Aspergillus fumigatus Activates Thrombocytes by Secretion of Soluble Compounds

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During invasive aspergillosis, platelets might be involved in immune defense, but they also might contribute to the pathology of the disease. We tested the hypothesis that Aspergillus secretes factors that influence the activity and functionality of thrombocytes. Platelets were incubated with medium wherein Aspergillus fumigatus was grown. This fungal culture supernatant potently stimulated thrombocytes in a time- and dose-dependent fashion, inducing release of alpha and dense granules, membrane alterations, aggregation, and formation of microparticles. Fungus-induced platelet activation could be confirmed in vivo: thrombocytes from mice infected with A. fumigatus showed a higher activation level than platelets from noninfected animals. Two stimulating components in the fungal culture supernatant were identified: a fungal serine protease and the mycotoxin gliotoxin. Activation of platelets by fungal factors stimulates antifungal functions: platelets gain the capacity to interact with foreign particles, and they become able to inhibit fungal growth, thus supporting the host immune network. However, some consequences of platelet activation might also be harmful, including excessive inflammation and induction of thrombosis. These findings imply that measuring platelet activation in patients might be an interesting diagnostic parameter.

Keywords. invasive aspergillosis; secreted factors of Aspergillus fumigatus; thrombocytes; activation of platelets; innate immunity.

Aspergillus fumigatus is one of the most frequent causes of invasive mycoses in immunosuppressed individuals [1, 2]. More profound knowledge of the pathogenesis of aspergillosis and the interactions between A. fumigatus and immune cells might help to develop new therapeutic approaches.

Emerging evidence assigns a crucial role to thrombocytes as immune mediators that participate in the innate immune response against bacteria, viruses, and fungi [3–6]. Two roles can be attributed to platelets: (1) direct attack of the invading pathogen and (2) interaction with and stimulation of innate and adaptive immune elements. A prerequisite for these functions is the activation of thrombocytes, which is a multistep process that includes release of alpha and dense granules, membrane alterations, microparticle formation, and aggregation [7]. However, excessive activation of thrombocytes might promote thrombosis, a hallmark of an invasive Aspergillus infection, and thus contribute to the pathology of the disease [8, 9].

Platelet activation after fungal infection might occur directly, by contact with the fungal surface, and indirectly, by means of secreted fungal factors. The first mechanism is restricted to sites of fungal infection, whereas secreted fungal substances might affect platelets from a distance, thus putatively playing an even more important physiological role. It is well-known that Aspergillus secretes a broad spectrum of different factors during growth, including enzymes, mycotoxins, and other metabolites [10–12]. In the present study, we tested potential effects of secreted fungal factors on the activation and antimicrobial capacity of thrombocytes.
METHODS

Antibodies and Chemicals
Antibodies and annexin V were purchased from BioLegend, and the protease inhibitor cocktail was purchased from Roche Diagnostics. All other protease inhibitors, fluorescein isothiocyanate (FITC), gliotoxin, glucose, glutamine, and arginine were purchased from Sigma–Aldrich. Calcein white (Fungi-Fluor Kit) was from Polysciences.

*A. fumigatus* Isolates and Fungal Supernatant Preparation
A clinical isolate of *A. fumigatus* (A22) from a lung biopsy specimen of an immunosuppressed patient with respiratory insufficiency was used for the majority of experiments. Nine further clinical isolates from patients with cerebral and pulmonary aspergillosis, as well as 6 environmental isolates, were compared for their capacity to secrete platelet-activating factors. Moreover, ATCC46645 (C1) and the corresponding knockout mutant C1AgliP, which is deficient in gliotoxin peptide synthetase [13], were tested.

To obtain the fungal supernatant, $10^8$ conidia of the *A. fumigatus* strains were inoculated in 500 µL of Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Life Technologies) at 37°C. After 2 days, the fungus was removed by centrifugation and filtration, using Spin-X filters (Corning Life Sciences). The fungal supernatant was freshly used or frozen at −20°C for further disposal.

