

Human Platelets Produce Granulocyte-Macrophage Colony-Stimulating Factor and Delay Eosinophil Apoptosis

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SUMMARY: An association between eosinophils and platelets has been described in several diseases, most notably asthma. Although the mechanisms through which platelets influence eosinophil behavior are not well defined, platelets seem to contribute to the selective accumulation of eosinophils at sites of allergic inflammation by virtue of their ability to produce eosinophil chemotactic factors. We report here for the first time that platelets delay apoptosis, thus enhancing eosinophil survival. A marked inhibition of spontaneous apoptosis was observed using eosinophil:platelet ratios of 1:50, 1:25, 1:10, and 1:5. Moreover, promotion of eosinophil apoptosis by either pronase or dexamethasone was also inhibited greatly in the presence of platelets. The antiapoptotic effect mediated by platelets was dependent on the release of soluble products and was significantly inhibited by neutralizing antibodies directed to GM-CSF. Studies performed by flow cytometry, directed to analyze the cellular source of this cytokine, demonstrated that intracytoplasmic GM-CSF is present in resting platelets. Moreover, GM-CSF was found in platelet supernatants, at concentrations able to prevent eosinophil apoptosis. Our findings support a novel mechanism through which platelets may contribute to eosinophil accumulation at allergic inflammatory sites. (*Lab Invest* 2003, 83:589–598).

Eosinophils play a critical role in the pathogenesis of asthma and allergic diseases. During the course of allergic inflammation, eosinophils are selectively recruited in the skin or mucosa, and upon activation, they release potent inflammatory mediators, including toxic cationic proteins, oxygen metabolites, proteolytic enzymes, and several cytokines, which contribute to the initiation and maintenance of the allergic inflammatory response (Costa et al, 1997; Dombrowicz and Capron, 2001; Gleich, 2000). Resolution of this response is dependent, at least in part, on the rate of eosinophil apoptosis, which seems to be a crucial factor in determining the steady-state number of eosinophils at the site of inflammation (Simon and Blazer, 1995; Walsh, 2000). The uptake of apoptotic eosinophils is mediated by both professional and nonprofessional phagocytes, such as smooth muscle cells, lung fibroblasts, and airway epithelial cells (Haslett, 1999; Platt et al, 1998; Walsh, 2000; Walsh et al, 1999). It is interesting that this process not only prevents spillage of the eosinophil's histotoxic con-

tents but also induces the release of anti-inflammatory cytokines by macrophages, such as IL-10, transforming growth factor β (TGF- β), and prostaglandin E₂ (Haslett, 1999; Perssen and Erjefalt, 1997; Platt et al, 1998; Stern et al, 1996; Walsh, 2000; Walsh et al, 1999).

Signals from the microenvironment can delay or accelerate apoptosis of eosinophils. Cytokines such as IL-3, IL-5, IL-13, and GM-CSF enhance eosinophil survival, thus promoting the accumulation and persistence of eosinophils at sites of inflammation (Lopez et al, 1986; Luttmann et al, 1996; Rothenberg et al, 1988; Yamaguchi et al, 1991). By contrast, IL-4, TGF- β , IL-12, and glucocorticoids, as well as ligation of the type II integral membrane signaling receptor CD69 or the tyrosine phosphatase-linked panleukocyte receptor CD45, accelerate the spontaneous rate of eosinophil apoptosis (Alam et al, 1994; Blaylock et al, 1999; Nutku et al, 2001; Wallen et al, 1991; Walsh et al, 1996; Weidi et al, 1998).

Several lines of evidence suggest that the behavior of eosinophils in allergic diseases is influenced by platelets. From guinea pig models, it was suggested that platelets are a prerequisite component in allergic asthma, because platelet depletion reduced eosinophil infiltration into the lung after allergen exposure to sensitized animals (Lellouch-Tubiana et al, 1988). Consistent with these results are observations made in allergic rabbits that indicated that animals rendered thrombocytopenic showed a marked reduction in the number of eosinophils in the bronchoalveolar lavage fluid, as well as in the magnitude of the late asthmatic

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response and airway hyperresponsiveness (Coyle et al, 1990). Moreover, studies performed in allergic subjects suggested not only that platelet-dependent events determine exacerbation of asthma but also that platelets recovered from the bronchoalveolar lavage fluid of patients with asthma are often associated with eosinophils and also show evidence of degranulation (Gleich, 1990; Knauer et al, 1981; Morley et al, 1985; Page, 1988; Page and Coyle, 1989; Yasuba et al, 1991). The mechanisms through which platelets influence eosinophil behavior are not well defined. However, there are known mediators released by platelets, such as RANTES, ATP, PAF, and platelet factor 4, which induce chemotaxis of eosinophils (Burgers et al, 1993; Chihara and Nakajima, 1989; Kamayoshi et al, 1992; Wardlaw et al, 1986). They may contribute to the selective accumulation of eosinophils at sites of allergic inflammation.

No previous reports have examined the ability of platelets to modulate the rate of eosinophil apoptosis. We report here for the first time that platelets release GM-CSF enhancing eosinophil survival. Our findings support the notion that platelets may play a role not only in promoting selective eosinophil infiltration after antigen challenge but also in perpetuating the allergic inflammatory response by virtue of their ability to improve eosinophil survival.

