Activation of NF-κB transcription factor in human neutrophils by sulphatides and L-selectin cross-linking

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Received 22 August 2002; revised 6 January 2003; accepted 14 January 2003

First published online 24 January 2003

Edited by Masayuki Miyasaka

Abstract Sulphated galactocerebroside (sulphatide) has been established as a ligand for L-selectin and shown to trigger intracellular signals in human neutrophils. We have found that sulphatide activated transcription factor NF-κB in human neutrophils in a concentration-dependent manner whereas non-sulphated galactocerebroside did not demonstrate such an effect. The activation was inhibitable by pretreatment with primary monoclonal anti-L-selectin antibody (clone LAM1-116). Binding of the primary antibody to L-selectin was insufficient to induce NF-κB activation but cross-linking of L-selectin with a secondary antibody was effective. α-Chymotrypsin, the agent known to shed L-selectin, activated NF-κB by itself. The response to sulphatides was inhibited by jasplakinolide, an actin-polymerising agent known to downregulate surface expression of L-selectin, FcγRIIIb, CD43 and CD44. Recently we have reported that sulphatide stimulated the attachment of human neutrophils to collagen via Mac1 (CD11b/CD18) integrin [Sud'ina et al., Biochem. J. 359 (2001) 621–629]. We now show signalling from sulphatide to NF-κB activation and discuss its involvement in neutrophil adhesion.

Key words: Neutrophil; Galactocerebroside; Sulfatide; NF-κB transcription factor

1. Introduction

The transcription factor NF-κB plays a pivotal role in gene expression of inflammatory mediators such as cytokines and adhesion molecules. NF-κB activation in human neutrophils (polymorphonuclear leukocytes, PMN) has been shown to be induced by the inflammatory stimuli lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF) [1]. A temporal activation of NF-κB was observed in phagocytosing neutrophils [2]. It is known that cell attachment onto a surface can induce activation of NF-κB, as had been shown on fibroblast and smooth muscle cells adhering to fibronectin [3]. It is not yet known whether ligation of an adhesion molecule such as L-selectin can activate NF-κB.

Sulphatide has been established as a ligand for L-selectin: sulphatides specifically bind to L-selectin receptors on neutrophils, while galactocerebrosides do not [4]. Sulphatide is a typical component of myelin membranes and is also expressed on plasma membranes. It is secreted by both myeloid and tumour cells [5]. In neutrophils, sulphatides elicit several functional responses, e.g. they stimulate oxidative metabolism and induce massive superoxide production [6]. The role of sulphatides as L-selectin signalling molecules was demonstrated also by increasing cytosolic free calcium and enhancing expression of TNF and interleukin-8 genes in human neutrophils [7]. We have previously reported that sulphatides stimulated adhesion of human neutrophils to surfaces coated with collagen and fibronectin [8]. Since this may proceed through an activation for NF-κB we have now studied its activation in neutrophils and found it triggered by sulphatides.

Sulphatide has been reported to activate leukocytes through L-selectin-dependent and -independent pathways [9,10]. Because of evidence that peripheral blood granulocytes can bind sulphatide after L-selectin shedding [10], we examined whether L-selectin might transmit signal to NF-κB activation. In the current study we show that L-selectins, when cross-linked, are able to activate NF-κB in neutrophils.

Recent evidence has demonstrated that L-selectin ligation and cross-linking upregulate Mac-1 surface expression [11,12]. In a recent publication [8] we have shown that sulphatide induced Mac-1-mediated attachment of human neutrophils to a collagen-coated surface. To determine if L-selectin was necessary for sulphatide-induced attachment, we determined PMN attachment to a collagen-coated surface after pretreatment of PMN with α-chymotrypsin, phorbol myristate acetate (PMA) and jasplakinolide, all known to induce L-selectin shedding [7,10,13–15]. We found that the adhesive response to sulphatide was abrogated in jasplakinolide-treated cells, partly abolished in PMA-treated cells and slightly diminished in α-chymotrypsin-treated cells. We conclude that L-selectin-independent signalling contributes to sulphatide-induced attachment of PMN to collagen.

