Recombinant Human (rh)IL-4-Mediated Apoptosis and Recombinant Human IL-6-Mediated Protection of Recombinant Human Stem Cell Factor-Dependent Human Mast Cells Derived from Cord Blood Mononuclear Cell Progenitors

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Recombinant Human (rh)IL-4-Mediated Apoptosis and Recombinant Human IL-6-Mediated Protection of Recombinant Human Stem Cell Factor-Dependent Human Mast Cells Derived from Cord Blood Mononuclear Cell Progenitors

Carole A. Oskeritzian,* Zhiliang Wang,* Jarema P. Kochan,‡ Margaret Grimes,† Zhongmin Du,* Hyeun-Wook Chang,* Steven Grant,* and Lawrence B. Schwartz2*

Although stem cell factor (SCF) appears to be the major growth factor for human mast cells, other factors undoubtedly play important roles in the development, survival, and function of these cells. The current study examined the effects of recombinant human (rh) IL-4 and rhIL-6 on rhSCF-dependent development and survival of human mast cells derived in vitro from cord blood progenitor cells. After 4–8 wk of culture with rhSCF and various amounts of rhIL-4, a dramatic decline in mast cell numbers was observed with rhIL-4, the EC50 being about 0.1 ng/ml. Numbers of other cell types remained high. Mast cells derived from cord blood progenitors after 7 wk of culture with rhSCF alone displayed an MC T phenotype and expressed Kit, FcεRI, and IL-4R on their surface. Mast cells examined after purification by immunomagnetic sorting became apoptotic within hours after exposure to rhIL-4, a phenomenon blocked by anti-IL-4 Ab. Because rhIL-4-dependent apoptosis but not the loss of mitochondrial membrane potential was prevented by the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(Z-VAD)-fluoromethylketone, mitochondrial perturbation most likely preceded caspase activation. Consistent with this conclusion was the observation that both apoptosis and loss of mitochondrial membrane potential (∆ψm) were inhibited by cyclosporin A in combination with aristolochic acid. rhIL-6 protected cord blood mast cells from rhIL-4-induced apoptosis. Thus, IL-4 can cause both maturation and apoptosis of human mast cells, the latter effect being abrogated by IL-6. The Journal of Immunology, 1999, 163: 5105–5115.

S

tem cell factor (SCF,3 Kit ligand, Steel factor, mast cell growth factor) is the major growth factor for both human and rodent mast cells. In humans, SCF is the only growth factor identified thus far that by itself in vitro will cause hematopoietic progenitor cells from fetal liver (1), cord blood (2), peripheral blood (3, 4), and bone marrow (5) to become mast cells. The influence of IL-3 on mast cell development, unlike that in rodent systems, is negligible in most in vitro human systems (4, 6), though it may enhance proliferation of multipotential progenitors and promote mast cell survival (7). Whereas mast cells derived from fetal liver with recombinant human (rh)SCF alone reportedly lack surface FcεRI (8), those derived from cord blood, peripheral blood, and bone marrow, in most but not all cases, express surface FcεRI when rhSCF is the sole exogenous growth factor. rhIL-4 influences human mast cell development in various ways. For example, when added to the human mast cell leukemia line, HMC-1, rhIL-4 down-regulates surface Kit expression (9). rhIL-4 added with rhSCF to fetal liver cells at the beginning of culture results in diminished surface levels of Kit on the mast cells that develop, a modest decline in the numbers of mast cells obtained (10), and induction of expression of functional surface FcεRI that is associated with a 10-fold increase in cellular levels of FcεRI transcripts (11). Mast cells derived from cord blood progenitors in the presence of rhIL-4 together with rhSCF and rhIL-6 showed increased chymase expression (12), whereas those derived from fetal liver progenitors in the presence of rhIL-4 and rhSCF showed a decrease in the number of the chymase+MC T type of mast cell, with no change in number of the chymase+MC TC type of mast cell (11). For mast cells derived from human cord blood in the presence of rhSCF and rhIL-6, rhIL-4 induces homotypic aggregation dependent upon LFA-1 and ICAM-1 (13), and detectable levels of surface FcεRI (14). rhIL-13, like rhIL-4 but weaker, down-regulates Kit expression and up-regulates LFA-1 and ICAM-1 on HMC-1 cells, but has a negligible effect on the development of mast cells from cord blood progenitors exposed to rhSCF (15).

The present study examines the effects of rhIL-4 on the development of rhSCF-dependent human mast cells from cord blood mononuclear cells (CBMC) and shows that rhIL-4 can induce apoptosis via a mitochondrial-dependent pathway in the mast cells that develop in the absence of rhIL-6, whereas cord blood-derived mast cells...
mast cells are protected from rhIL-4-mediated apoptosis if developed in the presence of rhIL-6.

