CHAPTER VI

ACTIVATION OF $\beta_1$ INTEGRINS INCREASES APOPTOSIS OF CYTOKINE-TREATED HUMAN NEUTROPHILS
Activation of $\beta_1$ Integrins Increases Apoptosis of Cytokine-Treated Human Neutrophils

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1. ABSTRACT

Apoptosis of neutrophils at the inflammatory site is thought to be of major importance in the resolution of inflammation. Pro-inflammatory cytokines generally protect neutrophils from apoptosis, but adhesion through $\beta_2$ integrins may induce a respiratory burst in neutrophils, which increases apoptosis. In contrast, in many other cell types, adhesion via $\beta_1$ integrins increases cell survival.

Recently, it has been described that neutrophils express functionally active $\beta_1$ integrins. Therefore, we studied whether these adhesion molecules play a role in neutrophil apoptosis, using the $\beta_1$ integrin subunit (CD29)-activating mAb 8A2.

We found that activation of $\beta_1$ integrins increases apoptosis of neutrophils that had been treated with TNF-\(\alpha\), IFN-\(\gamma\) or GM-CSF, cytokines that normally protect against apoptosis. This effect was also present when $\beta_2$ integrins were blocked, and in cells of patients with chronic granulomatous disease (CGD), incapable of undergoing a respiratory burst. Activation of caspases was necessary for apoptosis of neutrophils that had been treated with $\beta_1$ integrin-activating mAbs.

These data show the existence of a novel intracellular pathway which, starting with $\beta_1$ integrin activation, counteracts anti-apoptotic signaling induced by cytokines, and allows neutrophils to undergo caspase-mediated apoptosis.

2. INTRODUCTION

The life span of neutrophilic granulocytes (neutrophils) is, compared with other leukocytes, very short. After approximately 6 hours in the circulation, neutrophils migrate to the tissues, were they presumably die within a few days by apoptosis (programmed cell death). This
Pi Integrin Activation in Neutrophil Apoptosis

Mechanism has been proposed to play an important role in the resolution of the acute inflammatory response, when large numbers of neutrophils are present in the tissues.

However, the short life span of neutrophils can be prolonged by the action of pro-inflammatory cytokines, such as GM-CSF, IFN-γ or TNF-α. It has been shown that this is caused by a delay of apoptosis. In some cases, an upregulation of Bcl-2 family members by cytokine treatment was associated with decreased cell death. In contrast, survival is decreased if a respiratory burst takes place. The respiratory burst, which produces a large amount of reactive oxygen intermediates (ROI) necessary for the intracellular killing of microbes, is also induced by certain pro-inflammatory cytokines. This complicates the picture for this cell type, and it appears that the sum of these different stimuli decides whether the cell dies or survives.

Yet another mechanism that could presumably influence neutrophil survival is adhesion. Adhesive interactions have been shown to mediate anti-apoptotic pathways in numerous cell types. For most cultured cells, but also for leukocytes closely related to neutrophils, such as eosinophilic granulocytes, adhesion is essential for cell survival. The most important family of adhesion molecules is formed by the integrins, α/β heterodimers that bind to various cellular ligands and extracellular matrix molecules. The fact that integrins only bind their ligands if they are in an activated state provides a mechanism for control of integrin-mediated cell adhesion. Activation of integrins is induced by signals from within the cell, and can be mimicked in vitro by the addition of divalent cations or monoclonal antibodies that by binding to an integrin subunit lock the molecule in its high-affinity state.

Neutrophils express large amounts of β2 integrins (CD18) on their surface. Especially CD11b/CD18 (Mac-1, CR3) is abundantly expressed and plays an important role in neutrophil biology through its binding to various ligands, such as complement factors, adhesion molecules (ICAM-1) and matrix molecules (fibrinogen, fibronectin). In contrast to other cell types, adhesion of neutrophils seems to be a pro-apoptotic signal, because binding via the integrin CD11b/CD18 has been linked to reduced survival.

