detected at 8 hrs, while at 24 hrs a decrease in caspase-3 and caspase-8 was evident.

Surprisingly, an increase in the basal level of pro-caspase-8 was seen when IgE-activated CBMC were treated with TRAIL. The levels of pro-caspase-3 remained constant suggesting that the increase is specific for pro-caspase-8. The change in the pro-caspase-8 expression intensity was not seen in cells that were treated with TRAIL only. Moreover, no change in the basal level of pro-caspase-8 expression was observed in cells treated with either human myeloma IgE alone or followed by activation with anti-IgE. This suggests that a synergy between FcεRI and TRAIL-R signaling leads to upregulation in pro-caspase-8 expression and cleavage.

IgE-dependent activation influences both pro- and anti-apoptotic proteins expression

We then analyzed whether the observed upregulation in caspase activation was because of changes that FcεRI

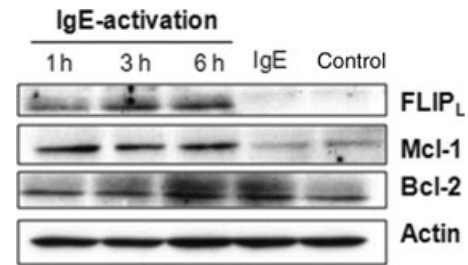


**Figure 2.** The change in the expression of anti- and pro-apoptotic molecules in CBMC as a result of IgE-dependent activation. CBMC were activated for the indicated periods of time. Nonactivated or IgE-sensitized CBMC were used as the controls. Expression of anti-apoptotic proteins (A) and pro-apoptotic proteins from the bcl-2 family (B) was assessed by immunoblot analysis using specific antibody. Actin was used as a control for loading normalization. Data are representative of three independent experiments performed with CBMC from three different donors.

cross-linking might induce in the apoptotic signaling machinery.

The influence of IgE-dependent activation on the pattern of anti-apoptotic protein expression was assessed by immunoblot analysis (Fig. 2). It can be seen that 1 h of activation brings about an increase in the expression level of the anti-apoptotic proteins FLIP and MCL-1. Moreover, at longer activation times, i.e. at 6 h, an increase in Bcl-2 was observed as well. The increase in Bcl-2 level can also be seen in mast cells incubated with myeloma IgE alone. The changes in Bcl-xL levels were not conclusive because of high variability amongst the donors (data not shown).

The expression of pro-apoptotic proteins was investigated in CBMC that were treated with myeloma IgE alone or followed by FcεRI cross-linking for 1 h and analyzed immediately after. Among the pro-apoptotic proteins from the Bcl-2 family that were investigated only BIM expression was increased (see below, Fig. 3C). The expression of BID and BAX, that were also present at basal levels, was unchanged by activation. Furthermore, BAD and BAK were undetectable under these conditions (data not shown).

TRAIL influences the upregulation of MCL-1, FLIP and BIM induced by IgE-dependent activation of CBMC

We further evaluated whether TRAIL influences the activation induced upregulation of FLIP, MCL-1 and BIM in CBMC.

For this purpose, mast cells from three different donors were first activated with anti-IgE for 1 h and then incubated with TRAIL for 4 h.

The treatment with TRAIL caused a decrease in FLIP level in activated cells (Fig. 3A). However, there was no change in the expression levels of the anti-apoptotic MCL-1 and the pro-apoptotic BIM (Fig. 3B,C).

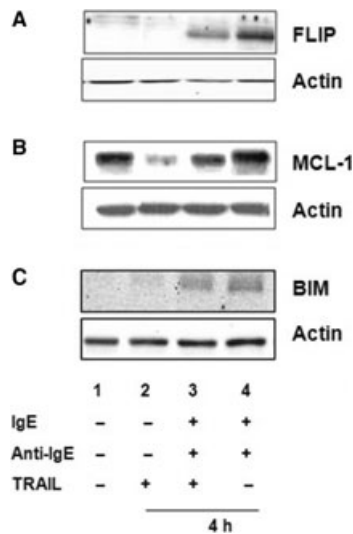


**Figure 3.** TRAIL decreases the level of MCL-1 but does not influence the increase in FLIP and BIM induced by IgE-dependent activation of CBMC. CBMC were activated with anti-IgE for 1 h. IgE-sensitized CBMC were used as the controls (lane 1). Nonactivated cells/activated cells were then incubated with TRAIL (500 ng/ml) for 4 h (lanes 2 and 3). Activated cells without TRAIL treatment, incubated under the same conditions were used as a negative control (lane 4). The expression of FLIP (A), MCL-1 (B) and BIM (C) was assessed by immunoblot analysis using specific antibody. Actin was used as a control for loading normalization. Data are representative of three independent experiments performed with CBMC from three different donors (B).