Materials and Methods

Culture of CBMC, fetal liver cells, and dispersed lung cells

Umbilical cord blood was obtained at the time of delivery and collected in heparin-treated tubes. The experimental protocol was approved by the Human Studies Committee at Virginia Commonwealth University (Richmond, VA). Cord blood was diluted 1:1 in PBS, layered over Histopaque (density = 1.077 g/ml; Sigma, St. Louis, MO) and centrifuged at 1000 x g for 20 min at room temperature to remove erythrocytes. CBMC were collected from the interface, washed twice in PBS, and submited to another density-dependent centrifugation step as described above. Cells at the interface were collected and washed three times in PBS, and suspended in RPMI 1640 supplemented with 10% heat-inactivated controlled process serum replacement medium-1 (Sigma), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM HEPES, 50 µM 2-ME, 200 U/ml penicillin, and 100 µg/ml streptomycin. Cells were dispensed into 24-well plastic tissue culture plates (Costar, Cambridge, MA) at 10^6 cells/2 ml/well in the presence of rhSCF (100 ng/ml; a gift from Amgen, Thousand Oaks, CA) alone or together with different concentrations of rhIL-4 (a gift from Amgen) and with rhIL-6 (50 ng/ml; Genzyme, Cambridge, MA). Cells were cultured at 37°C in a 6% CO2 incubator. Half of the culture medium was replaced once a week. Cells were harvested at different time points and subjected to cytofluorometry and to flow cytometry.

In some cases, fetal liver-derived mast cells and cord blood-derived cells were cocultured. Fetal liver-derived mast cells were prepared as described previously (1); complete medium containing rhSCF (50 ng/ml) was added every 3–4 days over a 4- to 6-wk time span. Cord blood cells were cultured as above with rhSCF (100 ng/ml) and rhIL-4 (20 ng/ml), also for 4–6 wk. Cells were then washed twice with PBS, counted, and cultured with rhIL-6 (100 ng/ml) in the presence and absence of rhIL-4 (20 ng/ml) for 6 days at 10^6 total cells/ml; the cells consisting of fetal liver-derived cells alone, cord blood-derived cells alone, or a 1:1 mixture of fetal liver and cord blood-derived cells. Total cell numbers and viabilities and the number of Kit^+^ cells were determined in each case.

Surgical lung tissue samples (10–60 g from patients with lung cancer or emphysema) were obtained from the Department of Pathology at Virginia Commonwealth University or through the Cooperative Human Tissue Network (Columbus, OH) as approved by the Human Studies Institutional Review Board at Virginia Commonwealth University. Each sample was minced extensively and then digested with a combination of type I collagenase and type I-S hyaluronidase (Sigma; 1.5 and 0.75 mg/ml, respectively) in MEM (density 0.5% in 0.5 N HCl). Total cell viability was determined by trypsin blue exclusion. Erythrocytes were eliminated from the cell preparation by centrifugation over a continuous 5% Percoll gradient (Pharma- cia, Piscataway, NJ). After each purification step (D1, D2, and Percoll gradient), the viability and the mast cell content of each fraction was monitored as described above. Cells (15–20% mast cells) were then cultured overnight in an incubator at 37°C with 6% CO2 in MEM + 2% FCS containing penicillin (200 U/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM). Four milliliters of enzyme solution was applied per gram of tissue and incubated for 30 min at 37°C with gentle shaking. The dispersed cells (D1) were separated from residual tissue by filtration through a 70-µm mesh nylon sieve. The remaining tissue was minced and digested again as above. Cells from the second digestion (D2) were collected and filtered as above. Filtered cells were washed twice in MEM + 2% FCS (500 x for 8 min at room temperature). Depending on their respective mast cell yields, D1 and D2 were pooled. Mast cells were detected on cytospins stained with acidic carbazole, and MC T cells (tryptase+^, chymase+^) that stain blue with Fast Blue RR (Sigma) (20). Because no chymase+^ MC^T^ cells were detected, only G3 was used in double-staining experiments aimed at examining apoptosis in mast cells or of IL-4R expression in mast cells. In the latter case, slides were first incubated with PBS containing 10% normal rabbit serum, 1% BSA, and 0.01% thimerosal for 1 h at room temperature. Slides were then washed in PBS (500 x for 5 min) and incubated overnight in PBS containing 1% goat polyclonal IgG anti-human IL-4R (10 µg/ml; R&D Systems, Minneapolis, MN) or nonimmune goat IgG as a negative control (10 µg/ml). Slides were washed three times for 5 min each time in 0.01 M Tris buffer, pH 7.4, containing saline and 0.05% Tween 20 (TTBS), rinsed with H2O, and incubated for 1 h at room temperature with a biotin-conjugated rabbit anti-goat IgG (200 µg/ml; Dako). Slides were then washed three times for 5 min in TTBS, rinsed with H2O, and incubated with streptavidin-peroxidase conjugate (20 µg/ml) for 1 h at room temperature. Slides were washed as described above, rinsed with H2O, and incubated with 3-amo-
In situ detection of apoptotic mast cells

