Heparan Sulfate Proteoglycans Are Involved in Opiate Receptor-Mediated Cell Migration

Nicole C. Kaneider, Stefan Dunzendorfer, and Christian J. Wiedermann*

Division of General Internal Medicine, Department of Internal Medicine, University of Innsbruck, Innsbruck, Austria

Received August 7, 2003; Revised Manuscript Received September 28, 2003

ABSTRACT: Opioid receptors are expressed in cells of the immune system, and potent immunomodulatory effects of their natural and synthetic ligands have been reported. In some studies, the opiate receptor antagonist naloxone itself displayed immunomodulatory actions. We investigated effects of naloxone on leukocyte chemotaxis. Cell migration was tested in micropore filter assays using modified Boyden chambers, and receptor expression was investigated using radiolabel binding assays. Naloxone induced peripheral blood nonadherent mononuclear cell and neutrophil chemotaxis at nanomolar concentrations and deactivated their migration toward β-endorphin, angiotensin II, somatostatin, or interleukin-8 but not toward RANTES, vasoactive intestinal peptide, or substance P. Ligand binding studies showed no alteration in the binding of interleukin-8 to neutrophils by naloxone. Cleavage of heparan sulfate from proteoglycans on the cells' surface completely inhibited chemotactic and deactivating properties of naloxone but not other attractants. Chemotactic properties were abolished by pretreating cells with heparinase, chondroitinase, sodium chlorate, and anti-syndecan-4 antibodies, indicating the involvement of syndecan-4. The extent of migration toward naloxone was diminished by pretreatment with dimethylsphingosine, a specific sphingosine kinase inhibitor. As syndecan-4 signaling in leukocyte chemotaxis involves activation of sphingosine kinase, results indicate that naloxone interacts with syndecan-4 function in cell migration and suggest a role for heparan sulfate proteoglycans as coreceptors to members of the δ-opiate receptor family.

Endogenous opioids are known for their immunomodulatory signals that regulate the trafficking of immune response cells (*1*−*4*). Classical pharmacological and biochemical studies have defined three classes of opiate receptors (δ, μ, and κ) that differ in their affinity for various ligands and their distribution within the immune system (*5*). In inflamed tissue, they can be found in lymphocytes, monocytes, macrophages, and neutrophils (*6*). Agonists to the μ-, δ-, and κ-opioid receptors induce a chemotactic response in various leukocyte subsets (*7*), and functional opiate receptor expression in leukocytes may have species specific variability (*5*, *6*, *8*). Recent results in neutrophils identified nociceptin and the novel opiate receptor, opiate receptor like-1 (ORL-1),[1] as an additional potent opiate neutrophil attractant and chemotaxis receptor, respectively (*9*). ORL-1 was cloned from highly conserved regions of the δ-opioid and somatostatin receptor and identified as a new member of the opiate receptor family (*10*).

Observations suggest that δ-like opioid peptides released from embryonic mixed brain cells could be responsible for the infiltration of the developing central nervous system by macrophage precursors (*11*). Opioid receptors may function to promote cellular migration by regulating chemokine receptor expression (*12*). Activation of constitutively expressed μ-opioid receptors inhibits microglial cell chemotaxis toward C5a (*13*), and migratory effects of opioids have been reported not only for glia or leukocytes but also for several other normal and malignant cell types (*14*−*16*), further indicating the potential importance of opoid-dependent regulation of leukocyte trafficking.

Opioids themselves may act as chemoattractants for leukocytes, but addition of opioid agonists to chemokines would hinder the chemoattractant ability of the chemokines (*4*). Opioid and chemokine receptors, both of which are shown to regulate leukocyte migration, are members of the G_i protein-linked seven-transmembrane receptor family. These receptors, as well as the chemokine and endogenous opioid peptide ligands, are widely distributed in brain tissue and the periphery. Pretreatment with opioids, including morphine, heroin, met-enkephalin, the selective μ-agonist [D-Ala^2,*N*-Me-Phe-4,Glyol^5]enkephalin, or the selective δ-agonist [D-Pen^2,D-Pen^5]enkephalin, leads to the inhibition of the chemotactic response of leukocytes to complement-derived chemotactic factors (*17*) and to several chemokines (*18*). The chemotactic activities of both μ- and δ-opioid receptors are also desensitized following activation of chemokine receptors. Szabo et al. (*4*) showed that cross-deactivation of opioid receptors by chemokines and vice versa is accompanied by phosphorylation of G protein-coupled receptors.

* To whom correspondence should be addressed: Department of Internal Medicine, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria. Phone: 0043-512-504-3255. Fax: 0043-512-504-4201. E-mail: christian.wiedermann@uibk.ac.at.