Results

Platelets Delay Spontaneous Apoptosis, Enhancing Eosinophil Survival

For analyzing whether platelets were able to modulate eosinophil apoptosis, cells were cultured at 37° C for 48 hours with unstimulated platelets, at eosinophil:platelet ratios of 1:25, 1:10, and 1:5, and apoptosis was revealed by fluorescence microscopy. Figure 1 shows that platelets markedly decreased eosinophil apoptosis. We next examined whether delayed apoptosis resulted in the enhancement of eosinophil survival. To this aim, eosinophils (1×10^6 /ml) were cultured for 96 hours at 37° C in the absence

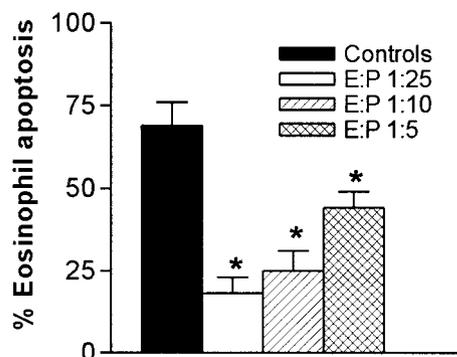


Figure 1.

Platelets delay spontaneous apoptosis of eosinophils. Eosinophils (1×10^6 /ml) were cultured at 37° C for 48 hours in the absence (controls) or presence of washed platelets (eosinophil:platelet ratios 1:25, 1:10, and 1:5). Then, the percentages of apoptotic cells were determined by fluorescent microscopy. Results are expressed as the mean \pm SEM of eight experiments; * $p < 0.01$, platelets versus controls.

(controls) or presence of unstimulated platelets (eosinophil:platelet ratio 1:50), and the percentages of eosinophil viability were determined by fluorescence microscopy as described in "Materials and Methods." It was found that platelets dramatically increased eosinophil survival (survival of eosinophils = 9 ± 6 versus $62 \pm 12\%$, control versus platelet-treated; $n = 11$, $p < 0.001$).

All of these experiments were carried out using washed platelets. To rule out the possibility that washing procedures used for platelet purification might influence the ability of platelets to prevent eosinophil apoptosis, we performed another set of experiments using platelets purified by gel filtration. It was found that gel-filtered platelets (GFP), used at an eosinophil:platelet ratio of 1:50, prevented apoptosis in a comparable way as washed platelets did (apoptosis of eosinophils at 48 hours = 74 ± 9 versus $23 \pm 5\%$, control versus GFP-treated; $n = 5$, $p < 0.001$).

During the course of apoptosis, phosphatidylserine (PS), a negatively charged phospholipid, becomes exposed at the cell surface (Homburg et al, 1995). Eosinophil apoptosis was then analyzed by flow cytometry using FITC-labeled annexin-V, which specifically binds to PS, in combination with propidium iodide staining. Figure 2A shows that the presence of platelets resulted in a marked reduction in the percentage of annexin-V-positive cells. Apoptosis was also examined by propidium iodide staining and flow cytometry. In agreement with the observations described above, we found that the proportion of eosinophils that display a hypodiploid DNA peak (ie, apoptotic cells) was markedly lower for eosinophils cultured in the presence of platelets than in control cells (Fig. 2B).

Although the above experiments were performed using unstimulated platelets, the isolation and culture of platelets was shown to be associated with the induction of a significant degree of activation as measured by the expression of P-selectin, a granule protein that becomes localized at platelet surface after activation (McEver, 1994). In fact, we found that after 30 minutes of incubation at 37° C in culture medium supplemented with 1% human serum albumin (HSA; Sigma, St. Louis, Missouri), $87 \pm 16\%$ of platelets showed moderate levels of P-selectin expression (mean fluorescence intensity = 36 ± 14 , $n = 4$) which, as expected, were significantly lower ($p < 0.01$) than those showed by platelet stimulated during 5 minutes at 37° C without stirring by thrombin (1 U/ml) or collagen (0.20 mg/ml; mean fluorescence intensity = 169 ± 23 and 96 ± 17 , respectively, $n = 3$). We then performed additional experiments to determine whether platelet-activating agents, such as thrombin and collagen, might improve the ability of platelets to delay eosinophil apoptosis. In preliminary studies, we observed that thrombin increased the rate of eosinophil apoptosis by acting directly on eosinophils themselves (not shown). Therefore, we carried out subsequent experiments using collagen, which did not induce any direct effect on eosinophils. Cultures were performed at different eosinophil:platelet ratios, in the