2. Materials and methods

2.1. Reagents

Sulphatides and galactocerebrosides were obtained from Sigma (Deisenhofen, Germany); recombinant human TNF was from Collaborative Biomedical Research (Bedford, MA, USA). All oligonucleotides were kindly provided by Dr E.A. Romanova (Moscow State University, Russia). T4 polynucleotide kinase was purchased from SybEnzyme (Novosibirsk, Russia); poly(dI-dC) was from ICN (Cleveland, OH, USA); [γ-^{32}P]ATP was from Isotope (Obninsk, Russia).

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00061-9

Diisopropyl fluorophosphate (DFP) and 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF) were from Sigma (Deisenhofen, Germany); leupeptin and pepstatin A were from Fluka (Buchs, Switzerland); phenylmethylsulphonyl fluoride (PMSF) was from Nacalai Tesque (Kyoto, Japan). Acid-soluble collagen type I was from Boehringer Mannheim (Mannheim, Germany). Monoclonal anti-human L-selectin antibody (mAb, clone LAM1-116) and monoclonal mouse IgG1 (clone MOPC31C) were from Alexis (Laufelfingen, Switzerland). Secondary, cross-linking goat anti-mouse IgG antibody ($F(ab')^2$ fragment) was from Sigma (Steinheim, Germany). All other reagents were molecular biology grade.

2.2. Neutrophil isolation and stimulation

PMN were isolated from freshly drawn citrate-anticoagulated donor blood. Leukocyte-rich plasma was prepared by 0.75% dextran T-500 sedimentation of erythrocytes at room temperature. Granulocytes were prepared from leukocyte-rich plasma by density gradient centrifugation on a bilayer gradient of Ficoll-Paque (1.077 and 1.123 g/ml densities). Granulocytes were washed twice in phosphate-buffered saline (PBS) buffer (0.144 g/l KH_2PO_4, 9 g/l NaCl, 0.795 g/l $Na_2HPO_4{\cdot}7H_2O$, pH 7.4), resuspended at 10^7/ml in Dulbecco's PBS (PBS buffer containing 1 g/l glucose and 0.098 g/l $MgSO_4$) without calcium chloride, and stored at room temperature.

Purified cells were resuspended in Hanks' buffer (phosphate buffer pH 7.4, containing 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose), at a final concentration of 5×10^6 cells/ml, and incubated at 37°C in silicone-coated flasks with gentle agitation. Then sulphatides, galactocerebrosides or TNF were added to cells and incubated for 20 min. In experiments with antibodies, aliquots of cells were incubated with 7.5 μg/ml anti-L-selectin or control mAb for 20 min at 37°C. In cross-linking experiments, aliquots of cells were first incubated with 7.5 μg/ml anti-L-selectin, then centrifuged, resuspended in Hanks' buffer and incubated with 15 μg/ml goat anti-mouse $F(ab')^2$. The incubations were terminated by adding ice-cold Dulbecco's PBS supplemented with DFP (1 mM, final concentration), prior to centrifugation at $1500\times g$ for 5 min at 4°C.

2.3. Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear extracts were prepared by a modified Dignam procedure [16], as follows. The pelleted cells were washed and resuspended in ice-cold hypotonic buffer (buffer A: 10 mM HEPES, pH 7.5, 10 mM KCl, 3 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol (DTT)) containing the following protease inhibitors: 1 mM PMSF, 2 mM DFP, 1 mM AEBSF, and 10 μg/ml (final concentration) each of aprotinin, leupeptin, pepstatin A, and bestatin. After 20 min incubation on ice, 0.1 volume of 2% NP-40 was added, the cells were vortexed and immediately centrifuged at $14\,000\times g$ for 1 min at 4°C. The pelleted nuclei were resuspended in ice-cold nuclear buffer (20 mM HEPES, pH 7.5, 25% glycerol, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM DTT, 1% NP-40) containing the protease inhibitors described above. After 20 min incubation on ice the suspension was twice frozen and thawed in liquid nitrogen and then centrifuged ($16\,000\times g$, 15 min, 4°C). The supernatant (nuclear extract) was aliquoted and stored in liquid nitrogen. Total protein concentration was determined according to the method of Bradford.

