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Facilitation of apoptosis by cyclosporins A and H, but not FK506 in mouse bronchial eosinophils

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Abstract

This study was undertaken to clarify whether or not binding to cyclophilin is a prerequisite for cyclosporin A-induced modulation of apoptotic cell death in eosinophils. Eosinophils were isolated from bronchoalveolar lavage fluid of mice challenged with inhaled allergen after sensitization. Apoptosis was determined by analysing the DNA content. At 72 h, 99% of the cells had died without addition of cytokines, whereas 55–60% of the cells survived in the presence of 10 U/ml recombinant murine interleukin 5 or recombinant murine granulocyte macrophage-colony stimulation factor (GM-CSF). Apoptotic cell death at 72 h in the presence of 10 U/ml interleukin 5 was increased by addition of cyclosporin H, an analogue of cyclosporin A without cyclophilin binding activity, in a concentration-dependent (0.3 to 3 μM) manner. The increase in apoptosis elicited by cyclosporin A and cyclosporin H took place also in the presence of 10 U/ml GM-CSF but to a lesser extent. There was a significant augmentation of apoptosis in eosinophils cultured in the presence of each cytokine for 72 h or longer. Tacrolimus (FK506) failed to augment apoptotic cell death. Thus, it is unlikely that binding of cyclosporin A to cyclophilin accounts for the increased apoptosis induced by cyclosporin A and its analogue in eosinophils. The increase in apoptosis induced by cyclosporin A, but not FK506, in activated eosinophils from the airways may be attributed to the anti-asthmatic effects of cyclosporin A. © 1997 Elsevier Science B.V.

Keywords: Cyclosporin A; Cyclosporin H; FK506; Eosinophil; Apoptosis

1. Introduction

Programmed cell death without leakage of cellular contents into the extracellular space is a pathophysiologically important factor in regulating the number of airway granulocytes, which show a high turnover (Carson and Ribeiro, 1993; Simon and Blaser, 1995). The airway function of asthmatic patients is improved following glucocorticoid treatment, which is accompanied by a decrease in the number of airway eosinophils and an increase in apoptosis of eosinophils in the airways (Woolley et al., 1996). Thus, the apoptosis of eosinophils and their subsequent ingestion by macrophages may represent a mechanism whereby the longevity and removal of activated eosinophils is controlled (Stern et al., 1992).

Eosinophil survival is only achieved by the continued presence of growth factors (Her et al., 1991; Stern et al., 1992; Simon and Blaser, 1995). The life span of eosinophils is in the range of two to five days, but is prolonged when the cells are activated (Kumar and Busse, 1995). Interleukin 5, which is critical in the terminal differentiation of eosinophils into mature eosinophils (Yamaguchi et al., 1988; Clutterbuck et al., 1989), saves them from apoptotic death and consequently prolongs their life span at inflammatory sites (Her et al., 1991; Yamaguchi et al., 1991). Interleukin 5, interleukin 3 and granulocyte macrophage-colony stimulation factor (GM-CSF) prevent spontaneously occurring apoptosis in human eosinophils (Her et al., 1991; Yamaguchi et al., 1991) and murine lung eosinophils (Tsuyuki et al., 1995). It has been recently demonstrated that lung eosinophils express the Fas receptor which, upon binding of an anti-Fas monoclonal antibody, transmits a death signal to the eosinophils (Tsuyuki et al., 1995). The susceptibility of the Fas receptor in

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eosinophils derived from eosinophilic patients is more a matter of cell regulation than receptor expression (Hebestreit et al., 1996). It is not clear yet whether the Fas ligand/Fas interaction occurs physiologically and, in the case of asthma, whether a defect in this interaction is a trigger or consequence of eosinophil activation and migration into the airways.