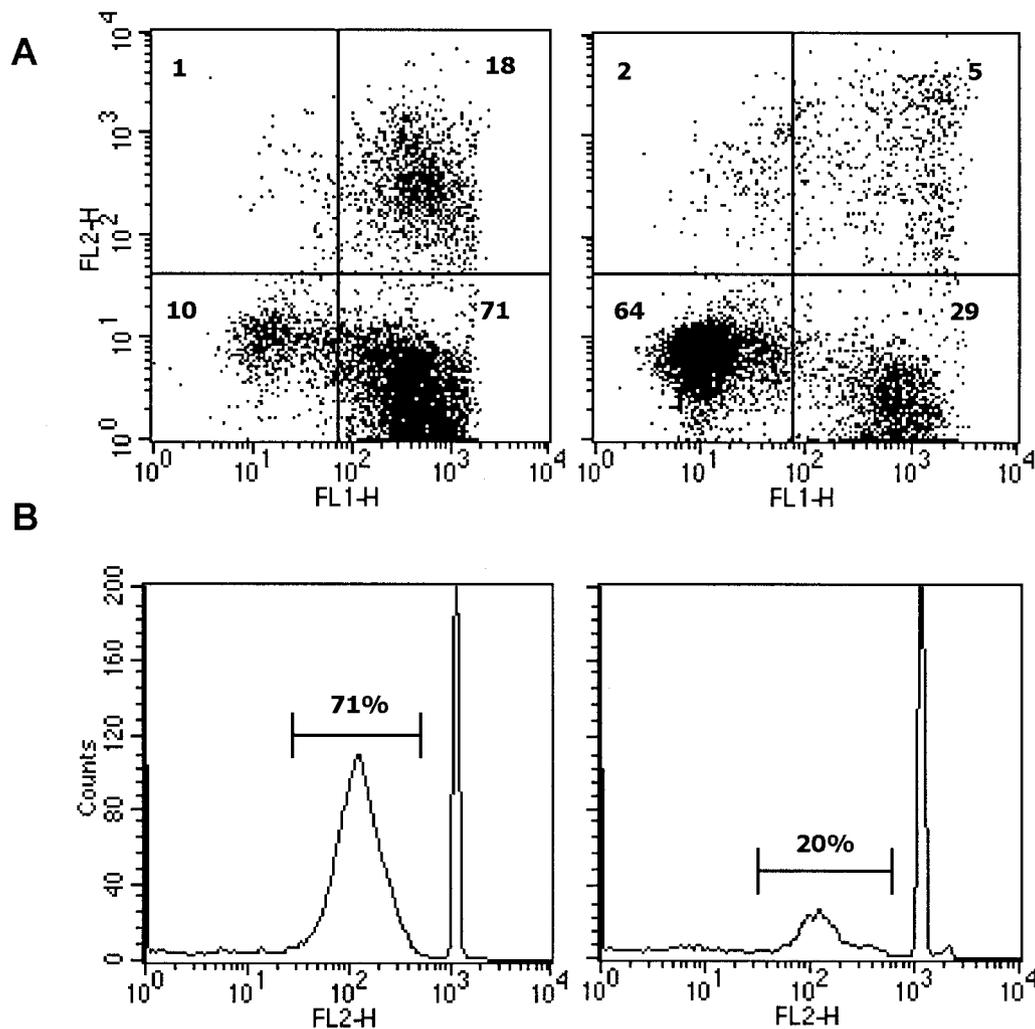


Figure 2.

Inhibition of eosinophil apoptosis mediated by platelets measured by annexin-V binding assay or evaluation of propidium iodide fluorescence of individual nuclei. Eosinophils ($1 \times 10^6/\text{ml}$) were cultured for 48 hours at 37°C in the absence (left) or presence (right) of platelets (eosinophil:platelet ratio 1:50). A, Cells were labeled with annexin-V FITC (FL1-H) and propidium iodide (FL2-H) for 20 minutes at 4°C , and the percentages of apoptotic cells were analyzed by two-color flow cytometry. B, Apoptosis was analyzed by propidium iodide staining of individual nuclei, as described in "Material and Methods." A representative experiment is shown ($n = 3$).

absence or presence of collagen (0.20 mg/ml), and apoptosis was revealed at 48 hours by fluorescence microscopy. Results in Figure 3 showed that collagen did not improve the ability of platelets to delay eosinophil apoptosis.

Platelets Inhibit Promotion of Eosinophil Apoptosis Induced by Pronase and Dexamethasone

Having shown that platelets delayed the spontaneous rate of eosinophil apoptosis, we then analyzed whether platelets were also able to inhibit the action of apoptosis-promoting agents. We have previously reported that exposure to proteolytic enzymes such as pronase markedly enhances human neutrophil apoptosis (Trevani et al, 1996). Because preliminary experiments showed that this phenomenon was also observed in human eosinophils, we then investigated whether platelets prevented stimulation of eosinophil apoptosis triggered by pronase. We also

investigated whether platelets were able to prevent stimulation of eosinophil apoptosis by dexamethasone (Wallen et al, 1991). These experiments are described in Figure 4. We found that platelets efficiently inhibited promotion of apoptosis induced by either pronase or dexamethasone.

Inhibition of Eosinophil Apoptosis Is Dependent on the Release of Antiapoptotic Factor(s) by Platelets

In an attempt to determine whether the ability of platelets to increase eosinophil survival was dependent on the release of soluble factors, we carried out incubations using semipermeable cell culture inserts. As described in "Materials and Methods," eosinophils were placed into the upper compartment, while culture medium (controls) or platelets (eosinophil:platelet ratio = 1:50) were added to the lower compartment. Both compartments were separated by a TransWell insert, which allows the passage of macromolecules