Preparation of Platelet-Rich Plasma and Whole-Blood Samples
All studies were approved by the local ethics committee. Blood was obtained with informed consent from healthy volunteers. Venous blood was taken with a trisodium citrate blood collection system (Sarstedt); platelet-rich plasma was prepared from whole-blood by centrifugation at 135g for 15 minutes at room temperature.

Platelets were incubated with medium, 0.1 U thrombin (Sigma–Aldrich), or fungal supernatant for up to 90 minutes at 37°C. In all experiments, incubation of platelets in RPMI medium, which does not induce any activation, represents the negative control, indicating the background activity of quiescent cells. To demonstrate that thrombocytes were not affected by preparation and are capable of being activated, thrombin was used as positive control. In some samples, the fungal supernatant was preincubated with a cocktail containing inhibitory compounds against different classes of proteases. To determine which class of proteases are involved, the following specific protease inhibitors were also used: E64 (which inhibits cysteine proteases), pepstatin (which inhibits aspartyl proteases), and chymostatin and 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF; both inhibit serine proteases). Furthermore, purified gliotoxin was used in some experiments.

Analysis of Alpha Granule and Dense Granule Release During Platelet Activation
Platelet activation by fungal supernatant or by thrombin was examined by detection of different platelet activation markers, using a FACS Canto flow cytometer (Becton Dickinson). Thrombocytes were gated by FITC-conjugated antibodies against CD41 or CD42a, which are antigens present on all platelets.

Activation-induced secretion of alpha granules and dense granules was quantified by measuring CD62P (P-selectin) and CD63, respectively, on the surface with specific antibodies.

To analyze adenosine triphosphate (ATP) release, thrombocytes were incubated with medium, thrombin, or increasing concentrations (v/v) of fungal supernatant. After 60 minutes, the culture medium was harvested, and the ATP content was quantified by luminometry, using a commercial kit (Promega) according to the manufacturer’s instructions, as well as a luminometer (Bio-Rad).

Analysis of Membrane Alterations and Microparticle Formation
Phosphatidylserine (PS), which is exposed on the surface of activated platelets [14], was detected by the binding of annexin V. Thrombocytes were incubated with FITC-conjugated annexin V according to the manufacturer’s instructions (BioLegend) and were subsequently analyzed by fluorescence-activated cell sorter (FACS) analysis.

To determine microparticle formation, platelets were incubated with medium or fungal supernatant for 90 minutes. Afterward, newly formed microparticles positive for the platelet marker CD41 were gated in the samples according to their size, using the FACS Canto cytometer.

Aggregation Assay
This feature of activated platelets was measured with the Multiplate analyzer (Verum Diagnostica). Platelet-rich plasma was preincubated at 37°C, followed by addition of either fungal supernatant or 32 µM thrombin receptor activating peptide (TRAP-6) (Verum Diagnostica). Aggregation was continuously recorded for 30 minutes. The impedance change was expressed in arbitrary aggregation units (AU) and plotted against the time. Platelet aggregation was quantified as the area under the curve (AUC), expressed in units (U).

Animal Experiments
Experiments were conducted using 7-week-old female Balb/c mice (Charles River Laboratory). Mice were treated in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and with Austrian law. Animal...
experiments were approved by the ethics committee of the Austrian Federal Ministry of Science and Research.

Six animals per experimental group were used. Half of the mice were infected intravenously with $2 \times 10^6$ conidia of *A. fumigatus*. Blood was taken at day 3, and thrombocytes were analyzed for exposure of CD62P on their surface.

**Interaction of Thrombocytes With Particles and Inhibition of Fungal Growth**

Platelet-rich plasma was labeled with antibodies against CD42a (BD Pharmingen). A 10-fold amount of either FITC-labeled latex beads (Sigma) or FITC-labeled conidia was added. After 60 minutes, cells were fixed with 1% formalin, and CD42a-positive thrombocytes that were also positive for FITC were quantified by flow cytometry.