Cyclosporin A and tacrolimus (FK506) exert a selective inhibitory effect on T-lymphocytes by binding with immunophilin, cyclophilin and FK506-binding protein to form heterodimeric complexes, resulting in the inhibition of calcineurin catalytic activity (Fruman et al., 1994). Airway eosinophilia is inhibited by cyclosporin A in rodents (Elwood et al., 1992; Norris et al., 1992) and lung function in patients with chronic severe asthma was improved by cyclosporin A in a randomized, double-blind, crossover trial (Alexander et al., 1992). In a previous study of eosinophils derived from bronchoalveolar lavage fluid of sensitized rats that had inhaled allergen (Kitagaki et al., 1996), cyclosporin A as well as a glucocorticoid was found to increase apoptosis, as determined by using either DNA content assay, DNA electrophoresis or morphological analysis. However, further study of this new aspect of cyclosporin A pharmacology is needed to determine the mechanism for the anti-eosinophilia. The first step of cyclosporin A action is assumed to involve immunophilin. Does binding of cyclosporin A to immunophilin modulate the cell death pathway?

The present study was carried out to clarify: (1) whether cyclosporin A and FK506, a potent T-cell inhibitor with immunosuppressant activity (Peters et al., 1993), increase apoptotic cell death in the presence of specific eosinophil survival factors such as recombinant murine interleukin 5 and recombinant murine GM-CSF in the mouse eosinophils and (2) whether binding to cyclophilin is a prerequisite for modulation of the apoptotic cell death in eosinophils. In the latter experiments cyclosporin A and cyclosporin H, an analogue of cyclosporin A substituted from L-methylvaline to D-methylvaline at position 11 without cyclophilin binding activity (Handschumacher et al., 1984; Von Wartburg and Traber, 1986) and FK506, which has FK506-binding protein binding capacity (Peters et al., 1993), were used.

2. Methods

2.1. Animals and materials

Male BALB/c mice weighing 21–25 g were purchased from Japan SLC (Japan). The animals were housed in plastic cages in an air-conditioned room at 24°C. Food and water were available ad libitum.

The drugs used were cyclosporin A (Sandoz Pharma, Switzerland), cyclosporin H (Sandoz) and tacrolimus (FK506). The drugs were diluted first in dimethyl sulfoxide (Wako, Japan) to a concentration of 10–30 mM and subsequently diluted in culture medium. The control group was treated with vehicle solvent containing the highest concentration of dimethyl sulfoxide used to prepare the drug solutions.

2.2. Induction of allergen-induced pulmonary eosinophilia

Pulmonary eosinophilia in mice was induced according to the method reported previously (Yamaguchi et al., 1994). Briefly, the mice were given subcutaneous injections containing 10 μg ovalbumin (grade V, Sigma, USA) mixed with alum (5 μg) and pertussis vaccine (10^9) (Wako) in a volume of 0.5 ml saline on day 0 and 13. The mice were exposed to an aerosol containing ovalbumin at a concentration of 10 mg/ml in saline generated by a nebulizer (type of nebulizer: Hospitak, USA) for 60 min on days 20, 22 and 24.

2.3. Separation of eosinophils from bronchoalveolar lavage fluid

Mice were killed with an intraperitoneal injection of 150 mg/kg pentobarbital sodium (Abbott Laboratories, USA) on day 27. Bronchoalveolar lavage fluid was obtained by rinsing the lungs three times with 1 ml of calcium and magnesium-free Hanks balanced salt solution (HBSS) containing 10 mM EDTA and 10 mM HEPES at 37°C. The recovered cells were re-suspended at 1×10^7 cells/ml in modified HBSS. The suspension was loaded on the top of two layers of different densities of Percoll (1.075 and 1.0825 g/ml, Pharmacia, Sweden) and then centrifuged for 20 min at $400 \times g$. The cells located between 1.075 and 1.0825 g/ml were isolated. After three washes, the cells were reacted with anti-mouse Thy1.2 (clone 30-H12, Miltenyi Biotec, USA) and anti-mouse CD45R (clone RA3-6B2, Miltenyi Biotec) with microbeads for 15 min at 4°C. Thy1.2- and CD45R-positive cells were removed with a magnetic particle concentrator (MACS, Miltenyi Biotec). The proportion of eosinophils was determined by cytocentrifugation and stained with Diff-Quick solution (Kokusai Shinyaku, Japan). The purity of the eosinophils was more than 96.5%. The viability of the eosinophils, assessed using trypan blue staining, was more than 98% just after cell separation.