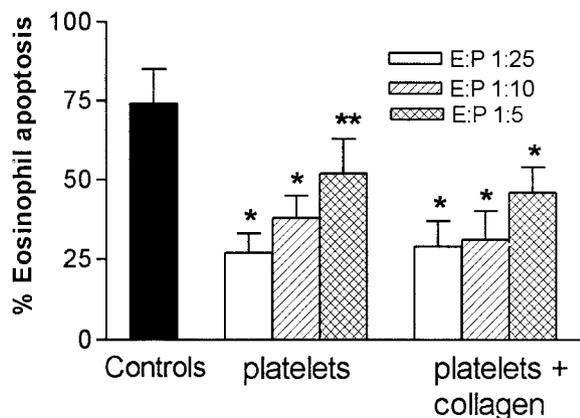


Figure 3. Collagen does not improve the ability of platelets to delay eosinophil apoptosis. Eosinophils (1×10^6 /ml) were cultured at 37° C for 48 hours with or without collagen (0.20 mg/ml), at different eosinophil:platelet ratios. Then, the percentages of apoptotic cells were determined by fluorescent microscopy. Results are expressed as the mean \pm SEM of five experiments. Eosinophils cultured with collagen in the absence of platelets show similar levels of apoptosis than control cells: 70 ± 9 ($n = 5$); * $p < 0.01$, ** $p < 0.05$, platelets versus controls.

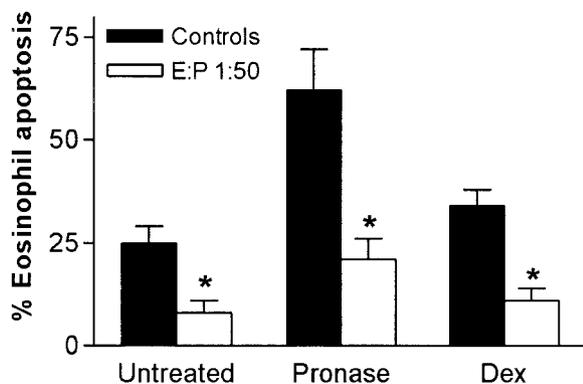


Figure 4. Platelets prevent promotion of eosinophil apoptosis induced by pronase and dexamethasone. Untreated or pronase-treated eosinophils (cell treated with 100 μ g/ml of pronase for 30 minutes at 37° C in saline, washed four times and resuspended in pronase-free medium supplemented with 1% HSA, at a concentration of 1×10^6 /ml, were cultured at 37° C for 24 hours in the absence (■) or presence of platelets (eosinophil:platelet ratio 1:50; □). Dexamethasone (1 μ M) was added at the beginning of the cell culture. Percentages of apoptotic cells were determined by fluorescent microscopy. In the absence of platelets both, pronase and dexamethasone significantly ($p < 0.05$) increased the percentages of eosinophil apoptosis. Results are expressed as the mean \pm SEM of six experiments; * $p < 0.01$, platelets versus controls.

but prevents cell to cell contact. Apoptosis was evaluated after 48 hours of culture by fluorescence microscopy. The results showed that, under these conditions, platelets were able to prevent eosinophil apoptosis in a comparable manner to that observed in mixed suspensions of eosinophils and platelets (see Fig. 1): % apoptosis = 78 ± 11 versus 26 ± 8 , untreated versus platelet-treated eosinophils, respectively (mean \pm SEM, $n = 4$, $p < 0.01$). This result indicates that direct contact between eosinophils and platelets is not required to prevent apoptosis. Supporting this observation, we also found that superna-

tants from unstimulated platelets were able to delay eosinophil apoptosis. In fact, when eosinophils (1×10^6 /ml) were incubated at 37° C for 48 hours with supernatants (20% vol/vol) harvested from platelet suspensions (5×10^8 /ml cultured alone for 18 hours at 37° C in medium supplemented with 1% HSA), a marked inhibition of eosinophil apoptosis was observed: % apoptosis = 74 ± 8 versus 27 ± 11 , untreated versus platelet supernatant-treated eosinophils, respectively (mean \pm SEM, $n = 7$, $p < 0.001$). It is interesting that results from Figure 5 indicate that these supernatants were also able to induce the activation of freshly isolated eosinophils, as shown by the induction of Ca^{2+} transient responses, suggesting that the inhibition of eosinophil apoptosis is associated with cellular activation.

Role of GM-CSF in the Delayed Eosinophil Apoptosis Mediated by Platelets

Upon activation, platelets release a wide range of inflammatory mediators, including different cytokines such as platelet-derived growth factor, TGF- β 1, and IL-1 (Deuel et al, 1982; Grainger et al, 1995; Hawrylowicz et al, 1989). Moreover, several members of the chemokine family, including platelet factor 4 (PF4), β -thromboglobulin (β -TG), ENA78, RANTES, and IL-8, are produced by platelets (Burgers et al, 1993; Chihara and Nakajima, 1989; Kameyoshi et al, 1992; Shao-Bo et al, 1996). The most important survival cytokines for eosinophils are IL-3, IL-5, and GM-CSF (Lopez et al, 1986; Rothenberg et al, 1988; Yamaguchi et al, 1991). Although no previous reports have examined whether platelets are able to produce these cytokines, it has