[1] Abbreviations: ORL-1, opiate receptor-like 1; HSPG, heparan sulfate proteoglycans; PBL, peripheral blood lymphocytes; IL-8, interleukin-8; RANTES, regulated on activation, normal T cell expressed and secreted; fMLP, formylpeptide; DMS, dimethylsphingosine; AT-II, angiotensin II; SST, somatostatin; SP, substance P; VIP, vasointestinal polypeptide; SEM, standard error of the mean.

10.1021/bi035295i CCC: $27.50
Published on Web 12/12/2003

In addition to the question of how ligation of opiate and chemokine receptors cross-deactivates each other's response (*4*, *18*), recent interest focuses on mechanisms that regulate immune cell function in a non-opioid-dependent fashion. Naloxone is a nonselective, competitive antagonist to the three opiate receptors (*6*) and, not surprisingly, is able to affect opiate receptor-mediated migration of monocytes and lymphocytes (*1*, *19*). However, the antagonist itself has been shown to exert immunomodulatory effects on human neutrophil respiratory burst activity (*20*, *21*). With regard to cell migration, as early as 1985, naloxone was described to preserve *in vitro* migration of gliadin-exposed leukocytes from patients with coelicac disease in the absence of opiate receptor agonists (*22*). Naloxone was found to prevent both opioid and opioid-unrelated increases in the extent of granulocyte migration *in vitro* (*23*). Besides being able to inhibit morphin-induced migration, the (−)- but not the (+)-naloxone isomer proved to abolish either casein-, serum-, or formylpeptide-induced chemotaxis. It was concluded that opioid-induced modulation of leukocyte migration is likely to be mediated through opiate receptors, but the antichemotactic effect of naloxone suggested an involvement of as yet unexplained mechanisms (*24*).

When stimulated with antithrombin, migration of neutrophils, monocytes, and lymphocytes is abolished by removal of heparan sulfate chains or addition of anti-syndecan-4 antibodies (*25*, *26*), and disruption of the syndecan-4 gene in primary fibroblasts abolishes migration during *in vitro* wound healing assays (*27*). It appears that cell migration directed by either extracellular matrix or soluble factors may be dependent on the heparan sulfate chains of syndecan-4. Syndecan-4 is a ubiquitous transmembrane proteoglycan that localizes to the focal adhesions of adherent cells and binds to a range of extracellular ligands, including growth factors and chemokines. The cytoplasmic domain of syndecan-4 interacts with a number of signaling and structural proteins, and both extracellular and cytoplasmic domains are necessary for regulated activation of associated transmembrane receptors (*28*).

Since naloxone itself not only inhibits leukocyte migration (*23*, *24*) but also hinders proliferation of leukemic cells (*29*), we investigated the possible involvement of syndecan-4 in direct effects of naloxone on leukocyte migration and in particular its functional interaction with the cell surface heparan sulfate proteoglycans (HSPG). We report on naloxone-dependent leukocyte migration that is abolished by removal of heparan sulfate chains from the cells' surface.

MATERIALS AND METHODS

Cell Preparations. Human neutrophils were obtained from the peripheral blood of healthy donors after discontinuous density gradient centrifugation on Percoll by dextran sedimentation and centrifugation through a layer of Ficoll-Hypaque, followed by hypotonic lysis of contaminating erythrocytes using a sodium chloride solution (*30*). Cell preparations yielded more than 95% neutrophils (by morphology in Giemsa stains) and more than 99% viability (by trypan dye exclusion). Except in binding experiments, where a binding buffer was used, all other neutrophil experiments were performed with RPMI 1640/0.5% BSA (Aventis Behring, Marburg, Germany).

Mononuclear leukocytes (MNL) were isolated from EDTA-treated peripheral blood by using Lymphoprep density gradient centrifugation (Nycomed Pharma AS, Oslo, Norway) as previously described (*31*). The MNL preparations were resuspended in RPMI 1640/10% FCS (Biological Industries, Kibbutz Beit Haemek, Isreal) and incubated at a density of 10^6 cells/mL at 38 °C for 30 min. Nonadherent MNL [peripheral blood lymphocytes (PBL)] were placed into new tissue culture dishes and incubated for an additional 90 min. PBL were then washed and resuspended in RPMI 1640/0.5% BSA.

Migration Assay. Migration of leukocytes into cellulose nitrate to gradients of soluble attractants was assessed using a 48-well Boyden microchemotaxis chamber (Neuroprobe, Bethesda, MD) in which a 5 μm pore-sized filter (Sartorius, Göttingen, Germany) separated the upper chamber from the lower chamber. β-Endorphin, vasoactive intestinal peptide (VIP), and substance P (SP) were from Neosystem (Strassbourg, France). RANTES was purchased from Peprotech (London, U.K.). Interleukin-8 (IL-8) was from Roche (Basel, Switzerland). Naloxone, naltrindole (δ-antagonist), norbinaltrophimine (κ-antagonist), and CTOP (μ-antagonist) were from Research Biochemicals International (Natick, MA).