2.4. Culture of eosinophils and drug treatment

The eosinophil suspension was adjusted to a concentration of 2×10^6 cells/ml in culture medium (RPMI-1640, Gibco, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES). Aliquots of 150 μl were placed into the wells of 24-well flat-bottomed plates (Corning, USA) containing 75 μl of recombinant murine interleukin 5 (Genzyme, USA) or recombinant murine GM-CSF (Genzyme) and 75 μl of each drug at the final

defined concentrations. The plates were maintained at 37°C in a 5% CO_2 atmosphere. Preliminary experiments to find the optimal anti-apoptotic effect with either cytokine indicated that more than 50% of cells survived in the presence of either cytokine (10 U/ml) for 72 h, while there was nearly 99% cell death in the absence of cytokine. Thus, drug effects were assessed in 72-h-cultures in the presence of 10 U/ml interleukin 5 or GM-CSF. To examine autocrine involvement, eosinophils were cultured in the presence of mouse anti-transforming growth factor (TGF)-β1, -β2 and -β3 (Genzyme) at the final concentration of 20 μg/ml; the antibody has been demonstrated to neutralize TGFβ in mice (Nunes et al., 1995).

2.5. Apoptosis assay

Flow cytometric analysis was performed to identify apoptotic cells according to a method described elsewhere (Nicoletti et al., 1991). Briefly, the $200 \times g$ centrifuged eosinophil pellets were gently re-suspended in 0.5 ml of hypotonic fluorochrom solution containing 100 μg/ml propidium iodide (Sigma) in 0.1% sodium citrate and 0.1% Triton X-100. The samples were incubated overnight in the dark at 4°C before propidium iodide fluorescence analysis of the individual nuclei using an EPICS-ELITE (Counter Corporation, USA) for 20 000 nongated events. Eosinophil apoptosis was expressed as the proportion of cells (% of total) displaying a 'hypodiploid' peak.

2.6. Assay of GM-CSF

GM-CSF in the supernatant of the cultured eosinophils stimulated by interleukin 5 was assessed by enzyme-linked immunosorbent assay (ELISA) (Endogen, USA). The lower limit of detection of the assay was 5 pg/ml murine GM-CSF, which is equivalent to 0.075 U/ml.

2.7. Statistical analysis

Data are given as means ± S.E.M. Statistical analysis of the differences among the groups was made with one-way