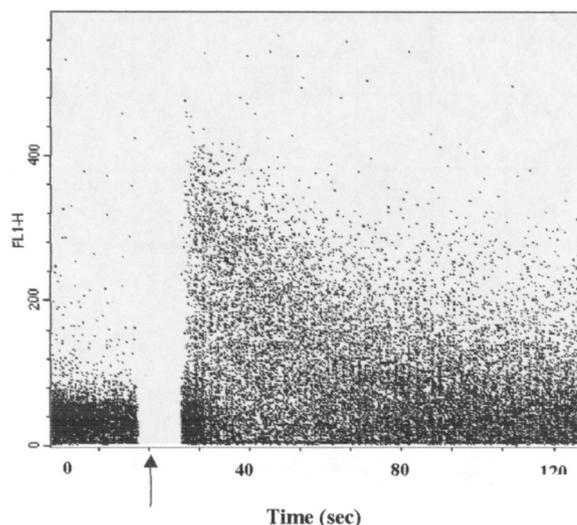


Figure 5. Platelet supernatants trigger rises of intracellular Ca^{2+} concentrations in eosinophils. Platelets (5×10^8 /ml in medium supplemented with 1% HSA) were cultured for 18 hours at 37° C, and platelet supernatants were then harvested. Eosinophils were loaded with fluo-3/AM as described in "Materials and Methods." The basal levels of fluo-3 fluorescence were recorded for approximately 15 seconds. Then, platelet supernatant (20% vol/vol) was added and fluorescence was recorded for 100 seconds. The increase in $[Ca^{2+}]_i$ was recognized as an increase in fluo-3 fluorescence. Results from a representative experiment ($n = 5$) are depicted.

been shown that megakaryocytes release both IL-3 and GM-CSF (Jian et al, 1994; Nimer and Uchida, 1995; Nimer et al, 1996; Wickenhauser et al, 1995). Thus, we speculated that inhibition of eosinophil apoptosis might involve the release of IL-3 and/or GM-CSF by platelets, and to test this hypothesis, we first analyzed whether blocking antibodies directed to IL-3 or GM-CSF were able to prevent the antiapoptotic effect mediated by platelets. Preliminary experiments were performed to determine the concentrations of anti-GM-CSF and anti-IL-3 antibodies needed to prevent inhibition of eosinophil apoptosis mediated by 5 to 20 ng/ml GM-CSF and IL-3, respectively. At concentrations of 2 $\mu\text{g/ml}$, both antibodies almost completely block the ability of these cytokines to delay eosinophil apoptosis (not shown). Taking this into account, supernatants harvested from unstimulated platelets cultured for 18 hours at 37° C were treated with 2 and 10 $\mu\text{g/ml}$ neutralizing antibodies directed to GM-CSF or IL-3, and after 10 minutes at 37° C, these supernatants were added to eosinophils, and apoptosis was revealed after 48 hours of culture by fluorescence microscopy. The results showed that anti-GM-CSF but not anti-IL-3 antibodies significantly prevented inhibition of eosinophil apoptosis by platelet supernatants (Fig. 6), supporting a role for GM-CSF in this phenomenon. It should be noted, however, that blocking antibodies directed to GM-CSF, even used at concentrations higher than that shown in Figure 6, did not abolish the antiapoptotic effect of supernatants (data not shown), suggesting that other mechanisms not related to the presence of GM-CSF might also be involved.

Because no previous studies have analyzed the ability of platelets to release GM-CSF, we performed another set of experiments to test whether platelets may be the cellular source of GM-CSF. Flow cytometric assays revealed the presence of intracellular GM-CSF in resting platelets (Fig. 7). Moreover, quantifica-

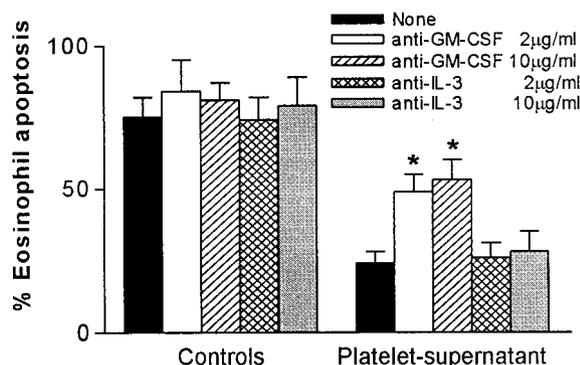


Figure 6.

Neutralizing antibodies to GM-CSF partially inhibit the delay of eosinophil apoptosis mediated by platelet supernatants. Neutralizing antibodies directed to GM-CSF or IL-3 were added to supernatants, obtained from platelets ($5 \times 10^8/\text{ml}$) cultured for 18 hours at 37° C. After 10 minutes at 37° C, these supernatants were added to eosinophils ($1 \times 10^6/\text{ml}$, 20% vol/vol), and apoptosis was revealed by fluorescent microscopy after 48 hours of culture at 37° C. Results are expressed as the mean \pm SEM of four experiments; * $p < 0.01$ versus eosinophils cultured with platelet supernatant in the absence of anti-GM-CSF antibodies.

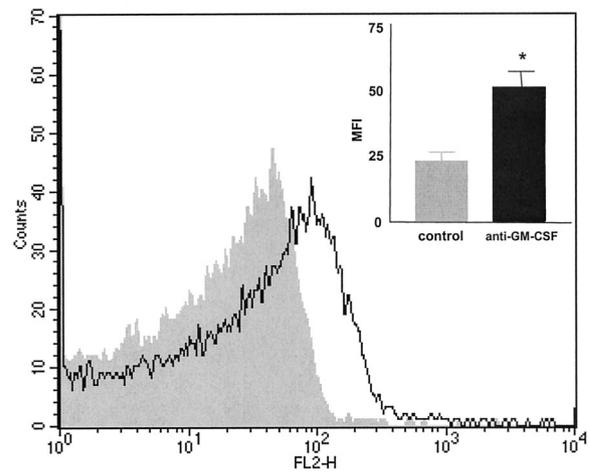


Figure 7.