For deactivation of migration, leukocytes were pretreated with naloxone for 20 min, followed by two washing steps, before chemotaxis was tested. In some experiments, cells were incubated with heparinase or chondroitinase ABC (Sigma Chemical Corp., St. Louis, MO) for a total time of 50 min prior to migration experiments (*30*). As leukocyte migration might be mediated via syndecan-4, chemotaxis experiments were performed in the presence of antibodies against the core protein of syndecan-4 (D-16) and the ectodomain of this proteoglycan (5G9) (both from Santa Cruz Inc., Wiltshire, England). Since sodium chlorate is able to modify proteoglycan sulfation, we tested naloxone chemotaxis after a 20 min pretreatment of cells with sodium chlorate (Merck, Darmstadt, Germany). Dimethylsphingosine (DMS), a specific inhibitor of sphingosine kinase, was from Biomol (Plymouth Meeting, PA). The time for migration into the filters was 30 min for neutrophils and 120 min for lymphocytes. After the migration time, the filters were dehydrated, fixed, and stained with hematoxylin eosin. The depth of migration of cells into the filter was quantified by microscopy, measuring the distance (micrometers) from the surface of the filter to the leading front of cells, before any cells had reached the lower surface (leading front assay). Data are expressed as the "chemotaxis index", which is the ratio of the distance of stimulated and random migration of leukocytes into the nitrocellulose filters. Mean distances of random migration were around 55 and 60 μm for neutrophils and PBL, respectively. Day-to-day variability of neutrophil migration toward the positive control IL-8 (1 nmol/L) resulted in a variability coefficient of 0.127 ($n = 4$); the variabilty coefficient for lymphocyte migration toward IL-8 (1 nmol/L) (*32*−*34*) was 0.243 ($n = 4$).

Binding Experiments. Human neutrophils (10^7 cells/mL) were incubated with sodium iodide [^{125}I]IL-8 (2000 Ci/mM) at a concentration of 10 fmol/L with or without an excess of unlabeled IL-8, or naloxone at various concentrations, in a total volume of 400 μL of PBS/0.5% BSA (binding buffer), for 90 min at 4 °C. After incubation, cells were washed twice