The NF-κB DNA binding activity was determined by an electrophoretic mobility shift assay (EMSA). The oligonucleotide used as a probe for EMSA was a 20-bp double-stranded construct 5′-GCCTCGGGATTTCCCATCTG-3′, 5′-CAGATGGGATTTCCCGGGAAATCCCGAGGC-3′ (S), κB site is underlined. Mutant oligonucleotide used in control experiments was 5′-GCCTCTCTATTTCCCATCTG-3′, 5′-CAGATGGGAAATAGAGAGGC-3′ (N). Nuclear extracts containing 8–12 μg of protein in 10 μl were incubated for 40 min on ice with 1 pmol of radiolabelled duplex (~200 000 cpm Cerenkov count) in 20 μl binding buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM DTT, 0.1% NP-40, 12% glycerol) supplemented with 20 μg bovine serum albumin, 2 mM DFP, and 2 μg poly(dI-dC). For competition or supershift experiments the binding was performed in the presence of 50× excess of unlabelled DNA duplex or 2 μg of specific polyclonal anti-p65 (Rel A) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The mixture was incubated for 20 min on ice prior to addition of ^{32}P-labelled specific DNA duplex. The resulting complexes were resolved by electrophoresis on 6% non-denaturing polyacrylamide gel. The bands on the gel were visualised on a phosphorimager (Molecular Dynamics PhosphoImager SI, USA).

2.4. PMN adhesion assay

Myeloperoxidase was used as a marker enzyme to measure PMN attachment to collagen adsorbed to plastic surfaces in static conditions. Plastic 24-well tissue culture plates were coated with type I collagen 75 μg/ml for 24 h. Before use they were incubated for 1.5 h in PBS with 0.2% human serum albumin, and then were thoroughly washed with PBS. PMN (5×10^5/well) were added to collagen-coated 24-well culture plates in 500 μl of Hanks' balanced salt solution with 10 mM HEPES. After 30 min incubation in a CO_2 incubator at 37°C to allow leukocyte adherence wells were washed two times with 500 μl PBS solution for removal of non-adherent PMN. The extent of adherence was measured after the addition of detergent and a myeloperoxidase substrate as described [17,18]. 300 μl of a solution of 5.5 mM *ortho*-phenylenediamine and 4 mM H_2O_2 in buffer (67 mM Na_2HPO_4, 35 mM citric acid, 0.1% Triton X-100, pH 5) were added to each well, and after 4 min, the reaction was stopped by the addition of an equal volume of 1 M H_2SO_4. Standard dilutions of PMN with or without tested compounds were used for calibration.

3. Results

To determine whether NF-κB was activated by sulphatides in neutrophils, the cells were cultured in the presence of 50 μg/ml sulphatides for 20 min prior to nuclear extract preparation

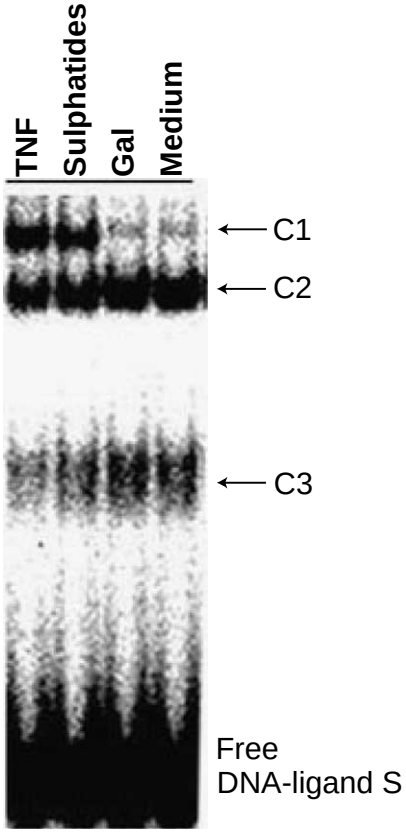

Fig. 1. Induction of NF-κB DNA binding activity in human neutrophils by sulphatides. Neutrophils were cultured for 20 min with 50 μg/ml of sulphatides or galactocerebrosides (gal). As a positive control, neutrophils were also stimulated with 10 ng/ml rh TNF. Nuclear extracts were then prepared and analysed in EMSA using a NF-κB DNA ligand S. Arrows indicate the positions of the three DNA–protein complexes. The amount of protein in the binding reaction was 5 μg. The result is representative of three separate experiments.