To analyze the effect of thrombocytes on fungal growth, conidia of *A. fumigatus* were incubated with medium, quiescent thrombocytes, or thrombocytes preincubated with fungal supernatant (ratio of platelets to conidia, 1:10); germination of the conidia was observed in a lifetime microscope (Nikon).

**Statistical Analysis**

Statistical analyses were performed with unpaired *t* tests, using GraphPad Prism software.

**RESULTS**

**Thrombocytes React on Contact With *A. fumigatus*–Derived Secretory Factors, With Release of Alpha Granules**

The reaction of platelets on the presence of *A. fumigatus*–derived culture supernatant was evaluated by incubation of thrombocytes with fungal supernatant, followed by measurement of the activation marker CD62P. CD62P is present in alpha granules and appears on the cell surface after fusion of these granules. The soluble factors released by *A. fumigatus* induced a strong increase in the membrane exposure of CD62P (Figure 1A). The extent was similar to that after addition of thrombin, which is one of the strongest platelet activators.

Time dependence of the *Aspergillus*-induced thrombocyte activation is shown in Figure 1B. A highly significant accumulation of CD62P on the surface was visible 5 minutes after

**Figure 1.** Release of alpha granules by thrombocytes, induced by culture supernatant of *Aspergillus fumigatus*. A, Platelet-rich plasma (PRP) was incubated with Roswell Park Memorial Institute medium, thrombin, or 20% (v/v) fungal supernatant (SN) from *A. fumigatus* for 90 minutes. Exposure of CD62P was assessed by flow cytometry. B, PRP was incubated with medium, thrombin, or 20% (v/v) fungal SN; CD62P was quantified at different time points. C, PRP was incubated with medium, thrombin, or different volumes (v/v) of fungal SN; CD62P was measured after 90 minutes. Each experiment was repeated at least 3 times with triplicate samples. ***P < .001.
addition of the fungal supernatant, and it increased constantly for up to 90 minutes. To study the dose dependence of platelet stimulation, different volumes of the fungal supernatant were added to the cells (Figure 1C). A significant rise in the CD62P signal on the cells was already visible with 2% (v/v) supernatant as the final volume of the sample.

**Thrombocytes Undergo a Broad-Spectrum Activation Program After Contact With A. fumigatus–Derived Secretory Factors**

To evaluate whether fungal supernatant also triggers fusion of platelet-dense granules with the plasma membrane, CD63 surface exposure and ATP release were checked (Figure 2A and 2B). A concentration of 1% (v/v) of fungal supernatant was sufficient to stimulate significant release of dense granules, as shown by the appearance of CD63 on the surface (Figure 2A); higher concentrations of *Aspergillus* supernatant triggered further upregulation of CD63. Furthermore, ATP, a substantial content of the dense granules, was released into the medium to a significant extent (Figure 2B).

Because activated platelets have been described to undergo membrane alterations, the exposure of phosphatidylserine after addition of the fungal supernatant was quantified by the binding of annexin V. A significant increase in annexin V binding was proven with 20% (v/v) *Aspergillus* supernatant (Figure 2C). Enhanced binding could also be measured with lower concentrations of supernatant, with 1% (v/v) as the lower limit (unpublished data).

Since budding of microparticles from activated platelets represents an important process to potentiate inflammatory processes, we analyzed their generation. Significant amounts of
microparticles appeared after addition of 1% (v/v) fungal supernatant (Figure 2D); higher concentrations did not further increase their formation.

Aggregation of the activated platelets might play a role in fungus-induced thrombosis. For that reason, the aggregation of the thrombocytes under influence of fungal secretory factors was monitored 3 times, using platelets derived from different blood donors. A representative experiment and the corresponding quantification of aggregation as the AUC are shown in Figure 3. A strong reaction could be triggered with the Aspergillus supernatant, compared with the control cells incubated only with medium. Only TRAP, a peptide known to be a potent inducer of aggregation, induced a higher effect.