The identification of apoptosis at a single cell level on slides was performed using the TACS assay in situ apoptosis detection kit according to the manufacturer (Genzyme). The principle of the technique is to detect DNA fragmentation generated during apoptosis. Biotinylated nucleotides are incorporated into the ends of DNA fragments using TdT. Labeled ends are detected using streptavidin-HRP and the peroxidase substrate, TACS Blue Label (Genzyme), which stains apoptotic cells blue. Pelleted cells were gently suspended in 10% neutral-buffered formalin at a concentration of 10^6 cells/ml and incubated for 10 min at room temperature. Fixed cells were centrifuged and resuspended in 80% ethanol. At room temperature, containing a solution of 2% H_2 O_2 for 5 min at room temperature. Slides were endogenous peroxidase activity, slides were placed in a Coplin jar containing a solution of 2% H_2 O_2 for 5 min at room temperature. Slides were then transferred to a solution of 1X labeling buffer containing TdT dNTPs (1X final concentration) and adjusted to 1 mM CoCl_2. Labeling was initiated by addition of 15 U of TdT per sample. The label was allowed to proceed for 15 min at 37°C and then was stopped by transferring the slides into a solution of TdT stop buffer. Slides were then rinsed for 1 min at room temperature in water, incubated with 10 ng of streptavidin-HRP for 10 min at room temperature, washed twice with water, and incubated with 50 μl of TACS Blue Label. To decide when to stop the staining reactions, the staining of control slides provided by the manufacturer and developed with the parallel were monitored microscopically. Typically, after about 7 min at room temperature the slides were washed four times in water, each wash lasting 5–10 s. Slides were then processed for the immunofluorescence staining of tryptase using TRITC-G3 and analyzed as described above. For each experiment, four different slides of each culture condition were processed and a total of 100–200 cells were analyzed per slide. Slides were read by one observer in a blinded fashion. DNase-free deionized water was used throughout the procedure.

Where indicated, annexin V (Genzyme) also was used to detect apoptotic cells. Harvested cells to analyze were pelleted by centrifugation and washed in cold PBS. Cell pellets were resuspended in a 1:100 dilution of annexin V-biotin, and developed according to the manufacturer’s instructions. As a negative control, cells from the same sample were incubated with MOPC-biotin.

Measurement of changes in mitochondrial membrane potential (Δψ_m)

Purified Kit^+ cord blood-derived mast cells (10^7/250 μl of complete RPMI 1640 medium) were loaded with 40 nM of the lipophilic, catonic, and fluorescent dye, 3,3′-dihexylxylcarbocyanine iodide (DiOC_3(3)); Molecular Probes, Eugene, OR) (21, 22) for 20–25 min at room temperature before being analyzed. DiOC_3(3) accumulates in intact mitochondria, but levels are reduced in cells undergoing the permeability transition accompanied by loss of Δψ_m. mitochondrial retention of DiOC_3(3) was determined up to 6 h after treatment in the presence of rhSCF (100 ng/ml) alone or with rhIL-4 (20 ng/ml), using a FACScan (Becton Dickinson) and CYCLOPS software. In addition, the following treatments (all reagents were purchased from Biotal Research, PA, and were dissolved in DMSO) were assayed on the same purified cord blood-derived mast cells before the treatment with rhIL-4. The pan-caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) was added to the cells at 25 μM, 1 h before rhIL-4 (23). Cyclosporin A (CyA) was added to the cells at 2.5 μM and FK506 at 5 μM, each one together with 50 μM arachidonic acid (AA) and 30 min before treatment with rhIL-4 (24). This dose of CyA in combination with AA was the highest found to be nontoxic to the cells. A portion of the cells from each experimental group was assessed for apoptotic DNA fragmentation, as described above (n = 4–6).

Results

Effect of rhIL-4 on development of mast cells from CBMC

Expression of cell surface FceRI and Kit were analyzed weekly by flow cytometry on CBMC cultured either in the presence of rhSCF alone (100 ng/ml) or in combination with rhIL-4 (20 ng/ml). These conditions were chosen to mirror those used to develop mast cells from fetal liver cells in our lab (11) and as reported by others for development of mast cells from CBMC (2) and peripheral blood and bone marrow cells (5). The dose of rhIL-4 was previously shown to increase expression of FcεRI on rhSCF-dependent mast cell development from fetal liver cells while also causing a modest decline in mast cell numbers (11). IL-6 has been shown to enhance rhSCF-dependent development of cord blood-derived mast cells in other labs, and additional treatment of such cells with rhIL-4 been
shown to promote features such as chymase and FceRI expression (7, 12–14, 25, 26). The effect of rhIL-6 in our system on the IL-4 response will be considered below.

Kit+ and FceRI+ cells appeared 2 wk after cultures were initiated with rhSCF alone or together with rhIL-4. As shown in Fig. 1A, inclusion of rhIL-4 caused a marked (7- to 8-fold) increase in cell numbers at 2, 4, and 8 wk of culture relative to conditions using rhSCF alone. Although the percentages of Kit+ and FceRI+ cells were significantly greater in the absence of rhIL-4 than in its presence at 2, 4, and 8 wk of culture (Fig. 1B), absolute numbers of Kit+ and FceRI+ cells were not different in the presence and absence of rhIL-4 at 2 wk. However, by 4 wk and also at 8 wk of culture, the numbers of Kit+ cells and FceRI+ cells were significantly lower in the presence than in the absence of rhIL-4. In fact, almost no Kit+ or FceRI+ cells could be detected at 8 wk. These results suggested that rhIL-4 acted to expand nonmast cell populations and to decrease numbers of mast cells after they had formed but did not prevent mast cell formation. Also, the effects of rhIL-4 on Kit+ and FceRI+ cells occurred in parallel. If addition of rhIL-4 was delayed until day 7 or day 14, and the cells were examined at 4 or 8 wk, almost no FceRI+ and Kit+ cells were detected, suggesting that rhIL-4 was affecting the survival of newly formed mast cells (data not shown).