Flow cytometric analysis of intracellular GM-CSF in platelets. Washed platelets (1×10^7) were fixed and permeabilized and subsequently stained with 0.25 mg of PE-rat anti-human GM-CSF mAb (PE-BVD2-21C11 Ab). To demonstrate specificity of staining, the binding of the PE-rat anti-human GM-CSF was blocked by preincubation of the fixed/permeabilized platelets with 2.5 mg of unlabeled rat anti-human GM-CSF mAb (unlabeled-BVD2-21C11 Ab) before staining (control, gray area). A representative experiment is shown. Inside the Figure, the mean values of mean fluorescence intensity \pm SEM of four experiments are shown; * $p < 0.05$ versus control.

tion of GM-CSF in the supernatants of platelets ($5 \times 10^8/\text{ml}$) collected either after 18 hours of culture at 37° C in RPMI medium supplemented with 1% HSA or after platelet disruption by repeated freezing and thawing revealed the presence of GM-CSF (29 ± 7 and $43 \pm 10 \text{ pg/ml}$, respectively, mean \pm SEM, $n = 7$) at concentrations able to inhibit eosinophil apoptosis (Okada et al, 1998; Tai et al, 1991). Moreover, we also observed that supernatants from platelets disrupted by freezing markedly prevented eosinophil apoptosis and that this effect could be partially attributable to the presence of GM-CSF, because neutralizing antibodies directed to GM-CSF significantly inhibited this antiapoptotic effect: % apoptosis after 48 hours of culture at 37° C: 78 ± 17 , 23 ± 12 , and 52 ± 13 , for eosinophils cultured in medium alone and in medium supplemented with 20% vol/vol of platelet supernatants, in the absence or presence of 10 $\mu\text{g/ml}$ neutralizing antibodies directed to GM-CSF, respectively (mean \pm SEM, $n = 4$, $p < 0.05$, eosinophils cultured with platelet supernatants in the absence and presence of anti-GM-CSF antibodies).

Finally, we analyzed the relationship between platelet activation and GM-CSF secretion. Washed platelets ($5 \times 10^8/\text{ml}$ in RPMI medium supplemented with 1% HSA) were incubated at 37° C for 15 minutes in the absence or presence of collagen (0.20 mg/ml). Supernatants were collected, and the levels of GM-CSF were evaluated by ELISA. We observed that activation by collagen resulted in a marked increase in the secretion of GM-CSF: 10 ± 7 versus $38 \pm 12 \text{ pg/ml}$, for platelets incubated in the absence or presence of collagen, respectively (mean \pm SEM, $n = 5$, $p < 0.01$).

Discussion

Eosinophils are the hallmark of allergic inflammation. They are selectively recruited at allergic inflammatory sites and contribute to the initiation and maintenance of the allergic response by virtue of their ability to release a considerable array of inflammatory mediators (Costa et al, 1997; Dombrowicz and Capron, 2001; Gleich, 2000). Eosinophil numbers in inflammatory sites can be related to a balance between incoming recruited cells and apoptotic death followed by phagocytic clearance, two different but interrelated processes that determine the tissue load of eosinophils (Walsh, 2000).

We report here for the first time that platelets delay eosinophil apoptosis, increasing cell survival. Not only spontaneous apoptosis but also promotion of apoptosis induced by agents such as pronase and dexamethasone were markedly inhibited by platelets. Inhibition of apoptosis was dependent on the release of soluble factors by platelets, and it was partially prevented by neutralizing antibodies directed to GM-CSF.

Aside from their well-established role in hemostasis, platelets seem to play an important role in several inflammatory processes. A large body of evidence supports not only that platelets are present at allergic inflammatory sites but also that platelet-dependent events determine exacerbation of allergic responses (Burgers et al, 1993; Chihara and Nakajima, 1989; Coyle et al, 1990; Gleich, 1990; Kameyoshi et al, 1992; Knauer et al, 1981; Lellouch-Tubiana et al, 1988; Morley et al, 1985; Page, 1988; Page and Coyle, 1989; Wardlaw et al, 1986; Yasuba et al, 1991). The mechanisms involved have not been well defined; however, attention has been focused on the ability of platelets to promote eosinophil accumulation by releasing a number of eosinophil chemotactic factors (Burgers et al, 1993; Chihara and Nakajima, 1989; Kameyoshi et al, 1992; Wardlaw et al, 1986). Our results support the notion that platelets may contribute to perpetuate the allergic inflammatory response not only by inducing the selective recruitment of eosinophils at allergic inflammatory sites but also by increasing eosinophil survival.