Nevertheless, in unactivated/resting mast cells, the treatment with TRAIL alone decreased the basic levels of MCL-1 (Fig. 3B).

The observed changes were not statistically significant because of high variability among the donors, as it was found from densitometry analysis (data not shown).

#### TRAIL induces mitochondria activation through BID cleavage

The influence of TRAIL on MCL-1 expression suggested the possible involvement of an intrinsic apoptotic pathway in the regulation of TRAIL induced apoptosis of human mast cells. Therefore, we further examined whether this pathway can be directly activated by TRAIL. For this purpose, we measured the changes in mitochondria membrane potential. A significant enhancement in mitochondrial depolarization was observed 8 and 18 h after CBMC treatment with TRAIL (Fig. 4A). The involvement of mitochondria in TRAIL induced CBMC apoptosis was confirmed by BID truncation that was already detected 4 h after TRAIL addition (Fig. 4B). Treatment with TRAIL also augmented the expression levels of nontruncated BID. However, the increase was not significant in comparison with the control levels, as is apparent from the densitometry analysis (Fig. 4C), which

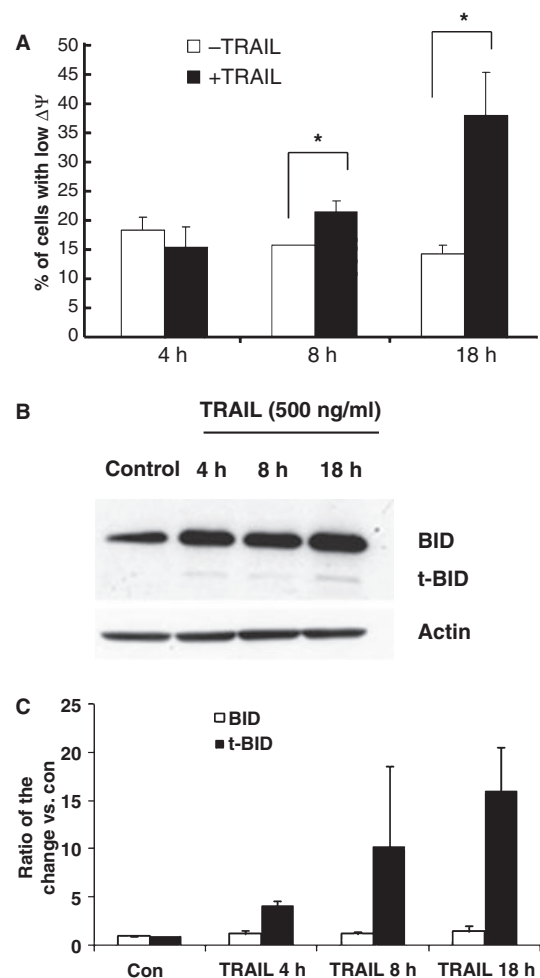


**Figure 4.** TRAIL induces activation of mitochondria pathway. CBMC treated with TRAIL (500 ng/ml) for 4, 8 and 18 h. Untreated cells cultured in the same conditions were used as a control. The loss of  $\Delta\Psi_m$  was measured using the fluorescent probe JC-1. The percentage of cells with low  $\Delta\Psi_m$  is indicated. Data are the mean  $\pm$  SD of three experiments performed in duplicates. (\*)  $P < 0.05$  compared with TRAIL-untreated cells (A). BID/Truncated BID (t-BID) expression was assessed by immunoblot analysis using specific antibody. Actin was used as a control for loading normalization (B). The level of BID/t-BID was quantified by densitometry, normalized to actin and represents fold change in the expression level in comparison with control (con). Data are mean  $\pm$  SD of three independent experiments (C). Data are representative of three independent experiments performed with CBMC from three different donors.

summarizes changes in nontruncated BID and truncated BID expression. These findings suggest that TRAIL-induced apoptosis of CBMC involves not only an extrinsic but also an intrinsic apoptotic pathway. Moreover, IgE-dependent activation caused a decrease in the level of BID levels, not immediately, but 4 h after activation. Interestingly, this decrease in BID expression induced by IgE-activation was abrogated by the treatment with TRAIL for 4 h (Fig. 5).