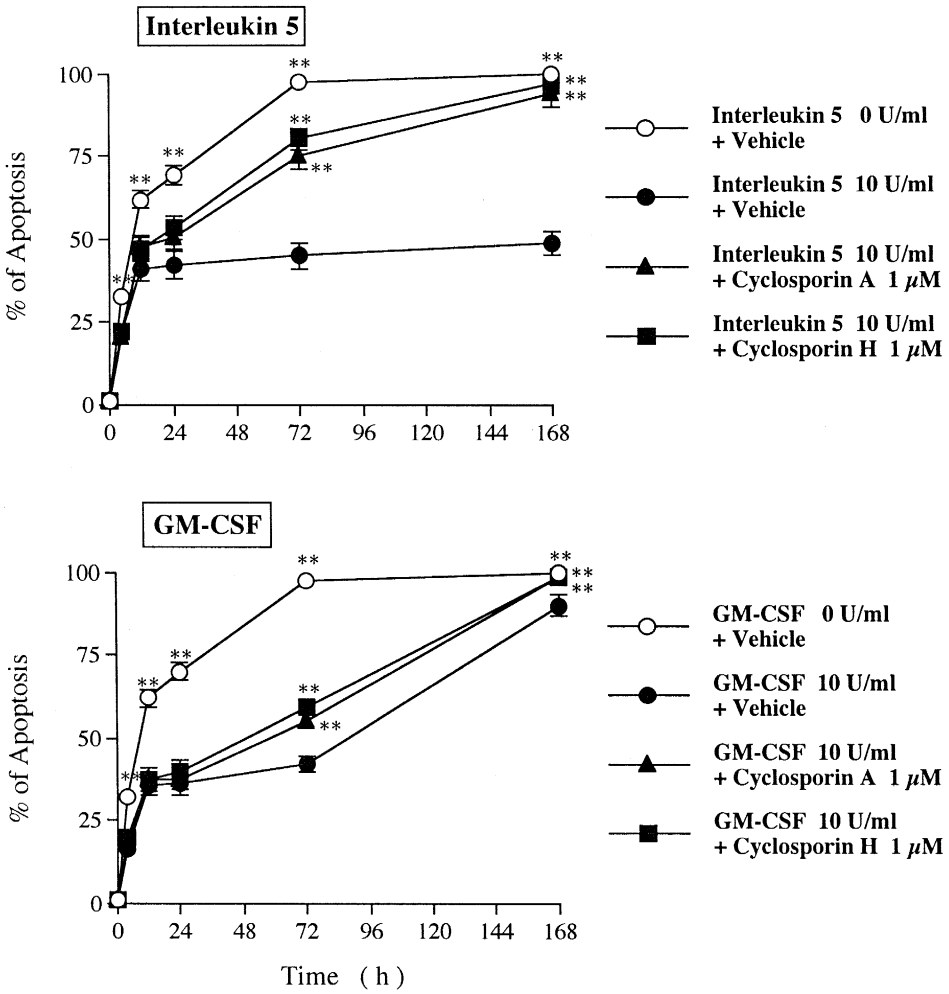

Fig. 1. Time-course of apoptotic cell death facilitated by cyclosporin A and cyclosporin H in eosinophils in the presence and absence of interleukin 5 or GM-CSF at 10 U/ml. Each point represents the mean + S.E.M. of 5 independent experiments. * Significantly different from values for the eosinophils treated with 10 U/ml cytokine plus vehicle, $P < 0.05$; ** $P < 0.01$.

analysis of variance followed by the Bonferroni multiple comparison test.

3. Results

3.1. Time-course of apoptotic cell death facilitated by cyclosporin A and cyclosporin H in the presence of 10 U / ml interleukin 5 or GM-CSF

Examination of eosinophils from bronchoalveolar lavage fluid of mice, challenged with antigen showed that cell death occurred spontaneously. Cell death was determined as being due to apoptosis by DNA florescence analysis. Interleukin 5 significantly inhibited apoptosis as early as 4 h, but the inhibition was more marked at 72 h (Fig. 1). In a similar fashion, GM-CSF attenuated cell death, the effect at 4 and 168 h being greater and smaller than that of interleukin 5, respectively (% of surviving eosinophils after interleukin 5 and GM-CSF: 4 h, 78.4 ± 1.87 and 83.3 ± 0.91, $P < 0.05$; 168 h, 50.9 ± 3.17 and 10.6 ± 3.08, $P < 0.01$).

In order to define the kinetics of the enhanced eosinophil apoptosis elicited by drugs, apoptosis was studied for 168 h, using a single concentration of drugs (1 μM). At 4, 12 and 24 h in culture, there were no significant changes in the cell death rate between control and drug-treated groups (Fig. 1). Cyclosporin A or cyclosporin H significantly increased apoptotic cell death at 72 h in culture compared with the prolonged survival seen with 10 U/ml of either cytokine alone. There was no difference in the kinetics of the increase in apoptosis elicited by cyclosporin A and cyclosporin H. The increase in cell death was more prominent in cultures incubated in the presence of 10 U/ml interleukin 5 than 10 U/ml GM-CSF.