We also show here for the first time that platelets are a source of GM-CSF. Studies performed by flow cytometry demonstrated that intracellular GM-CSF is present in resting platelets. Moreover, GM-CSF was found in platelet supernatants, at concentrations able to prevent eosinophil apoptosis. Our results support the notion that GM-CSF released by platelets accounts, at least in part, for the inhibition of eosinophil apoptosis mediated by platelets. Eosinophils are also able to produce GM-CSF; however, in agreement with previous work showing that in resting eosinophils GM-CSF mRNA is rapidly degraded, preventing mRNA accumulation and GM-CSF protein production (Esnault and Malter, 2001; Moqbel et al, 1991), we did not detect any production of GM-CSF in eosinophils cultured alone. This observation does not rule out the possibility that eosinophil activation induced by platelets may trigger the production of GM-CSF. In fact, it

has been shown that the prevention of eosinophil apoptosis by mast cells is dependent on the induction of GM-CSF production by eosinophils themselves (Levi-Schaffer et al, 1998). However, this mechanism does not seem to be involved in our experimental model, because kinetic studies showed that neutralizing antibodies directed to GM-CSF strongly suppressed the ability of platelet supernatants to delay eosinophil apoptosis when added at the beginning of the cell culture. By contrast, these antibodies exert a weak suppressor effect when added 30 minutes after the exposure of eosinophils to platelet supernatants (S. Raiden, unpublished results). Because the induction of GM-CSF production by stimulated eosinophils requires at least 2 hours of culture (Moqbel et al, 1991), this result suggests that GM-CSF released by platelets is responsible for the increase of eosinophil survival.

We have previously reported that platelets are also able to delay neutrophil apoptosis (Andonegui et al, 1997). In contrast with the observations made in eosinophils, we found that inhibition of neutrophil apoptosis was dependent on direct cell to cell interactions between platelets and neutrophils. In fact, we observed similar levels of apoptosis using viable or fixed platelets, whereas platelet supernatants did not mediate any significant effect. Because GM-CSF prolongs survival of both neutrophils and eosinophils (Haslett, 1999; Lopez et al, 1986), it is not clear why platelet supernatants, which contain GM-CSF, were unable to prevent neutrophil apoptosis. One possible explanation is that the ability of GM-CSF to delay neutrophil apoptosis may be inhibited by the simultaneous presence of other factors and/or cytokines in cell cultures. In fact, we recently demonstrated that in the presence of TNF- α , GM-CSF was unable to delay apoptosis of neutrophils; rather, it induced a significant increase in their apoptotic rates (Salamone et al, 2001). This finding was confirmed by Daigle et al (2002), who showed that stimulation of death domain-containing receptors of the TNF/nerve growth factor family disrupted antiapoptotic pathways initiated by survival factors such as GM-CSF in neutrophils. Additional studies are required to define the differences between the mechanisms through which platelets enhance survival of both eosinophils and neutrophils.

There is compelling evidence that links platelets and eosinophils in the development of allergic processes. However, the pathways through which platelets influence eosinophil behavior, as well as the impact of these pathways on the course of allergic processes, are not well defined. A better knowledge of these pathways will help toward the development of new strategies for the treatment of allergic diseases.

Materials and Methods

Reagents

Acridine orange, ethidium bromide, propidium iodide, collagen, thrombin, pronase, rhGM-CSF, and rhIL-3 were purchased from Sigma. The mAb anti-human

CD16 (clone 3G8, mouse IgG1) used in eosinophil purification was obtained from Immunotech (Marseille, France). Neutralizing antibodies directed to human GM-CSF and IL-3 were from Pharmingen (San Diego, California). FITC mAb to P-selectin was also from Immunotech.

Blood Samples

Blood samples were obtained from healthy donors who had not taken medication for at least 10 days before the day of sampling. Blood was obtained by venipuncture of the forearm vein, and it was drawn directly into plastic tubes containing 3.8% sodium citrate (1/9 vol/vol).

Purification of Eosinophils

Peripheral blood polymorphonuclear cells were obtained by dextran sedimentation and Ficoll-Hypaque gradient centrifugation, as previously described (Boyum, 1968). Contaminating erythrocytes were removed by hypotonic lysis. After washing, the fraction containing polymorphonuclear cells were resuspended in RPMI 1640 and were incubated with saturating concentrations of anti-CD16 mAb, for 45 minutes at 4° C. Then, cells were washed twice and eosinophils were isolated by negative selection using magnetic beads coated with anti-mouse IgG antibodies (Dynabeads M450; Dynal, Oslo, Norway), according to the manufacturer's instructions. Eosinophils were collected at a purity higher than 94% and at a viability of 97 to 100%, as assessed by trypan blue staining, and were resuspended in RPMI 1640 (Life Technologies, Grand Island, New York) supplemented with 1% of HSA, 100 µg/ml streptomycin, and 100 µg/ml penicillin (Gibco Laboratories, Grand Island, New York).

Preparation of Washed Platelets

Platelet-rich plasma (PRP) was prepared by centrifugation of the citrated blood samples at 180 ×g for 10 minutes. Then, PRP was centrifuged in the presence of EDTA (5 mmol/L) for 15 minutes at 1500 ×g, and platelets were washed twice (1500 ×g for 15 minutes) in 0.015 mol/L Tris, 0.145 mol/L NaCl, 0.002 mol/L EDTA, 0.1% glucose, and 0.05% BSA (pH 6.5; Tris-EDTA) and finally resuspended at the desired concentration in RPMI 1640 supplemented with 1% of HSA, 100 µg/ml streptomycin, and 100 µg/ml penicillin. The purity of platelet preparations was higher than 99.9% as judged by microscopic observation.

Preparation of GFP

PRP, in the presence of PGE1 (0.1 µmol/L), was gel-filtered on Sepharose 2B (Pharmacia, Uppsala, Sweden) as previously described (McCrae et al, 1990), using an elution buffer containing 0.137 mol/L NaCl, 0.027 mol/L KCl, 0.001 mol/L MgCl₂, 0.0033 mol/L NaH₂PO₄, 0.1% glucose, 0.3% HSA, and 0.020 mol/L

HEPES (pH 7.4). The purity of GFP was higher than 99.9% as judged by microscopic observation.