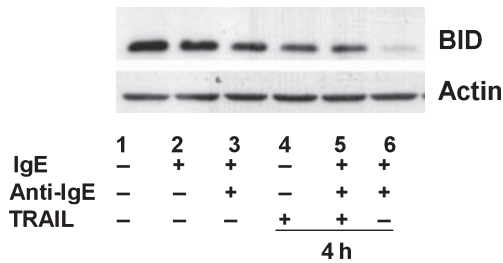


**Figure 5.** The reduction in BID expression mediated by CBMC IgE-dependent activation is abrogated by TRAIL. CBMC were activated with anti-IgE for 1 h. Nonactivated and IgE-sensitized CBMC were used as the controls. Nonactivated/activated cells were either lysed immediately (lanes 1–3) or treated with TRAIL (500 ng/ml) for 4 h (lanes 4 and 5). Activated cells without TRAIL treatment incubated in the same conditions were used as a negative control (lane 6). The expression of BID was assessed by immunoblot analysis using specific antibody. Actin was used as a control for loading normalization. Data are representative of three independent experiments performed with CBMC from three different donors.

IgE-dependent activation does not influence the decrease in human mast cell viability induced by SCF deprivation

We investigated the influence of IgE-dependent activation on pro-apoptotic stimulus carried out by SCF withdrawal.

For this purpose, human mast cells were first activated and therefore incubated with optimal (100 ng/ml) and suboptimal concentrations of SCF (0 and 10 ng/ml) for 24 h. No significant influence of activation on the mast cell death induced by SCF deprivation was found (Fig. 6). The percentage of viable cells was similar in activated (IgE + anti-IgE) and IgE-treated (IgE)/nonactivated cells (control) when SCF was withdrawn ( $43.2 \pm 2.2\%$  and  $42.8 \pm 3.9\%$ / $43.2 \pm 2.2\%$  respectively). When cells were cultured in suboptimal concentrations of SCF (10 ng/ml), there was a slight but not significant ( $p > 0.05$ ) increase in viability in activated mast cells in comparison to IgE-treated/nonactivated mast cells ( $62.5 \pm 2.5\%$  in comparison with  $55.4 \pm 2.7/57.1 \pm 1.1\%$  respectively).

## Discussion

Although modulation of apoptotic signaling of mast cells by IgE-dependent activation was previously studied in mast cells of murine origin (18, 19), our present data are the first to be obtained with primary human mast cells. As a source of human mast cells, we have been using CBMC, which are widely used for human mast cells research *in vitro* (20–22) because of the scarcity of human tissue derived mast cells. CBMC share many features of mature tissue derived mast cells such as SCF dependence and responsiveness to IgE-dependent activation (23–25),

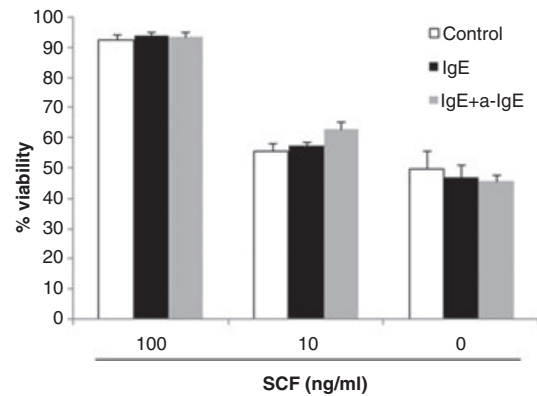


**Figure 6.** CBMC IgE-dependent activation has no significant influence on SCF withdrawal-induced CBMC death. CBMC were either incubated in culture medium (control) or sensitized with IgE. IgE sensitized CBMC were either incubated in culture medium (IgE) or activated by incubation with anti-IgE (5  $\mu$ g/ml) for 1 h at 37°C (IgE + a-IgE). Nonactivated and IgE-sensitized CBMC served as a negative control. Following activation cells were cultured in medium containing the indicated concentrations of SCF for 24 h. The viability was assessed by PI staining followed by PI exclusion analysis by FACS. Data are mean  $\pm$  SD of three independent experiments performed in duplicates.

and because of their tryptase content, they can be classified as mucosal type mast cells (26). However, *in vitro* and tissue derived mast cells might be different in some aspects such as maturity and proteases content (27). Moreover, we have reported that human lung derived mast cells express both TRAIL and FAS death receptors while CBMC were found to express only TRAIL death receptors (9).

Previously we reported that TRAIL-induced apoptosis is upregulated in CBMC as a result of Fc $\epsilon$ RI cross-linking and suggested that the upregulation is because of an increase in DR5 receptors expression (9). However, the increase was at the transcription level and no change in protein expression was observed. Therefore, we continued to search for the mechanisms that can explain this phenomenon.