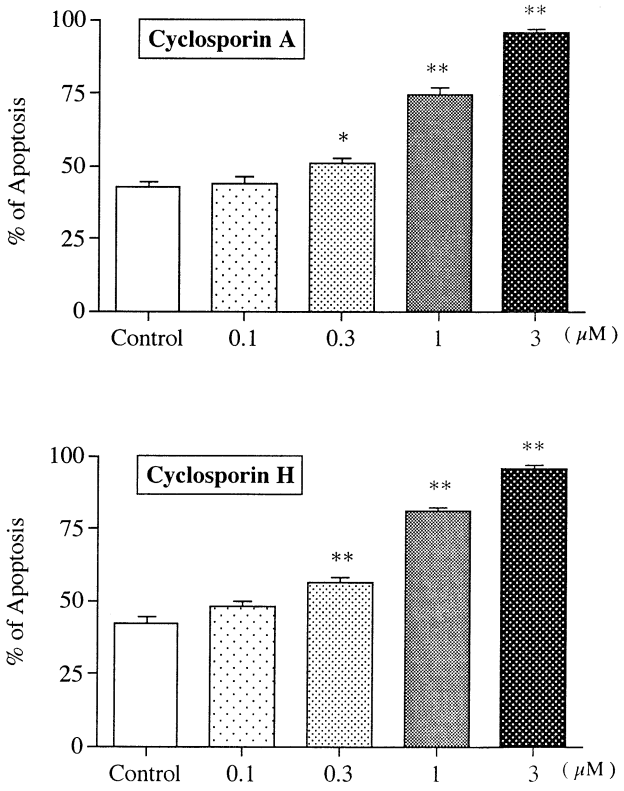

Fig. 2. Effects of cyclosporin A and cyclosporin H on apoptosis of eosinophils in the presence of interleukin 5. The eosinophils were cultured in the presence of 10 U/ml interleukin 5 for 72 h. Dimethylsulfoxide, used for drug dilution, was put into culture medium as control; see Section 2. Each point represents the mean + S.E.M. of 5 independent experiments. * Significantly different from control values, $P < 0.05$; ** $P < 0.01$.

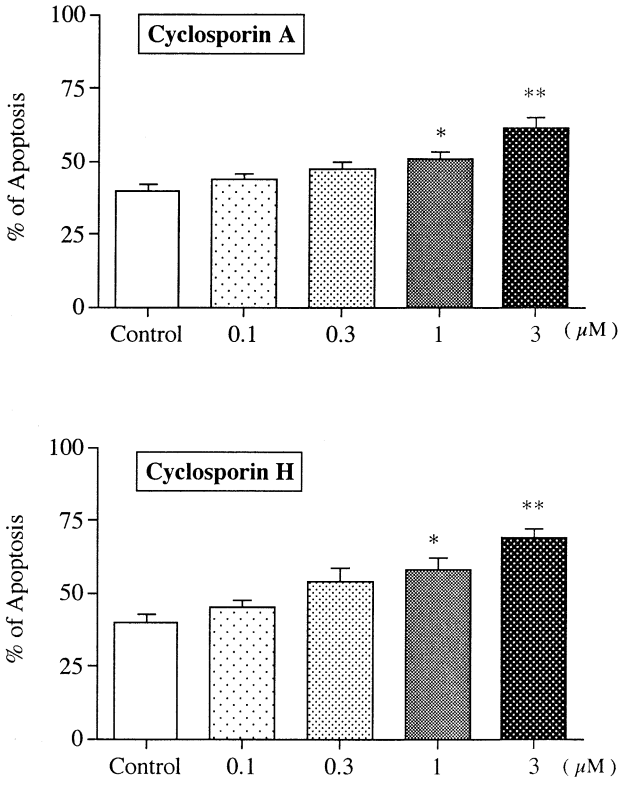

Fig. 3. Effects of cyclosporin A and cyclosporin H on apoptosis of eosinophils in the presence of GM-CSF. The eosinophils were cultured in the presence of 10 U/ml GM-CSF for 72 h. Each point represents the mean + S.E.M. of 4 independent experiments for cyclosporin A and 3 for cyclosporin H. * Significantly different from control values, $P < 0.05$; ** $P < 0.01$.