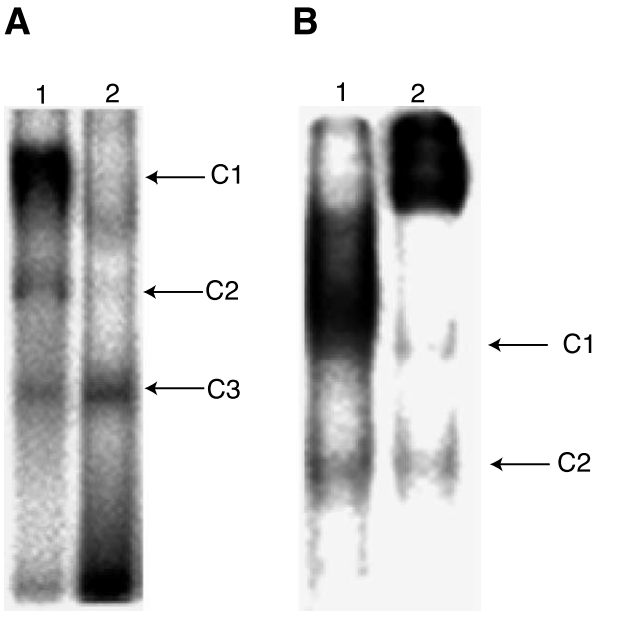

Fig. 2. Competition (A) and supershift (B) analysis of the DNA–protein complexes formed in response to sulphatides. A: Nuclear extracts were incubated with a ^{32}P-labelled NF-κB-specific DNA ligand S in the presence of a 50-fold excess of unlabelled non-specific DNA ligand N (lane 1), or specific DNA ligand S (lane 2). B: Nuclear extracts were incubated in the absence (lane 1) or in the presence (lane 2) of antibody raised against p65/RelA prior to the addition of labelled NF-κB-specific DNA ligand S and subsequent EMSA analysis.

and subsequent EMSA analysis. The efficiency of NF-κB binding to DNA (S) containing the κB recognition site was tested. As a negative control, neutrophils were stimulated with 50 μg/ml galactocerebrosides having the same glycolipid structure but lacking the sulphate group. As a positive control, the cells were stimulated with TNF, a stimulus previously shown to induce NF-κB in these cells [1].

As shown in Fig. 1, NF-κB binding activity (in C1 DNA–protein complex) was enhanced in nuclear extracts from sulphatide- and TNF-treated neutrophils. The non-sulphated analogue of sulphatide, galactocerebroside, had no effect on NF-κB activation. The specificity of DNA–protein interaction was confirmed by experiments with addition of excess unlabelled DNA duplex (S) containing a κB site as well as non-specific duplex (N). The specific DNA ligand reduced the amount of radioactive DNA–protein complex due to competitive binding, while the non-specific duplex did not (Fig. 2A).

NF-κB in neutrophils is well-characterised [1]. Therefore to prove the protein component of the DNA–protein complex we used only one type of antiserum, namely, antiserum against the p65 subunit of NF-κB. As shown in Fig. 2B, antiserum to p65 subunit completely supershifted the specific DNA–protein complex. This fact allowed us to conclude that the complex contained the p65 subunit of NF-κB.

Some studies [19,20] dealing with NF-κB in neutrophils underline the problem of NF-κB degradation in the course of preparing the nuclear extract. Our method and a spectrum of inhibitors employed allowed us to obtain the nuclear extract with the complete content of NF-κB protein. The specific DNA–protein complex from the nuclear extract of neutrophils comigrated with the specific DNA–protein complex from nuclear extracts of human umbilical vein endothelial cells (data not shown). Data presented in Fig. 3 show that sulphatides activated NF-κB in a concentration-dependent manner.

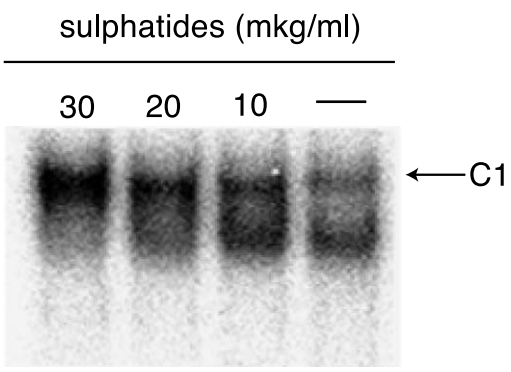

Fig. 3. Sulphatides activate NF-κB in a concentration-dependent manner. Neutrophils were incubated with different concentrations of sulphatides and then nuclear extracts were prepared and assayed for NF-κB binding activity to ^{32}P-labelled specific DNA ligand S by EMSA. The arrow indicates the position of the inducible complex. The amount of nuclear extract used was 4 μg of protein. The results are representative of three experiments.