In accordance with our previous findings, we found that IgE-dependent activation of CBMC enhanced TRAIL-induced 'initiator' caspase-8 cleavage as well as the total level of pro-caspase-8. The involvement of caspase-8 in TRAIL induced apoptosis is well characterized (28). However, the fact that IgE-dependent activation of mast cells can increase the death receptor activation-induced caspase-8 cleavage was not previously reported. Activated caspase-8 subsequently activates 'executioner' caspases such as caspase-3 by proteolytic cleavage (28). In accordance with the previous reports, we found that the enhanced cleavage of pro-caspase-8 brought about a subsequent increase in the levels of activated caspase-3.

In addition to the direct activation of mitochondria-independent apoptotic pathway in human mast cells, we found that TRAIL activates the intrinsic apoptotic pathway by caspase-8 mediated BID cleavage. This is interesting as activation of FAS, another member of TNF receptor family, was shown to induce apoptosis in MC/9 murine mast cell line without involvement of the mitochondrial pathway (29).

The involvement of an intrinsic pathway in TRAIL induced CBMC apoptosis is supported by the fact that TRAIL decreases the basic levels of MCL-1. MCL-1 levels are highly regulated by proteolytic degradation and phosphorylation (30, 31). In addition, the ability of TRAIL to induce caspase-3 and caspase-8 mediated MCL-1 degradation was recently reported in transformed cell lines (14). Interestingly, a TRAIL-mediated decrease in MCL-1 levels was inhibited by Fc $\epsilon$ RI cross-linking. This finding may be explained by the fact that Fc $\epsilon$ RI cross-linking stabilizes MCL-1 through phosphorylation. It was recently found that MCL-1 can be stabilized via phosphorylation performed by GSK-3 (32), a kinase, which is activated by IgE-dependent activation (33). This hypothesis still needs to be investigated. We have shown that MCL-1 was upregulated in human mast cells as a result of activation. Similar to MCL-1, an upregulation in the FLIP protein was also observed.

Treatment of the mast cells with TRAIL reduced the level of FLIP, though not to the baseline levels, suggesting that at least partially, TRAIL can overcome activation induced increase in FLIP expression. Both FLIP and MCL-1 were reported to play an important role in TRAIL-induced apoptosis (12). The upregulation of other pro-survival molecules such as Bcl-2 and Bcl-xL as a result of Fc $\epsilon$ RI ligation was previously reported in primary human monocytes, where it prevented FAS-induced apoptosis (34). However, in our study, upregulation of both FLIP and MCL-1 anti-apoptotic proteins did not prevent TRAIL induced apoptosis. This observation is similar to the previously reported findings that monomeric IgE, whose pro-survival effect is well established in murine studies, has no influence on the growth factor withdrawal induced apoptosis in human mast cells (35).

In addition to the upregulation of pro-survival molecules, IgE-dependent activation of mast cells downregulated the expression of pro-apoptotic BID. As the

decrease in the BID levels was not because of its cleavage-induced activation, this change is another anti-apoptotic signal that is induced by mast cell immunologic activation.

We have found that in spite of the pro-survival signaling induced by IgE-dependent activation, it did not have any significant influence on the viability of activated mast cells cultured with suboptimal concentrations of SCF. It is possible to speculate that as SCF is critical for human mast cell viability, its depletion provides a strong pro-apoptotic stimulus that cannot be overcome by activation. Similarly, it was previously shown that pro-survival cytokines production (i.e. IL-5 and TNF- $\alpha$ ) induced by monomeric IgE did not enhance survival of human CBMC after SCF withdrawal (35).

Together with the induction of anti-apoptotic signal transduction, we also found an increase in the pro-apoptotic BIM, but not in other pro-apoptotic proteins that were examined. The detected up-regulation in BIM can be a possible explanation of how Fc $\epsilon$ RI cross-linking elicits TRAIL-induced apoptosis and caspases activation.

However, the increase in pro-survival molecules, i.e. MCL-1 and FLIP, as well as the decrease in pro-apoptotic BID are also induced by the same trigger. The fact that IgE-dependent activation can, at the same time, upregulate both pro-apoptotic (BIM) and pro-survival (Bcl-xL) factors was previously reported in mast cells of murine origin (18). We therefore assume that the fate of activated mast cells in an inflamed tissue is probably dependent on several factors such as the kind of mast cell activation (IgE vs non-IgE dependent), growth factor/cytokine levels and death receptors/ligands expression.

It has been shown that in allergic inflammatory disorders, there is an increase in TRAIL expression. The elevated levels of soluble TRAIL were identified in broncho alveolar lavage of allergic asthmatics (36). In addition, TRAIL was found to be largely expressed in peripheral blood derived T cells and monocytes of atopic dermatitis patients in comparison to normal subjects (37). Thus, in conditions in which there is still an ongoing allergic response, we hypothesize that mast cell activation can act as a negative regulator of the response because of the up-regulation of the mast cell death ligand-induced apoptosis.

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