3.2. Effects of cyclosporin A, cyclosporin H and FK506 on eosinophil apoptosis in the presence of 10 U / ml interleukin 5 or GM-CSF in 72-h-cultures

Cyclosporin H as well as cyclosporin A at 0.3 μM or higher significantly facilitated apoptotic cell death in eosinophils as compared to the interleukin 5-induced prolongation of the cell survival (Fig. 2). When cells were incubated with GM-CSF, cyclosporin H or cyclosporin A still increased apoptotic cell death but the drug concentration required for a significant effect was higher (1 μM)

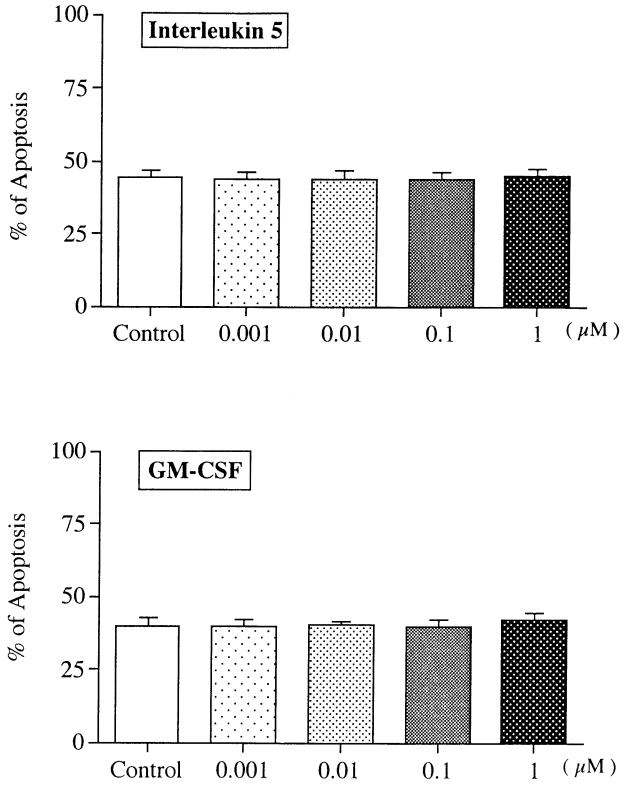

Fig. 4. Effect of FK506 on apoptosis of eosinophils in the presence of interleukin 5 or GM-CSF. The eosinophils were cultured in the presence of 10 U/ml interleukin 5 or GM-CSF for 72 h. Each point represents the mean + S.E.M. of 4 independent experiments for interleukin 5 and 3 for GM-CSF.

than that with interleukin 5 (Fig. 3). FK506 failed to augment apoptotic cell death in the presence of either interleukin 5 or GM-CSF (Fig. 4).

3.3. Effects of cyclosporin A on autocrine release of cytokines

This study was carried out to examine whether cyclosporin A modulates the autocrine release of eosinophil survival factor(s) and apoptosis-inducing factor(s). First, GM-CSF was not detected in significant amounts in the supernatant of cultured eosinophils 24 h after interleukin 5 was added to the medium. Second, in a preliminary experiment, anti-TGFβ did not reverse the increase in apoptosis elicited by 1 μM cyclosporin A in eosinophils cultured with 10 U/ml interleukin 5: apoptosis was increased by $67.7 \pm 3.8\%$ with cyclosporin A and $68.0 \pm 4.9\%$ with cyclosporin A plus anti-TGFβ ($n = 3$, no significant difference between them).