Another family of integrins, the β1 integrins (CD29), is widely expressed on leukocytes, but was previously believed to be of minor importance for neutrophils. However, recent reports have claimed expression of some β1 integrins, and suggested a role in adhesion and migration of neutrophils. Ligand binding to β1 integrins on eosinophilic granulocytes protects these cells from apoptosis, similar to other, non-leukocytic cell lines. Whether β1 integrins play a role in apoptosis of neutrophils has not been investigated, although adhesion of neutrophils to β1 integrin ligands, such as laminin (LN) or fibronectin (FN) has been shown to induce apoptosis of neutrophils.
To study the role of β₁ integrins in neutrophil apoptosis, we treated these cells with monoclonal antibodies that specifically bind to the β₁ integrin subunit and also activate the integrin. We found that activation of β₁ integrins inhibited anti-apoptotic pathways that were induced by three cytokines, i.e. TNF-α, IFN-γ and GM-CSF. Blocking of CD11b, which normally strongly inhibits neutrophil apoptosis, had no effect if β₁ integrins were activated. The respiratory burst was not involved in this mechanism, since it was also present in neutrophils from patients with Chronic Granulomatous Disease (CGD), a disease that is characterized by the absence of this burst. Furthermore, we found that the apoptotic pathway of caspase-3 activation, which has been described to be involved in the induction of neutrophil apoptosis, is also required for this newly defined mechanism that blocks cytokine-induced anti-apoptotic pathways.

3. MATERIALS AND METHODS

3.1 Monoclonal antibodies and reagents

MAb 44a (IgG₁, anti-CD11b) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). MAb 8A2 (IgG₁, anti-β₁ integrin (CD29)) was a gift from Dr J.M. Harlan (University of Washington, Seattle, WA, USA). IgG-isotype controls as well as HSA (human serum albumin) were purchased from the Central Laboratory of the Netherlands Blood Transfusion Service (CLB, Amsterdam, The Netherlands). TNF-α was bought from Calbiochem (La Jolla, CA, USA), GM-CSF from SanverTECH (Heerhugowaard, The Netherlands), IFN-γ from Boehringer Mannheim (Mannheim, Germany). PI (propidium iodide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and BSA (bovine serum albumin) from Organon Teknika (Boxtel, The Netherlands). Annexin-V-FITC was obtained from Bender MedSystems (Vienna, Austria) and the broad spectrum caspase inhibitor z-VAD-fmk from Alexis Biochemicals (Läuferlingen, Switzerland). All other reagents, if not otherwise stated, were purchased from Merck (Darmstadt, Germany).

Fab fragments were produced by papaine digestion of mAbs 8A2 and 44a. The purity of these fragments was confirmed by gel electrophoresis and subsequent silver staining. A dose-response curve showed a maximal promoting effect of 8A2-Fab fragments at a concentration of 3-5 µg/mL.

3.2 Isolation and treatment of granulocytes

Granulocytes were isolated from heparinized blood of healthy donors as described. Briefly, 20 mL of blood was further anticoagulated and diluted with 20 mL of 10% triso-
dium citrate/PBS. Mononuclear cells and platelets were removed by density gradient centrifugation over isotonic Percoll (Pharmacia, Uppsula, Sweden) with a specific gravity of 1.076 g/mL. Erythrocytes were lysed by short treatment of the pellet fraction with ice-cold isotonic NH4Cl solution (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4).

The remaining granulocytes were washed once in PBS and then resuspended in HEPES medium (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 42 mM K2HPO4·3H2O, 5.5 mM glucose and 0.4 % (wt/vol) HSA, pH 7.4).

Light microscopic evaluation of May Grünwald/Giemsa stained cytospins of the granulocyte fraction revealed a purity of more than 99%, with more than 95% neutrophils.

3.3 Apoptosis assay

The percentage of apoptotic cells was determined as described before2. Briefly, the neutrophils were washed once in HEPES and then resuspended at a concentration of 2 x 10⁶ cells/mL in Iscove’s modified Dulbecco’s medium (IMDM) (BioWhittaker, Brussels, Belgium) supplemented with 10% heat-inactivated FCS (Gibco BRL, Paisley, UK), penicillin (100 IU/mL, Yamanouchi, Tokyo, Japan), streptomycin (100 µg/mL, Gibco BRL) and glutamine (300 µg/mL). On 96-well plates (NUNC Brand Products, Roskilde, Denmark), aliquots of 100 µl of cell suspension were pre-incubated with monoclonal antibodies (15 minutes), then stimulated with cytokines and subsequently maintained overnight (17 hrs) in a 5% CO₂ incubator at standard conditions. If applicable, prior to incubation with mAbs the neutrophils were incubated for 15 minutes with 0.2 mM z-VAD-fmk.

After this incubation, the PMN were treated with EDTA/PBS (5 mM, 30 min., 37°C) to release adherent cells from the wells. The cells were then taken from the wells, and washed once in ice-cold HEPES medium with additional Ca²⁺ (2.5 mM). All further steps were performed in this medium. The cells were incubated with Annexin-V-FITC (1:500), which specifically binds phosphatidyl serine residues on the cell membrane of apoptotic cells. After 45 minutes, the cells were washed once, and stained with PI (5 µg/ml), a fluorescent dye that will bind to DNA once the cell membrane has become permeable. The cells were then immediately analyzed with a FACScan (Becton Dickinson, San Jose, CA, USA). Under all conditions tested, percentages of surviving cells (Annexin-Vneg, PIneg) were compared. Necrosis (Annexin-Vneg, PINeg) was minimal(<3%) under all circumstances.