**Fungus-Induced Thrombocyte Activation Also Occurs In Vivo**

We aimed to confirm that fungus-induced thrombocyte activation also occurs in vivo and thus might take place in infected patients. Therefore, we compared the effect of fungal secretory factors on thrombocytes in platelet-rich plasma with that on platelets present in a whole-blood sample. This approach enabled us to check whether this reaction also takes place in the interplay of thrombocytes with other immune cells and plasma-derived soluble factors. As shown in Figure 4A, thrombocytes could be activated in platelet-rich plasma and in whole blood by addition of fungal supernatant. In both preparations, the reaction of platelets could be triggered with *Aspergillus* supernatant concentrations of ≥1% (v/v). The maximal stimulation of the thrombocytes in whole blood needed higher volumes of the fungal secretory factors than in platelet-rich plasma, probably because of the additional interaction of these factors with other blood cells (Figure 4A).

Next, we compared environmental versus patient-derived isolates of *A. fumigatus* for their capacity to stimulate platelets. As shown in Figure 4B, the fungal supernatants of patient-derived isolates tendentially activated thrombocytes to a higher extent than the secreted factors of isolates found in the environment. However, the difference between the 2 groups did not reach significance.

To further approach the in vivo setting of invasive aspergillosis, we performed an animal experiment. Mice were either mock treated or intravenously infected with *A. fumigatus*, and blood samples were taken from the animals at day 3. The extent of platelet activation was analyzed with CD62P as the
relevant marker. Animals infected with *A. fumigatus* showed significantly more CD62P on the surface of their thrombocytes than the mock-treated control animals (Figure 4C).

A Fungal Serine Protease and Gliotoxin Contribute to the Thrombocyte-Activating Effect of Fungal Supernatant

The fungal supernatant represents a complex mixture of different compounds secreted by *A. fumigatus* during growth. We tried to get some hints about which substance(s) in the supernatant might be responsible for thrombocyte activation. Since the presence of nutrients in the medium regulates the synthesis and secretion of fungal factors, we supplemented the medium with glucose as a carbon source or arginine or glutamine as a nitrogen source. While glucose did not modulate the presence of platelet-activating factors in the supernatant, supplementation with amino acids reduced the stimulating effect (Figure 5A).

Since nitrogen is known to regulate the synthesis of fungal proteases, we investigated whether proteases in the fungal supernatant might mediate platelet activation via protease-activated receptors on thrombocytes. Preincubation of the *A. fumigatus* supernatant with a cocktail of protease inhibitors strongly reduced its capacity to stimulate platelets, as shown by CD62P quantification (Figure 5B). To further determine which class of proteases is involved, a panel of more specific protease inhibitors was used. E64 is a highly selective

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**Figure 4.** Platelet activation by *Aspergillus fumigatus* in different experimental models. *A*, Platelet-rich plasma (PRP) or whole-blood samples were incubated for 90 minutes with medium, thrombin, or increasing concentrations (v/v) of *A. fumigatus* supernatant. Exposure of CD62P on CD41-positive thrombocytes was assessed by flow cytometry. *B*, Supernatants (20% v/v) of patient-derived and environmental isolates of *A. fumigatus* were added to the platelets for 90 minutes. Exposure of CD62P on thrombocytes was studied by flow cytometry. These experiments (4 and 5) were repeated at least 3 times with triplicate samples. *C*, Balb/c mice were infected intravenously with *A. fumigatus* conidia. Blood samples were taken at day 3 after infection, and the presence of CD62P on thrombocytes was assessed by fluorescence-activated cell sorter analysis. Each group consisted of 6 animals. *P* < .05; **P** < .01; ***P*** < .001.
inhibitor of cysteine proteases, but it was unable to interfere with the platelet-activating effect of the fungal supernatant. Similarly, pepstatin, which inhibits aspartic proteases, could not abolish the platelet stimulation by fungal supernatant. In contrast, the inhibition of serine proteases by AEBSF or chymostatin significantly downmodulated the ability of the fungal supernatant to activate thrombocytes, thus indicating the contribution of 1 or several serine protease(s) (Figure 5C).