4. Discussion

Cyclosporin H, which has little binding affinity for cyclophilin, was effective in facilitating apoptotic cell death in a similar concentration range to cyclosporin A, which has high binding affinity for cyclophilin (Handschumacher et al., 1984). Cyclophilin appears to be abundantly expressed in all tissues, and the protein is assumed to be the major cytosolic receptor for cyclosporin A and to be responsible for the accumulation of cyclosporin A within cells (Sigal et al., 1991). If this is the case for eosinophils, the binding of cyclophilin may not be critical for apoptotic cell death. Consequently, it seems that calcineurin catalytic activity and the translocation in nuclear factor in activated T-cells, which are inhibited by cyclosporin A in T-lymphocytes (Flanagan et al., 1991), may not be involved directly in apoptotic processes in eosinophils. Cyclosporin H does not inhibit the rise in intracellular Ca^{2+} concentration induced by various stimuli such as complement C5a, leukotriene B_4 and NaF (Wenzel-Seifert and Seifert, 1993). These findings imply that cyclosporin H and cyclosporin A facilitate apoptotic cell death through a mechanism which does not involve the pathway of cyclophilin/calcineurin pathway in eosinophils. It remains to be determined to what extent the mechanism proposed for the inhibition of T-cell activation is applicable to the action of the cyclosporin A and its analogues on eosinophil apoptosis.

It was interesting that cyclosporin A and its analogue, but not FK506, facilitated apoptotic cell death in eosinophils derived from bronchoalveolar lavage fluid. FK506 has immunosuppressing activity in vitro that is approximately 100 times higher than that of cyclosporin A (Peters et al., 1993) and hence an insufficient concentration of FK506 would be unlikely to account for lack of apoptosis-facilitating activity. It could be that the binding of FK506 to FK506-binding protein, an immunophilin for FK506, changes the apoptotic program in the eosinophil. As discussed before for cyclosporin H and cyclophilin binding, the binding of FK506 to FK506-binding protein does not seem to play a primary role in apoptotic cell death in eosinophils. It has been reported that cyclosporin A inhibits T-cell receptor-induced apoptosis in T-cell hybridomas and thymocytes (Shi et al., 1989). By contrast, FK506 and cyclosporin A inhibit the proliferation of activated B-cells (Wicker et al., 1990) and induce programmed cell death in murine B-cell lines by interrupting signaling events needed to maintain the negative regulation of cell death (Gottschalk et al., 1994). Thus, the mechanism of the immunosuppressant modulation of cell death depends on the cell type and/or its stage of activation, thus leaving unanswered the question of what other factors underlie the different action of the two immunosuppressants on eosinophils.

Apoptotic cell death, determined with flow cytometric analysis, was found to be approximately 68% in mouse eosinophils cultured without survival factor(s), whereas it is 58% in rat eosinophils in 24 h culture (Kitagaki et al., 1996). Apoptosis of mouse eosinophils was increased by cyclosporin A in the presence of a single growth factor such as interleukin 5 or GM-CSF. These findings are

consistent with those for rat eosinophils in which cyclosporin A in concentrations from 0.1 to 1.0 μM augments apoptosis in the presence and absence of survival factors (Kitagaki et al., 1996). The activity of cyclosporin A against GM-CSF and interleukin 5 was different in the present study. The effective dose for prolongation of eosinophil survival by GM-CSF is ten times higher than that for interleukin 5 (Ohnishi et al., 1993). It is, thus, conceivable that if apoptotic cell death depends on the balance between survival factors and the apoptotic-driving force, the greater anti-apoptotic activity of GM-CSF than interleukin 5 at the same concentration accounts for the smaller apoptotic effect of cyclosporin A against GM-CSF. Alternatively, the post-receptor mechanism for interleukin 5 may be more susceptible to the modulating effect of cyclosporin A than is the mechanism for GM-CSF. It remains to be determined whether cyclosporin A modulates post-receptor activation. Cyclosporin A did not augment apoptosis in eosinophils cultured in the presence of cytokines at times earlier than 24 h. Wallen et al. (1991) reported that it took more than 2 days for glucocorticoids to induce apoptosis in eosinophils, although rat eosinophils cultured in the absence of survival factors underwent apoptotic cell death as early as 12 h (Kitagaki et al., 1996). Therefore, a disturbed balance between survival factors and cell death-driving forces may determine the timing of enhanced apoptosis. However, it should be remembered that we measured eosinophil apoptosis by using the DNA content assay, which reflects the final but not early processes of apoptosis with DNA condensation and fragmentation. Early signs of cell death might reveal the effect of cyclosporin A earlier.