To prove more definitely that L-selectin ligation can lead to NF-κB activation, NF-κB binding activity was determined in nuclear extracts from PMN incubated with primary anti-L-selectin antibody. We observed that binding of the primary antibody alone was insufficient to induce activation of NF-κB (Fig. 4). Accordingly, the effect of cross-linking L-selectin on NF-κB activation was examined. Cells were treated with primary anti-L-selectin mAb, washed, resuspended and incubated with goat anti-mouse Ig F(ab′)^2 fragment. After addition

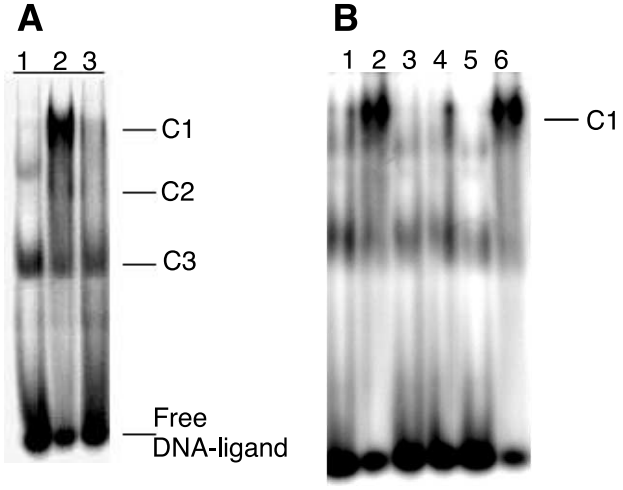

Fig. 4. A: Cross-linking of L-selectin leads to NF-κB activation. Neutrophils were incubated with 7.5 μg/ml anti-L-selectin (2,3) or control (1) mAb for 20 min at 37°C, washed, centrifuged, resuspended and incubated without (1,3) or with 15 μg/ml goat anti-mouse F(ab′)$_2$ fragment (2). Nuclear extracts were then prepared and analysed for NF-κB binding activity (see legend to Fig. 3). The amount of protein in the binding reaction was 5 μg. The result is representative of three experiments. B: Activation of NF-κB by sulphatides is inhibited by anti-L-selectin mAb. Neutrophils were incubated with 10 μg/ml anti-L-selectin (3,4) or control (5,6) mAb for 15 min at 37°C, then vehicle (1,4,5) or 50 μg/ml sulphatides (2,3,6) were added for 20 min at 37°C. Nuclear extracts were then prepared and analysed for NF-κB binding activity (see legend to Fig. 3). The amount of protein in the binding reaction was 9 μg. The result is representative of two experiments.

Table 1
Effect of α-chymotrypsin, jasplakinolide and PMA on sulphatide-stimulated attachment of human neutrophils to collagen

PMN–collagen interactions		PMN attachment to the surface (% of added cells)
Control		2.0 ± 0.7
Sulphatides, 50 μg/ml		73 ± 9
Galactocerebrosides, 50 μg/ml		2.2 ± 0.8
Anti-L-selectin		2.0 ± 1.1
L-selectin cross-linking		2.0 ± 0.9
Jasplakinolide, 500 nM		1.5 ± 0.5
Jasplakinolide (500 nM)+sulphatide (50 μg/ml)		2.0 ± 0.8
α-Chymotrypsin-treated cells	Control	3.4 ± 0.8
	Sulphatide	46 ± 5.8
PMA-treated cells	Control	33 ± 4.0
(10 min)	Sulphatide	13 ± 1.9
PMA-treated cells	Control	7.3 ± 1.2
(30 min)	Sulphatide	24 ± 3.1