The dose response of the inhibitory effect of rhIL-4 on the numbers and percentages of Kit+ and FceRI+ cells developing from CBMC is shown in Fig. 2. FceRI+ and Kit+ cells after 8 wk of culture with rhSCF and different doses of rhIL-4 decreased in parallel with increasing doses of rhIL-4. Essentially no receptor-positive cells were detected at 2 and 20 ng/ml of rhIL-4. Because the total numbers of cells remaining after 8 wk of culture were higher with increasing concentrations of rhIL-4, the EC$_{50}$ concentrations of rhIL-4 for mast cell number and mast cell percentage differed. The EC$_{50}$ for mast cell percentage was about 0.01 ng/ml, whereas the EC$_{50}$ for mast cell number was about 0.1 ng/ml.

Time course of inhibitory effects of rhIL-4 on FceRI+ mast cells

Because rhIL-4 appeared to act primarily on mast cells rather than mast cell progenitors, rhIL-4 (20 ng/ml) was added along with rhSCF to mast cells that had formed after 7 wk of culture of CBMC with rhSCF. Cells were examined daily for FceRI by flow cytometry over a 4-day span and the results of three independent experiments are shown in Fig. 3. In the presence of rhSCF alone at 100 ng/ml, the percentage of cells positive for FceRI was approximately the same on days 4 and 0 (data not shown). In the presence of rhIL-4 there was a progressive decline in the numbers and percentages of cells expressing FceRI. Despite the decline in mast cells, total cell numbers increased from a mean of 3.5 × 10$^5$ to a mean of 6.2 × 10$^5$ over the 4-day experiments.

Mast cells derived from CBMC express IL-4R

To determine whether rhIL-4 might directly exert its effects on cord blood-derived mast cells, the expression of IL-4R was analyzed by flow cytometry and immunocytochemistry. CBMC were cultured for 7 wk in rhSCF alone (100 ng/ml) and then divided into two portions. One portion was cultured again in rhSCF alone, the other in the presence of both rhSCF and rhIL-4 (0.002, 0.2, and 20 ng/ml). One wk later, cells from each experimental condition were analyzed for cell surface Kit and IL-4R by flow cytometry and cellular tryptase and IL-4R by immunocytochemistry (Fig. 4A). Interestingly, the percentages of cells cultured in rhSCF alone that expressed IL-4R were not significantly different from the percentages expressing Kit and tryptase, each being detected on about 50% of the cells. Dual labeling of cyto spun by immunocytochemistry indicated that >95% of the rhIL-4-4+ cells and >95% of the tryptase+ cells were double-positive when cells were cultured in rhSCF alone.

Upon exposure to rhIL-4, cell surface Kit+ and IL-4R+ cells and tryptase+ cells decreased in parallel in a dose-response manner to essentially zero at 20 ng/ml of rhIL-4. In contrast, cellular IL-4R+ cells showed a biphasic response, decreasing at 0.2 ng/ml and increasing at 20 ng/ml of rhIL-4. Possible explanations are that IL-4R expression was induced in a nonmast cell population, or a nonmast cell population already expressing IL-4R was expanded. Preliminary experiments indicate these cells are CD3+ T cells (H.-W. Chang and L. B. Schwartz, unpublished observations). Detection of IL-4R inside these cells rather than on their surface suggests that surface receptor may have been internalized with exogenously added rhIL-4. However, additional mechanisms, including desensitization to receptor coupling or alterations in the
Intracellular signaling pathways after receptor coupling, should be considered. To determine whether surface Kit, FcεRI, and IL-4R were expressed on the same cell population, double-labeling experiments were performed, as shown in Fig. 4B. In this figure representative dot blots are shown of CBMC that had been cultured for 7 wk with rhSCF (55% tryptase+ cells) and then exposed to rhSCF alone (Fig. 4B, upper) or to rhSCF and rhIL-4 (Fig. 4B, lower) for only 2 days before analysis. Without treatment with rhIL-4, Kit+ cells accounted for about 41% of the total, and nearly all were also FcεRI+ (Fig. 4B, upper left) and IL-4R+ (Fig. 4B, upper right). FcεRI+ or IL-4R+ cells with low levels or no detectable Kit accounted for about 38% of the cells. Although these cells could represent nonmast cell lineages, it is more likely that most of these cells represent mast cells that had internalized Kit-rhSCF complexes, as reported previously (27). As shown above, by 4–7 days after addition of rhSCF, nearly equal percentages of cells are positive for surface Kit, IL-4R, and FcεRI. Addition of rhIL-4 together with rhSCF for 2 days resulted in a dramatic decrease in the percentages of surface Kit+/IL-4R+ and Kit+/FcεRI+ cells (Fig. 4B, lower) to <1. The percentages of Kit+/FcεRI+ and Kit+/IL-4R+ cells increased to about 62%, but the total percentages of FcεRI+ and IL-4R+ cells decreased from about 78 to 63%. Again, it is likely that rhSCF-induced internalization of Kit accounted for the disproportionate decrease in Kit+ cells. It is also possible that rhIL-4 may have delayed recovery of surface Kit.