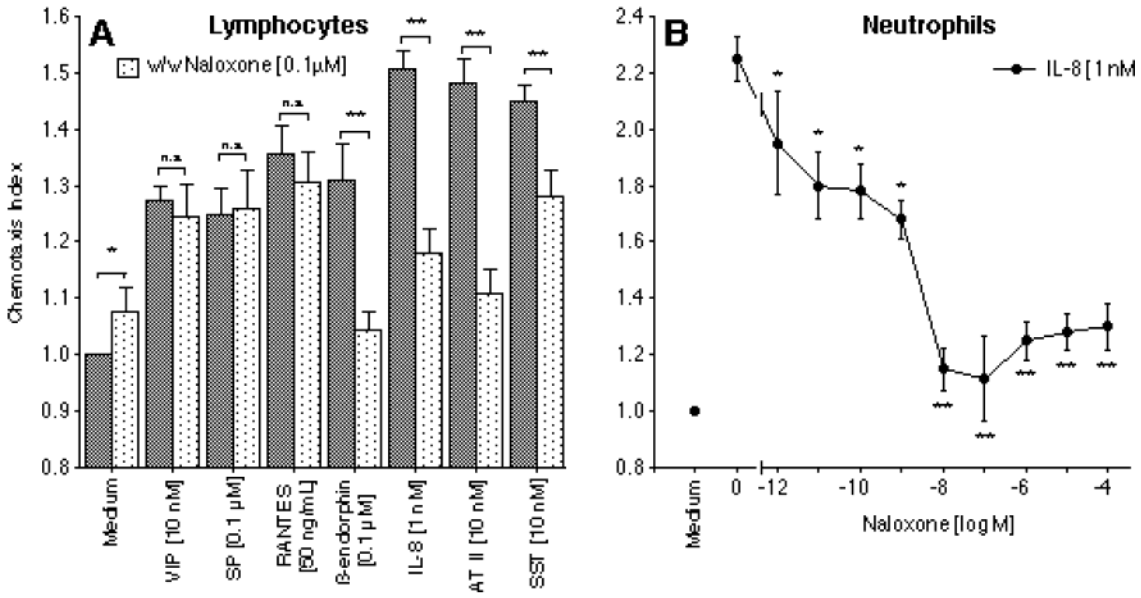

FIGURE 1: Deactivation of lymphocyte and neutrophil chemotaxis by naloxone. Cells were pretreated for 20 min with naloxone, and after washing had been carried out, PBL shown in the left panel (A) migrated toward various chemoattractants (VIP, SP, RANTES, β-endorphin, IL-8, AT-II, and SST) and neutrophils shown in the right panel (B) were attracted by IL-8. Migration time periods for lymphocytes and neutrophils were 90 and 30 min, respectively. Migration depth was quantified microscopically by the leading front assay. Data are expressed as the chemotaxis index, which is the ratio of the distance of stimulated and random migration of leukocytes into the nitrocellulose filters ($n = 7$). In the Mann Whitney U test after Kruskal Wallis ANOVA, one asterisk denotes a P of <0.05 and two asterisks denote a P of <0.01.

with PBS/0.5% BSA containing 0.5 mol/L saline and centrifuged at 200*g* for 10 min. The bottom of the tube containing the neutrophil pellet was counted in a Beckman γ counter. Results are expressed as counts per minute (cpm). Specific binding is defined as total binding minus nonspecific binding, which is the residual cpm bound in the presence of a 100-fold excess of unlabeled IL-8 (*30*).

Statistics. Data are expressed as means ± SEM of the CI or of cpm. Means were compared by the Mann Whitney test after Kruskal Wallis ANOVA. A P value of <0.05 was considered significant.

RESULTS

Selective Deactivation of Leukocyte Chemotaxis by Naloxone. RANTES and VIP, SP, or β-endorphin are prototypical lymphocyte attractant chemokines and neuropeptides, respectively (*19*, *31*, *35*). In addition, migratory responses of lymphocytes have been observed toward IL-8 (*36*, *37*), a well-established neutrophil attractant (*36*), somatostatin (SST) (*38*), and angiotensin II (AT-II) (*39*, *40*). IL-8, β-endorphin, SST, and AT-II exert their cellular effects via specific receptors that belong to the δ-opiate receptor family (*41*). We, therefore, tested the effects of naloxone on migration of PBL toward these various attractants.

PBL migrated toward the neuropeptides VIP (10 nM), SP (0.1 μM), or β-endorphin (0.1 μM), as well as toward IL-8 (1 nM), SST (10 nM), and AT-II (10 nM). RANTES (50 ng/mL) served as a positive control for PBL chemoattraction. All attractants induced significant PBL chemotaxis. Pretreatment of the cells with naloxone did not affect VIP-, SP-, or RANTES-induced chemotaxis of PBL but significantly hindered migration toward non-opioid ligands to the δ-opiate receptor family, SST, and AT-II or toward β-endorphin or IL-8 (Figure 1A). Noteably, in the absence of chemoattractants, naloxone itself exerted a significant chemotactic effect (Figure 1A).

The inhibitory effect of naloxone on cell migration was also observed in neutrophils that were attracted by IL-8 (1 nM); the effect was dose-dependent and strongest at equimolar concentrations of naloxone (Figure 1B). Similar results were obtained when leukocytes were exposed to a combination of naloxone and the attractants in the lower chambers (data not shown). When AT-II or SST was used for pretreatment followed by chemotaxis toward IL-8, deactivation of neutrophil migration also occurred (data not shown).

No Effect of Naloxone on Binding of IL-8 to Neutrophils. IL-8 is a potent chemokine that has a key role in the recruitment of neutrophils. IL-8 reacts in the recruitment of neutrophils via two distinct types of IL-8 receptors (*42*). As naloxone inhibited IL-8-induced chemotaxis, we wanted to exclude the possibility that it impairs binding of IL-8 to its binding sites.

Concomitant incubation of neutrophils with radiolabeled IL-8 and various concentrations of unlabeled IL-8 displaced the [^{125}I]IL-8 from its receptor on the cell surface in a typical manner. Naloxone, even at high concentrations (up to 1 mM), failed to affect binding of IL-8 to neutrophils (Figure 2).

Chemotactic Effects of Naloxone on Lymphocytes and Neutrophils. To test whether naloxone may directly stimulate migration in a well-defined assay system using isolated cells, the nonselective opioid receptor antagonist was tested for its ability to induce chemotaxis. Both cell types, PBL and neutrophils, migrated in a dose-dependent manner toward gradients of naloxone that was put into the lower wells of the chemotaxis chamber; maximal chemotaxis was induced in lymphocytes at 0.1 nM and in neutrophils at 0.1 μM (Figure 3). The selective opioid receptor antagonists naltrindole (δ-antagonist), nor-binaltrophimine (κ-antagonist), and