Culture of Eosinophils with Platelets

Unless otherwise stated, human eosinophils (1 × 10⁶/ml) were cultured in the absence or presence of platelets (eosinophil:platelet ratios = 1:50, 1:25, or 1:10) for different times at 37° C in 5% CO₂-95% humidified air. Then, the percentages of apoptotic eosinophils were determined by fluorescence microscopy and/or by flow cytometry. In all cases, platelets and eosinophils were isolated from the same donor for each assay. In experiments aimed to investigate the participation of soluble factors in the delayed apoptosis of eosinophils mediated by platelets, co-cultures of eosinophils and platelets were performed using TransWell culture inserts. Platelets or culture medium (controls) were placed in the lower compartment of 48-well flat-bottom plates at a total volume of 600 µl, and eosinophils were added into 0.4 µm pore size inserts (upper compartment; Costar, Cambridge, MA) at an eosinophil:platelet ratio of 1:50. With the use of this method, eosinophils in the upper compartment were physically separated from platelets in the lower compartment, whereas the passage of macromolecules across the semipermeable membrane was allowed.

Quantification of Eosinophil Apoptosis and Viability by Fluorescence Microscopy

Quantification was performed as previously described (Coligan et al, 1994), using the fluorescent DNA-binding dyes acridine orange (100 µg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 µg/ml) to differentiate between viable and nonviable cells. With this method, nonapoptotic cell nuclei show variations in fluorescent intensity that reflect the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained. For determining the percentage of eosinophils showing nuclear morphologic features of apoptosis, at least 200 cells were scored in each experiment. The percentage of necrotic cells was determined by counting ethidium bromide-positive cells.

Quantification of Eosinophil Apoptosis by Annexin-V Binding Assay

Annexin-V binding to eosinophils was performed using an apoptosis detection kit (Immunotech). Cells were labeled with annexin-V FITC and propidium iodide for 20 minutes at 4° C and analyzed by two-color flow cytometry (FACScan flow cytometer; Becton-Dickinson Immunocytometry System, San Jose, California) using CellQuest analysis software (Becton Dickinson), as previously described (Homburg et al, 1995).

Quantification of Eosinophil Apoptosis by Propidium Iodide Staining and Flow Cytometry

The proportion of eosinophils that display a hypodiploid DNA peak ie apoptotic cells, was determined using a modification of Nicoletti's protocol (Nicoletti et al, 1991). Briefly, cell pellets containing 2×10^5 eosinophils were suspended in 400 μ l of hypotonic fluorochrome solution (propidium iodide, 50 μ g/ml in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated for 2 hours at 4° C. The red fluorescence of propidium iodide in individual nuclei was measured using a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, California). The forward scatter and side scatter of particles were simultaneously measured. Cell debris was excluded from analysis by raising the forward scatter threshold.

Measurement of Fluctuations in Intracellular Ca^{2+} Concentrations

Changes in $[Ca^{2+}]_i$ were monitored using fluo-3/AM, as previously described (Kao et al, 1989). Briefly, eosinophils suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 were incubated with 4 μ M fluo-3/AM for 30 minutes at 30° C. Then, loaded cells were washed twice and resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 1% HSA. Aliquots of 50 μ l of this cell suspension were then added to 250 μ l of RPMI 1640 containing 1% HSA and warmed at 37° C. The samples were immediately loaded onto the flow cytometer, and the basal fluorescence (FL1) was recorded during 15 seconds. Then, supernatants from platelets (5×10^8 /ml) cultured for 18 hours at 37° C in RPMI medium supplemented with 1% HSA were added (50 μ l), and the fluorescence was recorded during an additional 100 seconds. Acquisition of samples was performed at 37° C, and fluctuations in $[Ca^{2+}]_i$ were recognized as alterations in fluo-3 fluorescence intensity over time. Data were analyzed by using CellQuest software (Becton Dickinson, Mountain View, California). Cells that raised their $[Ca^{2+}]_i$ in response to stimulation, to higher levels than that shown by 97% of resting cells, were considered to be activated.

Measurements of GM-CSF Production by Platelets

Washed platelets (5×10^8 cells/ml in RPMI 1640 medium supplemented with 1% HSA) were incubated for 18 hours at 37° C. Then, supernatants were harvested, aliquotted, and quickly stored at -70° C until used. The concentration of GM-CSF was assessed using a commercial enzyme immunoassay kit (R & D Systems, Minneapolis, Minnesota). The sensitivity of the cytokine test was 5 pg/ml.

Flow Cytometric Analysis of Intracellular GM-CSF in Fixed/Permeabilized Platelets

Washed platelets (10^7) were fixed and permeabilized using a commercial kit (Cytotfix/Cytoperm; Pharmingen) and subsequently stained with 0.25 μ g of PE-rat

anti-human GM-CSF mAb, clone BVD2-21C11 (Pharmingen). For demonstrating specificity of staining, the binding of the PE-BVD2-21C11 antibody was blocked by preincubation of the fixed/permeabilized platelets with 2.5 μ g of unlabeled BVD2-21C11 antibody (Pharmingen) before staining.

Statistical Analysis

Student's paired *t* test was used to determine the significance of differences between means, and $p < 0.05$ was taken as indicating statistical significance.

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