Mycotoxins are generated and released during fungal growth, with gliotoxin as the most prominent representative. To determine whether gliotoxin might be involved in stimulation of thrombocytes, we used an *A. fumigatus* mutant (C1ΔgliP) lacking an enzyme for gliotoxin synthesis. The corresponding wild-type strain C1 was used for control. The wild-type strain produced platelet-activating compounds to a similar extent as the *A. fumigatus* strain we used for all former experiments. However, the supernatant of the ΔgliP mutant lacked nearly all capacity to trigger thrombocyte activation (Figure 5C). Further evidence for a principal role of this mycotoxin in platelet activation was supplied by an additional experiment, in which the effect of the whole fungal supernatant was mimicked by purified gliotoxin (Figure 5D). Gliotoxin concentrations as low as 3 nM were sufficient to induce thrombocyte activation, as demonstrated by an increase in CD62P; 30 nM gliotoxin showed a similar effect as the tested fungal supernatant. Higher amounts of gliotoxin (up to 1000 nM) further enhanced the CD62P signal.

**Figure 5.** Influence of protease and gliotoxin on thrombocyte activation by culture supernatant of *Aspergillus fumigatus.* A, *A. fumigatus* was grown in medium alone or in medium supplemented with 1% glucose, 20 mM glutamine (Gln), or 10 mM arginine (Arg); supernatants were harvested after 2 days. Platelets were incubated with thrombin, medium, supplemented media, or fungal supernatants at a final concentration of 20% (v/v). B, Thrombocytes were incubated with medium, thrombin, or fungal culture supernatant (SN) in a final concentration of 20% (v/v). The SN was partly preincubated with a protease inhibitor (PI) cocktail (1-fold) or with the PIs E64 (40 µM), pepstatin (1 µM), 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride (AEBSF; 2 mM), or chymostatin (300 µM). C, Thrombocytes were incubated with medium, thrombin, or different fungal culture supernatants (SN), derived from the *A. fumigatus* wild-type strains A22 or C1 or from the gliotoxin-deficient mutant C1ΔgliP, in a final concentration of 20% (v/v). D, Platelets were incubated with medium, fungal supernatant (SN), or increasing concentrations of purified gliotoxin. In all experiments, exposure of CD62P on the thrombocytes was measured by flow cytometry after 90 minutes of incubation. Each experiment was repeated at least 3 times with triplicate samples.*P < .05; **P < .01; ***P < .001.
Supernatant-Stimulated Thrombocytes Show Enhanced Interaction With Fungal Pathogens

We aimed to clarify whether thrombocytes can fulfill antimicrobial functions and interact with the fungus after activation by *A. fumigatus* supernatant. Fungal hyphae were stained with calcofluor white, and CD42a-labeled platelets that were either mock treated or preactivated with fungal supernatant were added. Microscopic analysis clearly showed that quiescent thrombocytes bound to the hyphae to a moderate extent (Figure 6A); in contrast, the prestimulated platelets revealed a very high level of interaction with the hyphae (Figure 6B).

We further quantified the stickiness of supernatant-activated platelets to foreign particles by FACS analysis, mimicking a putative pathogen by using FITC-labeled latex beads. No differentiation between adhesion and internalization can be made by this method. As shown in Figure 6C, the supernatant-triggered platelets bound or even ingested the beads in a dose-dependent manner. The interaction with the larger *A. fumigatus* conidia was much lower but still significantly increased in supernatant-stimulated thrombocytes as compared to unstimulated thrombocytes (Figure 6D).