3.4 Statistics

Student’s t-test for paired samples (two-tailed) was used for statistical analysis.
4. RESULTS

4.1 Activation of β₁ integrins prevents cytokine-induced neutrophil survival

Figure 1A shows that treatment of neutrophils with TNF-α at low concentrations (0.1-1 ng/ml) induces neutrophil survival. At higher concentrations of TNF-α this effect is lost, due to the induction of CD11b-mediated adhesion and the consequent occurrence of a respiratory burst, which leads to apoptosis. This was previously illustrated by the absence of this pro-apoptotic effect of high-dose TNF-α when neutrophils were treated with blocking mAbs to CD11b or in neutrophils from patients with chronic granulomatous disease (CGD), which lack the ability to mount a respiratory burst (Chapter V).

To study whether recruitment of β₁ integrins influences neutrophil apoptosis, we treated neutrophils with Fab fragments of a mAb that specifically binds and activates the β₁ integrin subunit. This mAb, 8A2, has been shown to enhance adhesion of eosinophilic granulocytes and lymphocytes. Survival of non-stimulated neutrophils was not influenced by 8A2, but the anti-apoptotic effects of TNF-α were completely abolished (Fig 1A).

Since we found in the past that β₁ integrin activation influences the activation state of the CD11b/CD18 integrin on neutrophils (Chapter II), we studied the effects of Fab fragments of mAb 44a, which block CD11b. This antibody protects neutrophils from the pro-apoptotic effect of high-dose TNF-α (Fig 1B). After treatment with 8A2, the protective effect of 44a Fab fragments was lost (Fig 1B). Furthermore, we found that the ability of 8A2 to induce apoptosis was intact in cells from patients with a disease called LAD1/variant (not shown). These patients lack functionally active β₂ integrins.

Fig. 1A. Survival of human neutrophils after overnight incubation. Survival was assessed by FACS analysis of the percentage of Annexin-V⁻, PI⁻ cells. Control cells (●), treated with isotype-matched control mAbs, were compared to cells that had been pre-incubated with Fab fragments of β₁ integrin-activating mAb 8A2 (○). Treatment with TNF-α resulted in increased survival at 0.1 and 1 ng/ml TNF-α. Pre-treatment with 8A2-Fab abolished the anti-apoptotic effect of high TNF-α concentrations (0.1-10 ng/ml) (Fig. 1A, n=6, #p<0.01).
Theoretically, coating of the microwells with β1 integrin ligands that are present in the culture medium (e.g., fibronectin) could occur. This could lead to 8A2-induced binding to β1 integrin ligands. However, a combination of antibodies to VLA-5 and VLA-4, both ligands for fibronectin, did not reduce the pro-apoptotic effects of 8A2 (not shown).

One of the possible mechanisms for 8A2-induced apoptosis could be the induction of a respiratory burst. As outside-in signaling from membrane-bound integrins to the interior of the cell is a well-known biological phenomenon\textsuperscript{17}, we hypothesized that the signal provided by β1 integrin activation might be strong enough to induce large-scale production of ROI, which is a dominant pro-apoptotic signal in the neutrophil (Chapter V). To study this possibility we repeated our experiments with neutrophils from patients with chronic granulomatous disease (CGD). Also, in these cells, 8A2 increased apoptosis if the cells had been stimulated with TNF-α, excluding the possibility that oxygen metabolites mediated the pro-apoptotic effects (Fig 1C).
4.2 Activation of $\beta_1$ integrins reverses neutrophil survival induced by IFN-γ and GM-CSF

Apart from TNF-α, numerous other pro-inflammatory cytokines have been shown to protect neutrophils from apoptosis. Especially IFN-γ and GM-CSF, cytokines that are used in the treatment of various hematological disorders, are known for these anti-apoptotic effects (Chapter V). However, as with TNF-α, the strong anti-apoptotic effects of IFN-γ and GM-CSF were completely reversed if the neutrophils had been pre-treated with 8A2 (Fig 2).
4.3 Apoptosis after β1 integrin activation is mediated by caspases

The caspase cascade has been shown to be active in neutrophils. This intracellular pathway is characterized by the consecutive activation of a number of related enzymes termed caspases, until a so-called executioner caspase cleaves and inactivates proteins involved in cellular survival. Neutrophil survival is greatly enhanced if cells are treated with a broad-spectrum inhibitor of these caspases, z-VAD-fmk.