Mast cells incubated in the presence of rhIL-4 for 2 days undergo apoptosis

To evaluate whether apoptosis could account for the decreased numbers of mast cells observed in the presence of rhIL-4, cells were double labeled for apoptotic fragmentation of DNA in nuclei and for tryptase in mast cell secretory granules. Fig. 5A shows a fluorescence photomicrograph of tryptase+ mast cells, whereas Fig. 5B shows a light photomicrograph of the same field of cells stained for excessive DNA fragmentation; these cells had been cultured with rhSCF alone for 5–7 wk. As summarized in Table I, in two independent experiments none of the tryptase+ cells in preparations of cord blood-derived mast cells exposed to rhSCF alone appeared to be apoptotic, whereas 40–50% of the tryptase+ cells showed DNA fragmentation by the TACS Blue Label procedure, consistent with apoptosis. During this 2-day culture there were modest declines (20–30%) in total cell numbers with these unsorted preparations of mast cells. Fig. 5, C and D, are higher magnification photomicrographs of a single cell from a culture treated with rhSCF for 7 wk and rhSCF and rhIL-4 (20 ng/ml) for 2 days. In this case, the cell is tryptase+ (Fig. 5C) and appears to be undergoing apoptosis (Fig. 5D). The overall percentage of apoptotic cells 2 days after addition of rhIL-4 remained about the same as for those cells exposed only to rhSCF. However, the percentages of mast cells undergoing programmed cell death increased dramatically when rhIL-4 was added, increasing from 0 to 94% in one experiment and from 0 to 70% in a second experiment. Increased apoptosis of mast cells was associated with declines in the percentages and calculated numbers of tryptase+ mast cells, indicating a selective loss of mast cells during the 2-day incubation with rhIL-4.

To determine whether rhIL-4 acts directly against the cord blood-derived mast cells, such mast cells were purified and then incubated with rhIL-4. Kit+ cells from two independent 5- to 7-wk-old cultures of rhSCF-treated CBMCs were sorted using the immunomagnetic purification procedure and detached after 2 days.
of culture as described in Materials and Methods. Detached Kit1 cells were then cultured for 2 additional days in the presence of rhIL-4 (20 ng/ml) and rhSCF (100 ng/ml) or with rhSCF alone. The sorted cells were $95\%$ mast cells as judged by their tryptase staining. As reported in Table I, two independent experiments showed that the addition of rhIL-4 to purified cord blood-derived mast cells induced apoptosis among the mast cells. Within 2 days of culture, rhIL-4 caused 90\% of the mast cells to become apoptotic, coupled with 3- to 4-fold decreases in the percentages and 30- to 38-fold decreases in the total numbers of tryptase1 cells.

rhIL-4-mediated apoptosis of cord blood-derived mast cells is blocked by anti-IL-4 Ab

To validate that rhIL-4 was responsible for the apoptosis observed above, mast cells derived from cord blood progenitors in the presence of rhSCF were treated for 3 h under the conditions shown in Fig. 6. This period had been determined to result in apoptosis of about 30\% of the mast cells without substantially reducing the number of mast cells. As shown, the apoptotic effect of rhIL-4 was significantly reduced by neutralizing anti-IL-4 Ab but not by control Ab.

Loss of $\Delta \psi_{\text{m}}$ is a primary event during rhIL-4-induced apoptosis of cord blood-derived mast cells

Induction of apoptosis in many systems has been related to initiation of mitochondrial damage, including loss of the $\Delta \psi_{\text{m}}$ and/or release of cytochrome c into the cytoplasm (24). To determine whether an alteration in $\Delta \psi_{\text{m}}$ or activation of upstream caspase(s) was the primary event during rhIL-4-induced apoptosis of purified cord blood-derived mast cells, a general inhibitor of caspase activity and an antagonist of mitochondrial permeability transition were utilized. rhIL-4 applied at 20 ng/ml for 6 h resulted in a decreased $\Delta \psi_{\text{m}}$ in 32\% of the cells and apoptosis (by DNA fragmentation) in 18\% compared with medium controls (Table II). No alteration in $\Delta \psi_{\text{m}}$ or the extent of apoptosis was detected between rhIL-4-treated and untreated cells 1 h after addition of this cytokine, whereas by 3 h there were significant alterations in each

| Table I. Analysis of apoptosis in cord blood-derived mast cells* |
|-------------------------|------------------|
|                         | Unssorted Cells (%) | Kit1 Cells (%) |
|                         | rhSCF | rhSCF + rhIL-4 | rhSCF | rhSCF + rhIL-4 |
|                         | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 | Expt. 3 | Expt. 4 |
| Apoptotic cells         | 20 ± 3 | 30 ± 6 | 30 ± 1 | 27 ± 5 | 15 ± 4 | 10 ± 4 | 29 ± 3 | 35 ± 5 |
| Mast cells              | 50 ± 4 | 42 ± 5 | 15 ± 3 | 20 ± 6 | 95 ± 4 | 97 ± 3 | 30 ± 6 | 25 ± 4 |
| Apoptotic mast cells    | 0      | 0      | 94 ± 8 | 70 ± 8 | 2 ± 2 | 4 ± 3 | 92 ± 3 | 95 ± 4 |
| Cell recovery: total    | 86/43  | 100/42 | 71/11  | 70/14  | 90/86 | 77/75 | 9/3  | 7/2  |
| cells: total mast cells |        |        |        |        |      |      |      |      |