Cells were incubated on a collagen-coated plastic surface without (control) or with 50 μg/ml sulphatides or 50 μg/ml galactocerebrosides for 30 min at 37°C. In experiments with mAbs the cells were pretreated with 7.5 μg/ml anti-L-selectin, washed, resuspended and incubated on a collagen-coated surface without or with 15 μg/ml goat anti-mouse $F(ab')_2$ fragment. In experiments with α-chymotrypsin, neutrophils were incubated with 10 U/ml chymotrypsin for 10 min at 37°C. After washing, the cells were resuspended and incubated on a collagen-coated surface without or with 50 μg/ml sulphatides for 30 min at 37°C. In experiments with jasplakinolide neutrophils were incubated on a collagen-coated surface with 500 nM jasplakinolide or with 500 nM jasplakinolide and 50μg/ml sulphatides for 30 min at 37°C. In experiments with PMA neutrophils were incubated with 100 nM PMA for 10 or 30 min at 37°C. After washing, the cells were resuspended and incubated on a collagen-coated surface without or with 50 μg/ml sulphatides for 30 min at 37°C. Percentage of adherent cells was determined after washing of non-adherent cells as described in Section 2. PMN attachment is expressed as percentage of PMN adhering in relation to the total number of PMN added. Values shown are the means ± S.D. of three experiments, each run in duplicate.

of the cross-linking antibody, NF-κB activation was clearly indicated (Fig. 4A).

To distinguish L-selectin-dependent and -independent components in sulphatide signalling, neutrophils were preincubated with anti-L-selectin mAb (LAM1-116) prior to the addition of sulphatides. L-selectin ligation with primary antibody inhibited the sulphatide-mediated response (Fig. 4B). Neutrophils were incubated with α-chymotrypsin or jasplakinolide, which produced shedding of L-selectin [7,13–15], before the addition of sulphatides. Both treatments abrogated the response to sulphatide, but α-chymotrypsin itself activated NF-κB in PMN (Fig. 5). PMA could also shed surface L-selectin [10], but activated NF-κB [1].

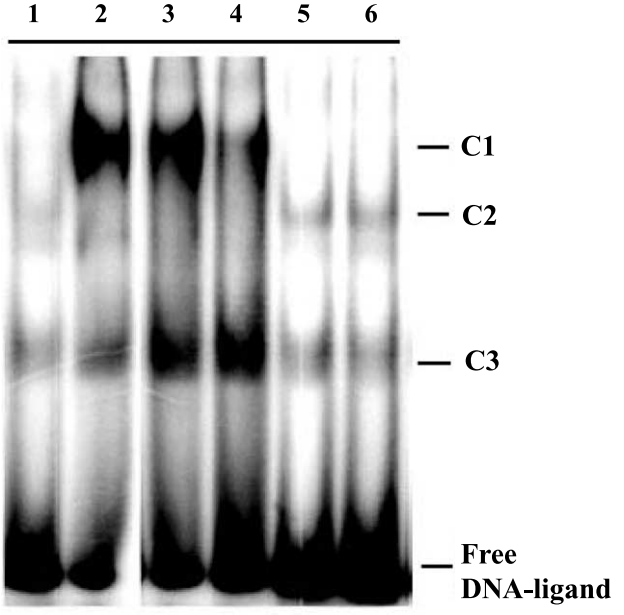

Fig. 5. α-Chymotrypsin and jasplakinolide pretreatments inhibit the response to sulphatides. Lines 1,2: Neutrophils were incubated without (1) or with (2) 50 μg/ml sulphatides for 20 min at 37°C. Lines 3,4: Neutrophils were incubated with 10 U/ml chymotrypsin for 10 min at 37°C. After washing the cells were untreated (3) or stimulated (4) with 50 μg/ml sulphatides for 20 min at 37°C. Lines 5,6: Neutrophils were incubated with 500 nM jasplakinolide+50 μg/ml sulphatides (5) or only with 500 nM jasplakinolide (6) for 20 min at 37°C. Nuclear extracts were then prepared and analysed for NF-κB binding activity (see legend to Fig. 3). The amount of protein in the binding reaction was 9 μg. The result is representative of two experiments.