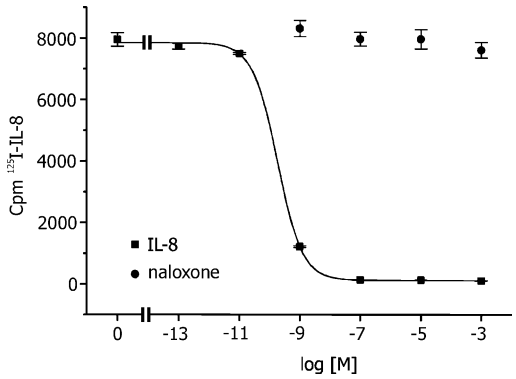

FIGURE 2: Competitive binding of ^{125}I-labeled IL-8 with neutrophils. Human neutrophils (10^7 cells/mL) were incubated with radiolabeled IL-8 for 90 min at 4 °C in the presence of increasing concentrations of unlabeled IL-8 or naloxone. Bound and unbound radiologand were separated by centrifugation, and specific binding was assessed. Results are expressed as mean ± SEM of counts per minute (cpm) ($n = 3$).

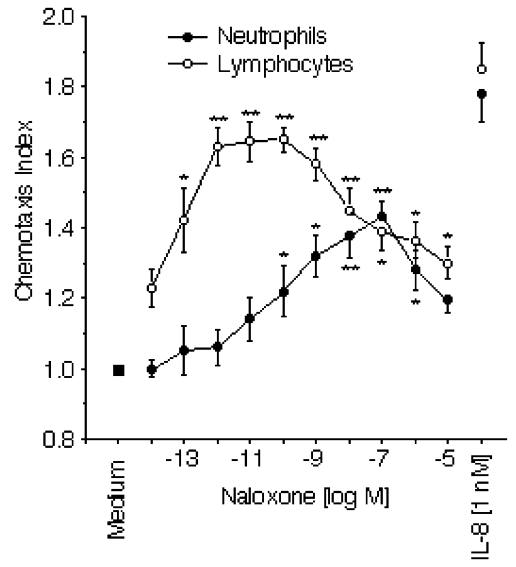

FIGURE 3: Lymphocyte and neutrophil chemotaxis toward naloxone. Untreated lymphocytes or neutrophils migrated toward various concentrations of naloxone in the lower wells of a Boyden microchemotaxis chamber. IL-8 served as a positive control. Migration time periods for lymphocytes and neutrophils were 90 and 30 min, respectively. Migration depth was quantified microscopically by the leading front assay. Data are expressed as the chemotaxis index, which is the ratio of the distance of stimulated and random migration of leukocytes into the nitrocellulose filters ($n = 5$). In the Mann Whitney *U* test after Kruskal Wallis ANOVA, one asterisk denotes a *P* of <0.05 and two asterisks denote a *P* of <0.01.

CTOP (μ-antagonist) that were tested in the same way failed to show such an effect (data not shown).

Inhibition of Naloxone-Induced Neutrophil Chemotaxis by Heparinase, Chondroitinase, and Sodium Chlorate. Syndecan-4 participates in focal adhesion by non-G protein-dependent activation of protein kinase C (*43*). Ligation of HSPG leads to directional migration of leukocytes (*25*, *26*). To investigate a role of this intact HSPG for naloxone-induced migration, neutrophils and PBL were pretreated for 50 min with heparinase I (50 nanounits/mL to 50 milliunits/mL) or chondroitinase (50 nanounits/mL to 50 milliunits/mL) at 37 °C, followed by washing. Experiments were performed with both heparinase I and chondroitinase to differentiate between the two HSPG, glypicans and syndecans. Glypicans carry heparan sulfate but not chondroitin sulfate side chains, whereas syndecans carry both (*44*).

Table 1: Effects of Heparinase on the Naloxone-Induced Deactivation of IL-8 Chemotaxis[a]

pretreatment	chemotaxis toward IL-8 (1 nmol/L)
medium	2.28 ± 0.3
heparinase (50 milliunits/mL)	2.28 ± 0.2
naloxone (100 nmol/L)	1.21 ± 0.1[b]
heparinase (50 milliunits/mL) followed by naloxone (100 nmol/L)	2.38 ± 0.1[c]

[a] Neutrophils were pretreated for 50 min at 37 °C with heparinase or medium. After being washed, cells were incubated with naloxone or medium for deactivation experiments. Then migration toward IL-8 was assessed. Data are expressed as the chemotaxis index, which is the ratio of the distance of stimulated and random migration of leukocytes into the nitrocellulose filters. Mann Whitney *U* test (after Kruskal Wallis ANOVA, $p < 0.05$); $n = 3$. [b] Effect of naloxone vs pretreatment with medium ($p < 0.05$). [c] Effect of heparinase on naloxone-induced chemotaxis deactivation ($p < 0.05$).