* CBMC were cultured for 5–7 wk in the presence of rhSCF alone (100 ng/ml). Unsorted cells were then cultured either with rhSCF alone (100 ng/ml) or in combination with rhIL-4 (20 ng/ml) for 2 days. Alternatively, the Kit1 mast cells were purified as described in Materials and Methods and submitted to the same culture conditions. Unsorted and sorted cells were each analyzed for apoptosis and tryptase in two independent cord blood preparations. Viability by trypan blue was $\geq 85\%$ in all cases except for Kit-purified cells treated with rhIL-4, which were $\sim 80\%$ viable. For each condition, four different slides were analyzed, 100–200 cells were counted per slide, and mean ± SD values are shown for each experiment.
induced directly by Bax (24). However, this effect was unrelated to agents known to prevent the mitochondrial permeability transition prevented by pretreatment with CyA in combination with ArA, as anticipated, but did not prevent the loss of Z-VAD.FMK abrogated apoptosis assessed by DNA fragmentation. To inhibit caspase activation, Z-VAD.FMK was added. A one-way ANOVA showed significant differences among these groups; the Tukey Test showed that the groups treated with rhIL-4 alone or with sheep IgG had statistically higher percentages of apoptosis than the other four groups (p < 0.001).

FIGURE 6. rhIL-4-mediated apoptosis of cord blood-derived mast cells is blocked with anti-IL-4 Ab. Three independent cultures of CBMC cultured for 6–8 wk in the presence of rhSCF (100 ng/ml) alone were examined after the Kit+ mast cells were purified to ≥90% using magnetic beads. Cells were washed and incubated for 3 h with SCF (100 ng/ml) alone or together with sheep IgG (40 μg/ml), sheep IgG anti-human IL-4 (40 μg/ml; Genzyme), rhIL-4 (20 ng/ml) preincubated with sheep IgG anti-human IL-4 (40 μg/ml), rhIL-4 (20 ng/ml) preincubated with sheep IgG anti-human IL-4 (40 μg/ml), and rhIL-4 (20 ng/ml). Apoptotic mast cells were then determined by double staining with TRITC-G3 and the TACS procedure, as described in Materials and Methods. Each bar shows the mean ± SE values for three experiments, each one performed in duplicate. The representative flow cytogram of an experiment in which 40% of the cells showed a decrease in Δψm 6 h after exposure to rhIL-4. To inhibit caspase activation, Z-VAD.FMK was added. Z-VAD.FMK abrogated apoptosis assessed by DNA fragmentation, as anticipated, but did not prevent the loss of Δψm (Table II and Fig. 7), indicating that the loss of Δψm was not dependent on activation of the upstream caspases, including caspase-8 (28). Apoptosis as well as the loss of Δψm induced by rhIL-4 were prevented by pretreatment with CyA in combination with ArA, agents known to prevent the mitochondrial permeability transition induced directly by Bax (24). However, this effect was unrelated to the ability of CyA to inhibit calcineurin, because FK506 (5 μM), another calcineurin inhibitor, in combination with ArA, did not prevent either of these events (Table II). Together, these findings suggest that exposure of cord blood-derived mast cells to rhIL-4 results in loss of Δψm, which in turn leads to activation of the apoptotic protease cascade.

Mast cells developed from fetal liver cells and those derived from lung do not undergo rhIL-4-mediated apoptosis

Fetal liver-derived mast cells (4–5 wk of culture with rhSCF alone) of 67 ± 20% purity (n = 5) were examined by flow cytometry for the presence of IL-4R. Only 1.9 ± 1.5% of the total number of cells were positive. As shown in Fig. 8, exposure of such mast cells to rhSCF (100 ng/ml) and IL-4 (20 ng/ml) for 6 days resulted in no significant change in the number of Kit+ mast

### Table II. Effect of inhibiting caspase activity and loss of Δψm on rhIL-4-induced apoptosis of purified CB-MC

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>rhSCF</th>
<th>rhSCF + rhIL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δψm (%)</td>
<td>Apoptotic mast cells (%)</td>
</tr>
<tr>
<td>None</td>
<td>22 ± 4.1</td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>Z-VAD.FMK</td>
<td>21 ± 3.7</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>CyA + ArA</td>
<td>21 ± 4.5</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>FK506 + ArA</td>
<td>21 ± 5</td>
<td>3.7 ± 1.3</td>
</tr>
</tbody>
</table>