Sulphatides and L-selectin cross-linking both activated NF-κB transcription factor. It is known that L-selectin cross-linking [12,21] and sulphatides [8] induce activation of β2 integrins. Do they signal in a similar way to PMN attachment onto a collagen-coated surface? Unlike sulphatides, the cross-linking did not induce PMN attachment to the surface (Table 1). To exclude binding of sulphatide to L-selectin in sulphatide–PMN interactions, we treated PMN with α-chymotrypsin, which is known to shed specifically L-selectin from the cell surface [7,13,14]. α-Chymotrypsin-treated cells preserved the ability to interact with sulphatides resulting in increased adhesion (about 60% of that of control-treated cells). Thus, sulphatides could activate PMN by L-selectin-dependent and -independent pathways. L-selectin-independent binding of sulphatides can be induced by the treatment of cells with PMA [10]. 10 min cell treatment with PMA corresponds to minimal L-selectin expression and minimal binding of sulphatide [10]. In these conditions PMA lowered the response to sulphatide from 73% (without PMA) to 13% (Table 1). 30 min cell treatment with PMA results in an increase of L-selectin-independent sulphatide binding [10] and an increase of PMN attachment (Table 1) that proves L-selectin-independent signaling to adhesion. Jasplakinolide, which is known to shed many surface receptors [15], totally abolished the response to sulphatide (Table 1).

4. Discussion

Activation of the NF-κB pathway in neutrophils occurs by the action of inflammatory stimuli like LPS, TNF and reactive

oxygen species. In vivo studies with sulphatides demonstrate that they have significant protective effects in neutrophil- and selectin-dependent models of lung injury [22]. In the current study we have for the first time demonstrated that sulphatide activated the transcription factor NF-κB and did it specifically since a non-sulphated analogue did not activate NF-κB.

Furthermore, we have shown that sulphatide signalling leading to NF-κB activation is L-selectin-dependent. This effect was not seen after treatment with galactocerebrosides which have the same glycolipid structure but lack the sulphate group and therefore do not bind L-selectin [23]. L-selectin ligation with primary anti-L-selectin mAbs inhibited the sulphatide-mediated NF-κB activation. α-Chymotrypsin selectively cleaves L-selectin [7,13,14], but no other neutrophil surface antigens from the cell surface, and we studied the effect of sulphatide on α-chymotrypsin-pretreated cells. We could not prove the lack of effect of sulphatide as α-chymotrypsin itself activated NF-κB. Jasplakinolide, known to downregulate cell surface expression of FcγRIIIb, CD43, CD44 and L-selectin [15], abolished NF-κB activation as well as sulphatide-induced attachment.

Using a primary antibody to L-selectin and cross-linking with a secondary antibody, we provide evidence that binding of the primary antibody alone to L-selectin is insufficient to activate NF-κB but cross-linking with a secondary antibody is required.

It is known that sulphatide induces TNF gene expression in human neutrophils [7] and TNF is a potential activator of NF-κB in human neutrophils. A number of κB binding sites is present in the regulatory region of mammalian TNF genes [24] and NF-κB binding is crucial for transcription of these genes [25]. Activation of the TNF promoter is mediated by NF-κB activation in cell response to many stimuli [26,27]. We propose that NF-κB activation promotes TNF gene expression in neutrophils treated with sulphatides. It is known that TNF is produced in a membrane-bound form and for TNF secretion the activity of sheddase is required [28]. In the fast response to sulphatides it is difficult to propose intermediate stages with TNF synthesis and shedding.

Our data suggest that NF-κB activation could be causally related to sulphatide-induced adhesion of neutrophils to solid surfaces coated by collagen. But the signalling pathways from sulphatides to NF-κB activation and to stimulation of the attachment seem to be distinct. α-Chymotrypsin, known to shed L-selectin, was unable to prevent sulphatide-stimulated attachment to collagen in agreement with the notion of L-selectin-independent binding of sulphatides to peripheral blood granulocytes [10]. We conclude that L-selectin-independent signalling contributes to sulphatide-induced adhesion. That jasplakinolide totally abolished the response to sulphatide one could propose from its ability to shed many surface receptors [15] and to stiffen cells blocking their ability to spread on the surface.

Based on the similar effects of sulphatide and L-selectin cross-linking on NF-κB activation, we expected also to find an increased attachment of PMN to the collagen-coated surface after L-selectin cross-linking as we observed after sulphatide. According to our data, this was not the case and we argue for a different signalling between adhesion by sulphatide and by L-selectin cross-linking.

Acknowledgements: This work was supported by DFG Research Group 'Endogene Gewebszerstörungen – Mechanismen der Autodestruktion', by the Russian Foundation 'Universities of Russia' (Grants UR.07.03.007 and UR.07.03.015) and by the Russian Foundation of Basic Research (Grant 01-04-48564). We also thank the Collective Use Center (Grant N 96-04-55009) for access to the PhosphoImager.

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