Chemotactic effects of naloxone (0.1 μmol/L) on neutrophils were found to be completely abolished by pretreatment with either heparinase I or chondroitinase, whereas chemotactic effects of formylpeptide or IL-8 remained unchanged (Figure 4A,B). The opiate receptor antagonist also lost its ability to deactivate neutrophil chemotaxis toward IL-8 (1 nM) when cells were treated with heparinase I prior to concomitant incubation with naloxone and the chemokine (Table 1).

To investigate the effect of sodium chlorate, which modifies sulfation of syndecans in cell culture, neutrophils were pretreated with sodium chlorate (10−40 mmol/L), and after cells had been washed twice, naloxone-induced (0.1 μmol/L) chemotaxis was tested. Neutrophil chemotaxis toward naloxone was significantly inhibited by sodium chlorate (Figure 4C).

Inhibition of Directed Neutrophil Migration toward Naloxone by Antibodies to Syndecan-4. Inhibition of naloxone-induced chemotaxis of leukocytes by both chondroitinase and heparinase I suggested syndecan involvement. It is known from previous studies that cell migration toward non-G protein-coupled receptors relates to function of syndecan-4 (*25*, *26*, *45*). Chemotaxis experiments with naloxone were therefore performed using two different antibodies to syndecan-4 (D-16 and 5G9) that block this function. Cells were pretreated with the two antibodies and then allowed to migrate toward naloxone (0.1 μmol/L). Antibodies to the syndecan-4 core protein and to the ectodomain of syndecan-4 inhibited directed migration of leukocytes toward naloxone in a concentration-dependent manner (Figure 4D). RANTES-induced chemotaxis was not affected by pretreatment of cells with these antibodies (data not shown).

Inhibition of Naloxone-Induced Leukocyte Chemotaxis by Dimethylsphingosine. Ligation of syndecan-4 elicits pertussis toxin-sensitive chemotaxis of leukocytes via protein kinase C (*45*). Activation of protein kinase C stimulates release of sphingosine 1-phosphate, a chemoattracting G protein-coupled receptor agonist (*46*). To study sphingosine kinase-dependent signaling of naloxone in leukocyte migration, cells were treated with DMS, an inhibitor of sphingosine kinase, an enzyme that synthesizes sphingosine 1-phosphate (*46*).