* CBMC were cultured for 5–7 wk in the presence of rhSCF alone (100 ng/ml). Kit+ mast cells were purified and then were cultured for another 6 h with either rhSCF alone (100 ng/ml) or in combination with rhIL-4 (20 ng/ml). Cells were analyzed promptly by flow cytometry to assess the Δψm, with the remaining cells being further processed for DNA fragmentation analysis as described in Materials and Methods. In some experiments, treatment with the cytokines was preceded by a 1-h incubation with Z-VAD.FMK (25 μM) or a 30-min incubation with either CyA (2.5 μM) plus ArA (50 μM) or FK506 (5 μM) plus ArA (50 μM). Mean ± SD values are shown.
cells. Examination of these cells for apoptosis by our double-labeling technique also revealed essentially no apoptotic mast cells in either case. To determine whether cord blood cells obtained after 4–6 wk of culture with SCF and IL-4 would alter the response of fetal liver-derived mast cells to IL-4, coculture experiments were performed. Such cord blood-derived cells were ≥80% CD31, with about one-third of those being CD4+CD8+ and the remainder being equally divided between CD4+CD8− and CD4−CD8+ cells. Less than 4% of the cells were mast cells. Equal numbers of fetal liver-derived cells (67% mast cells on average) and cord blood-derived cells (1% mast cells on average) were cocultured for 6 days in medium containing rhSCF (100 ng/ml) with or without rhIL-4 (20 ng/ml). As shown in Fig. 8, addition of rhIL-4 to rhSCF during this 6-day coculture period failed to decrease the number of mast cells as evidenced by the number Kit+ cells being unaltered. This result is dramatically different from that obtained with cord blood-derived mast cells (Table I). Thus, fetal liver-derived mast cells do not undergo IL-4-mediated apoptosis and do not develop this response under our experimental coculture conditions.

Lung-derived cells, enriched for mast cells by Percoll density-dependent sedimentation, were incubated with rhSCF in the presence or absence of rhIL-4 for 2 days and assessed by flow cytometry for surface Kit, FcεRI, and IL-4R. Total cell numbers recovered in the absence (5.8 × 10^5 ± 2.3 × 10^5) and in the presence (5.9 × 10^5 ± 2.3 × 10^5) of rhIL-4 were no different (n = 6) using paired Student’s t-test analyses corrected for the number of comparisons; neither were the respective percentages of FcεRI+ (32 ± 3 and 28 ± 6) and Kit+ (33 ± 4 and 29 ± 5), or the respective numbers of tryptase+ mast cells (1.5 × 10^5 ± 0.5 × 10^5 and 1.1 × 10^5 ± 0.6 × 10^5). Like cord blood-derived mast cells, essentially all human lung-derived mast cells appeared to be IL-4R+ by double-labeling with PE-YB5.B8.

**Mast cells developed with rhSCF and rhIL-6 are protected from rhIL-4-mediated apoptosis.**

Because the observation of apoptosis to rhIL-4 differs from prior reports in which rhIL-6 was used along with rhSCF to develop mast cells from CBMC, experiments were performed to examine the effect of rhIL-6 on rhIL-4-mediated apoptosis. The Kit+ cord blood-derived mast cells obtained in the presence of rhSCF with or without rhIL-6 were purified using immunomagnetic beads, detached during 1 wk of culture under the same conditions, and exposed to rhIL-4 for 3 h. Annexin V-biotin, which detects apoptosis at an earlier stage than the TACS procedure, as well as the TACS procedure, were used to examine apoptosis. As shown in Fig. 9A, rhIL-4 induced apoptosis in about 23% of the rhIL-6 naive cells, whereas the apoptosis percentage in rhIL-6-treated cells was about 5%, which was not significantly different from the rhIL-6 naive or
rhIL-6-treated cells that were not exposed to rhIL-4. Additional experiments showed that rhIL-6 could be added to cultures of cord blood-derived mast cells and rhSCF after 5–7 wk, and within 1 day provided protection from rhIL-4-mediated apoptosis (data not shown). Thus, rhIL-6 protects developing cord blood-derived mast cells from rhIL-4-mediated apoptosis.

Whether rhIL-6 protects cord blood-derived mast cells from rhIL-4-mediated apoptosis by suppressing surface IL-4R was examined in a time-course experiment. Purified Kit⁺ cord blood-derived mast cells cultured with rhSCF alone for 5–7 wk were incubated in the presence and absence of rhIL-6 for up to 1 wk and examined for surface IL-4R expression by flow cytometry. A uniphasic downward shift in MFI occurred in the presence of rhIL-6 within 24 h, decreasing the percentage of cord blood-derived mast cells being IL-4R⁺ from 90 to 45%, as shown in Fig. 9B. However, no further decrease in IL-4R expression was noted after 24 h.

**Discussion**

Human mast cells derived in vitro from CBMC in the presence of rhSCF alone underwent apoptosis when exposed to rhIL-4. Because this effect was not observed during the first 2 wk of culture, cells committed to a mast cell lineage that had begun to granulate appeared to be the most susceptible. Low doses of rhIL-4 were required for the effect on these mast cells, the EC₅₀ being about 0.1 ng/ml. As assessed by in situ detection of DNA fragmentation, almost all tryptase⁺ cells became apoptotic after exposure of 5- to 7-wk-old cord blood-derived mast cells to rhIL-4 with rhSCF for 2 days. Addition of rhIL-4 did not evoke a general cytotoxic effect, but instead caused a marked increase in the numbers of viable cells. However, because the numbers of rhSCF-dependent mast cells at 2 wk in the presence and absence of exogenous rhIL-4 were similar, we surmise that rhIL-4 did not divert mast cell progenitors to other lineages.