Inhibition of caspase cleavage protected 8A2-treated cells from apoptosis (Fig 3). Spontaneous apoptosis was also greatly reduced. Hence, pathways leading to apoptosis in neutrophils with activated β1 integrins requires caspase activation.

![Graph showing involvement of caspases in neutrophil apoptosis after β1 integrin activation.](image)

Fig 3. Involvement of caspases in neutrophil apoptosis after β1 integrin activation. z-VAD-fmk, an inhibitor of the caspase-pathway, strongly increased neutrophil survival (0.2 mM, dotted bars as compared to open bars for non-z-VAD-fmk-treated cells). 8A2 did not influence z-VAD-fmk-induced cell survival, indicating a role for the caspase pathway in apoptosis of 8A2-treated neutrophils (n=5, *p<0.05; #p<0.01).
5. DISCUSSION

The data presented provide the first evidence that $\beta_1$ integrins are involved in regulating survival of neutrophils. The strong anti-apoptotic effects of three cytokines, TNF-\(\alpha\), IFN-\(\gamma\) and GM-CSF, was abolished by activation of $\beta_1$ integrins by mAb 8A2. It was shown that an important, dominant pathway of neutrophil apoptosis, i.e. the generation of reactive oxygen species, is not involved in this process. In neutrophils from patients with CGD and in neutrophils from patients with LAD1/variant, the pathway leading to increased apoptosis by activation of $\beta_1$ integrins remained intact. Similar to the situation in spontaneous apoptosis, activation of caspases was required for apoptosis of neutrophils after $\beta_1$ integrins had been activated.

Expression of $\beta_1$ integrins on neutrophils has long been disputed\(^{20}\). Presumably, $\beta_1$ integrins are down-regulated during maturation of hematopoietic precursor cells toward a neutrophilic phenotype. Compared to other leukocytes, $\beta_1$ integrin expression on neutrophils is low, but in recent years a number of reports have shown expression of VLA-4, VLA-5, VLA-6 and VLA-9 on neutrophils\(^{10,11}\). Interestingly, it was shown that transmigration could upregulate $\beta_1$ integrins on neutrophils, enabling these cells to engage in adhesive interaction with ECM-ligands\(^{21}\). However, the functional significance of the presence of these adhesion molecules on the neutrophil’s surface is not completely elucidated. It seems that $\beta_1$ integrins have an additional role in transmigration and adhesion, together with the dominant integrin CD11b/CD18 (Chapter II). This $\beta_2$ integrin is highly expressed on neutrophils, and was shown to play a role in numerous essential biological functions of the neutrophil, including adhesion, migration, phagocytosis, respiratory burst and apoptosis. Nevertheless, $\beta_1$ integrin activation may lead to apoptosis without involvement of CD11b/CD18, as was shown in the present study with blocking mAbs or cells from LAD1/variant patients.

Apart from a straightforward function in adhesion and migration, integrins can serve as signaling molecules. Activation of integrins and ligand binding can pass signals to the cell, a process termed outside-in signaling\(^{17}\). Numerous biological effects of outside-in signaling have been reported in various cell types, among which are cellular activation, respiratory burst (in neutrophils) and apoptosis\(^{22}\). Especially $\beta_1$ integrins are capable of passing survival signals to the cells. However, recently it was found that engagement of $\beta_1$ integrins slows down the cell cycle in cultured cells\(^{23}\). Also, a novel signaling protein has been described that combines a “death domain” with a $\beta_1$ integrin-binding segment, coupling $\beta_1$ integrin signaling to a pro-apoptotic pathway\(^{24}\).
It seems that $\beta_1$ integrins can provide diverse signals to different cell types. To complicate the picture, we only found effects of 8A2 treatment if the neutrophils had been stimulated by pro-inflammatory cytokines. Hence, "spontaneous" apoptosis \textit{in vitro} was not affected. We can speculate that the sum of the stimuli provided by pro-inflammatory and anti-inflammatory cytokines, together with adhesive interactions, ultimately decides whether a neutrophil undergoes apoptosis or not. At the inflammatory site, stimulation by cytokines and binding to $\beta_1$ integrin ligands could well lead to activation of $\beta_1$ integrins on neutrophils. Our data suggest that $\beta_1$ integrin activation, by counteracting anti-apoptotic effects of pro-inflammatory cytokines, is one of the mechanisms that controls the life cycle of neutrophils during the inflammatory response.

\section{References}

Chapter VI