It is conceivable that facilitation of apoptotic death in eosinophils can be attributed to the anti-asthmatic properties of cyclosporin A and its analogues, as evidenced by the inhibitory effects of cyclosporin A in animals (Elwood et al., 1992; Norris et al., 1992) and in asthma patients (Alexander et al., 1992). Simon and Blaser (1995) have recently proposed that inhibition of programmed cell death of eosinophils is a key pathogenic event for eosinophilia in allergic diseases. One of the pathways suggested for eosinophil apoptosis is mediated through Fas (Tsuyuki et al., 1995; Hebestreit et al., 1996). It does not seem that activated eosinophils release soluble Fas ligand, and cyclosporin A-induced apoptosis in eosinophils is mediated by the interaction of Fas and Fas ligand in our culture system. Cyclosporin A inhibits apoptosis in T-cell hybridomas and thymocytes by inhibition of soluble Fas ligand release (Dhein et al., 1995). Murine B-cell lines are susceptible to anti-immunoglobulin-mediated apoptosis independently of induced growth arrest and the programmed cell death induced by cyclosporin A is suggested to be mediated via bcl-xl but not by bcl-2 (Gottschalk et al., 1994). It remains to be determined which genes are affected by cyclosporin A and its analogues in facilitating apoptosis.

Hematopoietic cytokines like interleukin 5 and GM-CSF increase the life span of eosinophils by inhibiting apoptosis and are upregulated in disease associated with blood and tissue eosinophilia (Walker et al., 1991; Yamaguchi et al., 1991; Her et al., 1991). The present findings are compatible with those of studies of in murine lung eosinophils incubated with cytokines (Tsuyuki et al., 1995), in which overexpression of certain cytokines inhibited apoptotic cell death (Simon and Blaser, 1995). Lyn and syk, intracellular tyrosine kinases, are required for the activation of anti-apoptotic pathways by the β subunit of interleukin 5 and GM-CSF receptors in human eosinophils (Yousefi et al., 1996). Although cyclosporin A could not be examined in mice eosinophils without addition of the cytokines because of the shorter life span of mice eosinophils as compared with rat eosinophils, the previous study with rats (Kitagaki et al., 1996) demonstrated that cyclosporin A facilitates spontaneously occurring apoptosis in eosinophils cultured without additional cytokines. Therefore, it is unlikely that cyclosporin A prevents activation of lyn and syk induced by ligation of the common β chain of interleukin 5 and GM-CSF receptors. Taken together with the autocrine synthesis of interleukin 5 and GM-CSF (Alam et al., 1994), cyclosporin A might inhibit the production of cytokines. However, in our system, GM-CSF was not detected by ELISA in the supernatant of cultured eosinophils stimulated by interleukin 5. Although the preliminary study showed concentration-dependent prolongation of eosinophil survival, 0.075 U/ml of GM-CSF, the lowest amount detectable, was not enough to prolong the survival. Alternatively, cyclosporin A might accelerate cell death indirectly by increasing the expression of transforming growth factor (TGF) β1, the induction of which (Prashar et al., 1995) leads to apoptosis in eosinophils (Alam et al., 1994). However, anti-TGFβ did not reverse the increase in apoptosis elicited by cyclosporin A in eosinophils treated with interleukin 5 for 72 h. Taken together, it does not seem that the autocrine release of hematopoietic factors is actively involved in the augmentation of apoptosis by cyclosporin A. However, the autocrine involvement of other factors for survival or apoptosis, which were not determined, can not be ruled out.

In conclusion, the facilitation of apoptotic cell death by cyclosporin A but not FK506 in activated eosinophils in the airways may be attributed to the anti-asthmatic properties of cyclosporin A and its analogues. It is unlikely that the binding of cyclosporin A to cyclophilin accounts for the facilitated apoptosis produced by cyclosporin A and its analogue in eosinophils whose survival has been prolonged by interleukin 5 or GM-CSF.

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