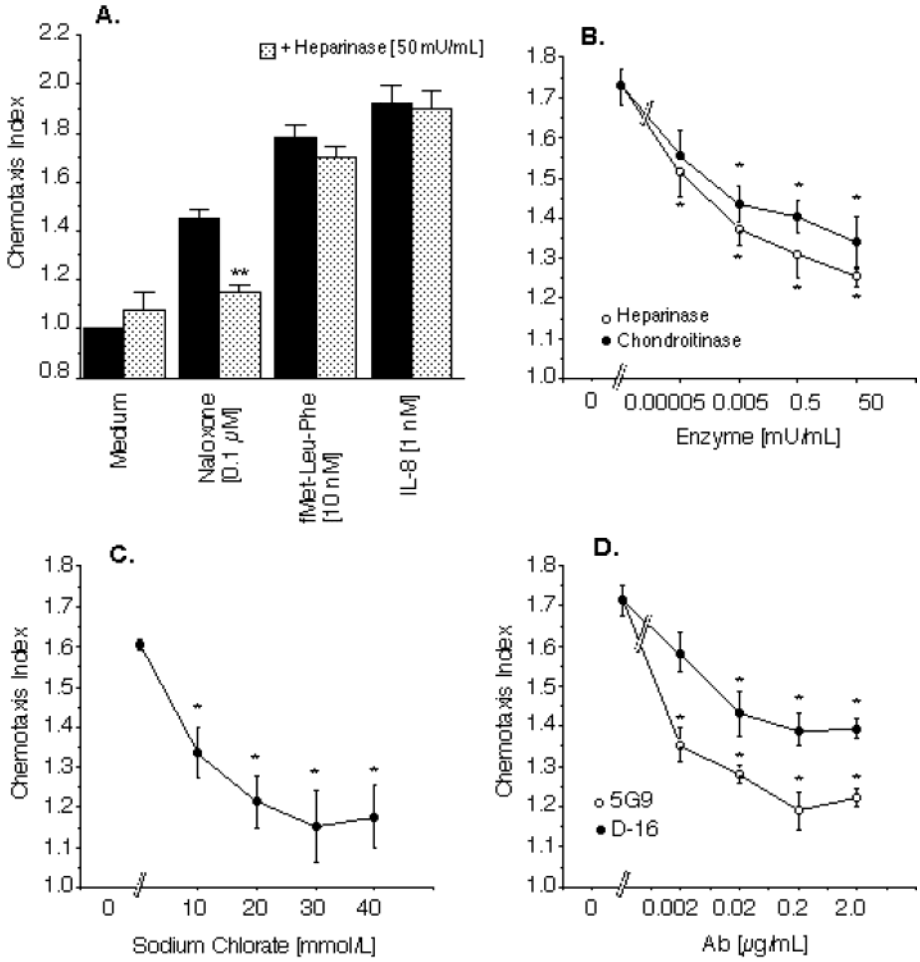

FIGURE 4: Effects of heparinase, chondroitinase, sodium chlorate, and anti-syndecan-4 antibodies on naloxone-induced chemotaxis of neutrophils. Panels A−C show the effects of heparinase, chondroitinase, and sodium chlorate, respectively. (A) After treatment of human neutrophils with heparinase and washing, cells migrated toward naloxone, formylpeptide (fMet-Leu-Phe), or IL-8. (B) Dose-dependent effects of enzyme pretreatment on naloxone-induced (100 nmol/L) migration of neutrophils. (C) Dose-dependent effect of pretreatment of human neutrophils with sodium chlorate on naloxone-induced (100 nmol/L) migration. Panel D shows the dose-dependent effects of preincubating human neutrophils with the monoclonal antibodies to either the syndecan-4 core protein (D-16) or the syndecan-4 ectodomain (5G9) on naloxone-induced (100 nmol/L) migration. Data are expressed as the chemotaxis index, which is the ratio of the distance of stimulated and random migration into the nitrocellulose filters ($n = 5$). Two asterisks denote a P of <0.01 in the Mann Whitney U test after Kruskal Wallis ANOVA.

Cells, preincubated with DMS (100 amol/L to 100 nmol/L) for 30 min, were allowed to migrate toward naloxone in the lower wells of the chemotaxis chamber. Treatment with DMS abbrogated naloxone- but not IL-8-induced chemotaxis in a concentration-dependent manner (Figure 5).

DISCUSSION

Cell motility is a prerequisite in developmental biology, host defense, and tissue repair. Leukocyte migration is crucial during the development of the immune system and in the responses to infection, inflammation, and tumor rejection. The relationship of the leukocyte with its environment occurs through different kinds of receptors that interact with ligands that are soluble, fixed on the membrane of other cells, or immobilized on the extracellular matrix. Leukocytes navigate toward their target sites by constantly orienting themselves in response to specific signals in their surroundings. Chemokines, cytokines, growth factors, and neuropeptides are key biological mediators that provide such signals for cell navigation (*47*).

Here we report that treatment of leukocytes with naloxone not only inhibited chemotaxis of the cells toward the well-known μ-receptor agonist, β-endorphin, but also hindered migration toward AT-II, SST, or IL-8. A common feature of these different ligands, from the respective neuroendocrine polypeptide and chemokine families, is that their putative receptors share significant sequence homology or belong to the family of δ-opiate receptors (*41*). Moreover, naloxone not only inhibited leukocyte migration toward this particular group of non-opioid attractants but also itself exerted chemotactic effects in neutrophils and lymphocytes. This suggests that receptor cross-deactivation (*30*) could be consistent with our finding that preincubation of monocytes or neutrophils with opiate receptor ligands, met-enkephalin or morphin, prevented their subsequent chemotaxis in response to the chemokine macrophage inflammatory protein-1α or IL-8 (*18*). Bidirectional, heterologous desensitization of opioid and chemokine receptors may occur regularly (*4*). Furthermore, enkephalin and endorphin are both synthesized in neutrophils, and enkephalin is also secreted upon stimula-

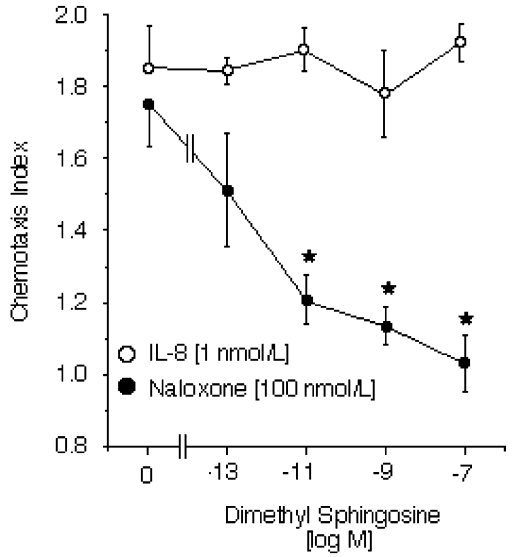

FIGURE 5: Effects of dimethylsphingosine on neutrophil migration. To investigate whether activation of sphingosine kinase is involved in naloxone-induced chemotaxis, neutrophils were incubated with dimethylsphingosine (DMS), a sphingosine kinase inhibitor, for 30 min. After washing had been carried out, cells were allowed to migrate toward naloxone (100 nmol/L) or IL-8 (1 nmol/L). Data are expressed as the chemotaxis index, which is the ratio of the distance of stimulated and random migration of leukocytes into the nitrocellulose filters ($n = 3$). One asterisk denotes a P of <0.05 in the Mann Whitney U test after Kruskal Wallis ANOVA.

tion (*48*, *49*). However, these data do not explain the mechanisms by which naloxone itself may induce chemotaxis or deactivate migration toward non-opioid mediators as it lacks agonistic activity of opiate receptors.