The effect of rhIL-4 appeared to be direct, because double labeling indicated that all Kit⁺ mast cells were FcεRI⁺ and IL-4R⁺ and because highly purified cord blood-derived Kit⁺ mast cells also underwent apoptosis when exposed to rhIL-4. However, indirect effects of rhIL-4 in the mixed cell cultures cannot be completely excluded. The apoptotic effect of rhIL-4 is unlikely to be due to diminished levels of surface Kit. Kit levels on rhSCF-dependent fetal liver-derived human mast cells, bone marrow-derived mast cells, and HMC-1 cells showed only about a 50% decline when cultured for 2–9 days with rhIL-4 (9, 29). This slow and modest IL-4-dependent decline in Kit levels alone cannot account for the rapid apoptosis (within hours) observed in the current study. In our case, the decline in Kit levels observed within the first 2 days of exposure to rhIL-4 and rhSCF was also observed with rhSCF alone, presumably due to internalization of the Kit-rhSCF complex, as reported previously (27), an effect from which mast cells recover as they synthesize new Kit. The dose response of rhIL-4 on cord blood-derived mast cells examined at 7 days indicated that equal percentages of tryptase⁺ Kit⁺, and FcεRI⁺ cells survived at each dose. Thus, rhIL-4-treated cells that survive beyond 2 days are likely to re-express surface Kit.

In contrast to cord blood-derived mast cells used in the current study, rhIL-4 did not have a substantial effect when added to fetal liver-derived rhSCF-dependent mast cells after the second week of culture. However, when rhIL-4 was added to fetal liver progenitors during the first week of culture, there was a decrease in the numbers of mast cells along with an induction of surface FcεRI expression (11). Fetal liver-derived mast cells express little, if any, IL-4R by 4–6 wk of culture, in contrast to the cord blood-derived mast cells studied herein. Coculture of fetal liver-derived mast cells with a predominant T cell population derived from CBMCs cultured for 4–5 wk with rhSCF (100 ng/ml) and rhIL-4 (20 ng/ml) did not alter the fetal liver mast cell response to rhIL-4, making it uncertain whether the IL-4-mediated apoptotic response is reversible. Also, human lung-derived mast cells, like those from fetal liver, do not undergo apoptosis when exposed to rhIL-4. Whether differences between fetal liver, adult lung, and cord blood-derived mast cells reflect differences in the tissue source of the progenitors, the maturational stage of the donor, or the accessory cell populations present at each site, remain to be understood. Many cells alter their functional responses to various cytokines as they mature, e.g., the NK cell response to IL-4 (30). Also, the plasticity of the IL-4 response is unknown. For example, whether the state of fetal liver-derived and adult lung-derived mast cells can be converted to one in which rhIL-4 induces apoptosis will be the subject of future experiments.

Whether the primary pathway leading to rhIL-4-triggered apoptosis in human cord blood-derived mast cells involved activation of the upstream caspase-8 or a loss of Δψₘ was examined pharmacologically. When upstream activator caspase-8 commits a cell to apoptosis, the general caspase inhibitor Z-VAD.FMK will prevent both apoptosis and downstream loss of Δψₘ (28). In contrast, when loss of Δψₘ commits a cell to apoptosis, leading to caspase-9 and the caspase-8 activation, Z-VAD.FMK does not prevent the loss of Δψₘ, even though apoptosis is abrogated. The loss of Δψₘ can be inhibited in some cells by a combination of CyA and the phospholipase A₂ inhibitor, ArA (24). CyA also forms a complex with cytosolic cyclophilin A that inhibits calcineurin (31, 32). However, calcineurin inhibition is not considered to be involved in preventing loss of Δψₘ, because FK506, another inhibitor of calcineurin, does not prevent the loss of Δψₘ (33), as also found in the current study. If loss of Δψₘ is the committing event toward apoptosis, then inhibiting loss of Δψₘ will inhibit apoptosis; if activation of caspase-8 is the primary event, then inhibiting loss of Δψₘ will not prevent apoptosis. Experiments shown in Fig. 7 and Table II favor loss of Δψₘ as the event that commits cord blood-derived mast cells to undergo apoptosis in response to rhIL-4. In agreement with previously reported results, cord blood-derived mast cells expressed both surface FcεRI and surface Kit when cultured with rhSCF alone (2, 34). Our results in the presence of rhSCF alone differ from reports showing rhIL-4 increases surface FcεRI expression, chymase production, and morphologic maturity of cord blood-derived mast cells developed in the presence of rhSCF and rhIL-6 (12, 14), and promotes the survival of such CB-MCs at risk for apoptotic death after withdrawal of rhSCF (7). The presence of rhIL-6 during the development of CBMC-derived mast cells may account for the different response to rhIL-4 observed between the current study and previous ones (12–14), because rhIL-6 protects cord blood-derived mast cells from rhIL-4-mediated apoptosis. The mechanism of this protective effect is not yet known, but is under investigation; for example, surface expression of IL-4R was diminished, but not eliminated by rhIL-6, even though the cells were protected from rhIL-4-induced apoptosis. Thus, different culture conditions appear to produce distinct mast cell responses to IL-4 (35, 36).

The growth, differentiation, recruitment, and activation of cells and apoptosis of cells often appear to be coregulated. For example, phorbol esters caused both differentiation and apoptosis of a human myeloid cell line (37), relating in part to the protein kinase C isotype repertoire (38), and the expression of a cyclin-dependent kinase inhibitor (p21) (39). For human peripheral blood eosinophils, rhIL-4 induces higher cellular levels of FcεRIα mRNA at 6 h.