Supernatant-Stimulated Thrombocytes Interfere With Fungal Germination and Growth

The inhibition of fungal germination and proliferation by activated thrombocytes was tested. Addition of quiescent platelets to *A. fumigatus* conidia delayed germination and hyphal...
elongation, as shown in Figure 7A. However, the presence of platelets that were activated by fungal supernatant nearly completely suppressed any germination of the *Aspergillus* conidia (Figure 7A). The supernatant of the ΔgliP mutant was much less effective than the corresponding wild-type strain at inducing the fungal-growth-inhibitory capacity of thrombocytes (Figure 7B).

Addition of the protease inhibitor AEBSF, which had been demonstrated to interfere with platelet activation, to the supernatant did not affect platelet activation, indicating that proteases do not participate in that activity (Figure 7C).

**DISCUSSION**

Thrombocytes were recently discovered to be part of the innate immune system and to harbor antimicrobial functions [5, 15–17]. The direct physical contact with fungal conidia or hyphae has been described to stimulate platelets [18–20]. We showed that they also sense the presence of fungus-derived soluble factors. The subsequent stimulation and antimicrobial activity are therefore not limited to the proximity of fungal hyphae but can occur all over the body in patients with invasive aspergillosis and, thus, may considerably affect the course of disease. However, the possibility of a disseminated thrombocyte reaction implies that platelets could also contribute to excessive inflammation and thrombosis [8].

The evidence that supernatant-induced thrombocyte activation also occurs in whole-blood samples strongly suggests the need for deeper insight into the role of thrombocytes in infected patients. Fungal isolates derived from infected patients have a tendency to be more potent platelet activators than environmental isolates. Data from a mouse model of invasive aspergillosis, showing the activation status of thrombocytes to be higher in infected animals than in noninfected animals, further underlines the in vivo relevance of our results, although this model allowed no differentiation between thrombocyte activation by direct contact with fungi and stimulation by released fungal factors.
Platelets are more resistant against the toxicity of gliotoxin than other immune cells, and gliotoxin concentrations of up to 1000 nM still induced activation. This might be because thrombocytes lack transcriptional capacity and thus are insensitive for NF-κB blocking. Thus, under conditions in which most other immune cells are functionally suppressed, platelets can still undergo activation and inhibit fungal germination and hyphal elongation. Gliotoxin concentrations of 3–1000 nM, which trigger thrombocyte activation, are also reached in vivo. Serum samples derived from patients with invasive aspergillosis contain 508–2405 nM gliotoxin [32].

Stimulated by fungal supernatant, thrombocytes gain a substantial antimicrobial function: they efficiently bind to fungal hyphae and interact with or even internalize foreign particles. The binding to hyphae can be hypothesized to be an important step in the immune defense against Aspergillus, since platelets store antimicrobial peptides [6] and serotonin, which also has antifungal activity [33]. Binding of platelets to hyphae under the influence of the fungal supernatant might help to directly target the release of antimicrobial peptides and serotonin to the site of fungal presence. Interaction of activated platelets with conidia is much lower than with hyphae, probably because of the different surface composition. Furthermore, our results show that contact with fungal supernatant enables thrombocytes to interact in general with foreign particles (eg, latex beads), which is a prerequisite for their phagocytic capacity [34]. Conidia that are larger than latex beads show moderate interaction with the platelets.

The second immunological function that is activated by fungal supernatant is the ability of platelets to inhibit fungal germination. Interestingly, fungal proteases seem to play no role in the induction of this platelet function, whereas gliotoxin is central for its stimulation. This finding supports the hypothesis that the modulation of platelets by the fungal supernatant is a multifactorial process.

A putative consequence of our study might be the identification of patients with invasive aspergillosis who are at particular risk for the development of thrombosis. Patients with high levels of CD62P on their platelets and strong annexin V binding might be considered to be predisposed to thrombus formation, with improved antithrombotic prophylaxis as a putative consequence. Further studies in vitro and in vivo will help to work toward this aim.

Notes

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