Naloxone binds to opiate receptors with high affinity but fails to activate receptor signaling, and it blocks signaling in response to opiate receptor agonists (*50*). There are reports that protein kinase C activity can be upregulated by naloxone, but this effect was observed only after naloxone-precipitated opiate withdrawal (*51*). Most such effects of naloxone are reported from *in vivo* experiments and could be ascribed to the removal of the regulatory effects exerted by endogenous opioid peptides in different organ systems, including the immune system, where activation T(H)2 and suppression T(H)1 cytokines with naloxone were observed (*52*). Endogenous opioids may be present in lymphocytes (*53*−*55*), but the presence or release from isolated neutrophils has not yet been reported. Since effects of naloxone on leukocyte migration were seen in neutrophils as well as in PBL, removal of autocrine regulatory effects of endogenous opioids by naloxone *in vitro* appears to be rather unlikely.

Because in our study naloxone inhibited chemotaxis of leukocytes toward IL-8, which is an established chemokine, we next focused on IL-8 receptors. Dimeric IL-8 was shown to be a heparin-binding molecule; beside specific receptor ligation, IL-8 binds to proteoglycans on leukocytes (*56*) and may, therefore, be present on isolated cells and released. Receptor binding studies in neutrophils, however, demonstrated that naloxone is unable to compete with the specific binding of IL-8 to its receptors. If IL-8 or other chemokine receptors are involved in the migratory effects of naloxone in leukocytes, trans-activation and cross-deactivation rather than direct effects on the receptors may take place.

Trans-activation of endothelial differentiation gene-1, which is a heterotrimeric guanine nucleotide binding protein-coupled receptor for sphingosine 1-phosphate, induces cell migration toward platelet-derived growth factor, which stimulates sphingosine kinase and increases the intracellular level of sphingosine 1-phosphate (*57*). Inhibition of sphingosine kinase suppressed chemotaxis not only toward platelet-derived growth factor (*57*) but also toward antithrombin which induces leukocyte migration via ligation of syndecan-4 and activation of protein kinase C (*45*). The core protein of syndecan-4, a member of the proteoglycan receptor family, can directly bind the catalytic domain of protein kinase C (*58*). Whether such receptor cross-communication is critical for naloxone-dependent cell motility was therefore explored.

To verify the involvement of heparan sulfate of syndecan-4 in naloxone-dependent migration of leukocytes, we used the specificity of heparinase I for highly sulfated polysaccharide chains containing linkages to 2-O-sulfated α-L-iodopyranosyluronic acid residues. The same heparinase I has recently been described to degrade the synthetic heparin pentasaccharide to a disaccharide and trisaccharide (*59*). Results of the study presented here showed that cleavage of heparan sulfate chains on glycosaminoglycans of leukocytes abrogated the effects of naloxone on both stimulation of directed migration as well as deactivation of chemotaxis. Modification of proteoglycan sulfation with sodium chlorate (*25*) similarly abrogated the migratory effects of naloxone. The chemotactic activities of formylpeptide and IL-8 were not affected by the modifications to neutrophil HSPG, indicating that chemotaxis receptor function and migratory capabilities of the cells are intact.

Since syndecans carry both heparan sulfates and chondroitin sulfates, and chemotaxis was significantly abolished by pretreatment with heparinase and chondroitinase, the suggestion is that of the two HSPG, i.e., syndecans and glypicans, syndecans are involved in naloxone-dependent signaling and induction of migration. Syndecan-4 exists on lymphocytes and on a variety of mature macrophage-like cells as well as on human neutrophils and human PBMC (*25*, *26*, *60*). It is involved in assembling and migration of various cell types, and syndecan-4 blockade with specific antibodies abolishes its function (*25*, *26*, *28*). In the experiments described herein, inhibition of cell migration toward naloxone with anti-syndecan-4 antibodies was found, suggesting that syndecan-4 mediates effects of naloxone on cell migration.

Sphingosine 1-phosphate is a novel leukocyte chemoattractant that induces immature dendritic cell and natural killer cell chemotaxis via G protein-coupled sphingosine 1-phosphate receptors (*61*, *62*). Although most chemoattractants of leukocytes use G protein-coupled receptors for signal relay, non-G protein-coupled receptors are also involved, including receptors for transforming growth factor-β (*63*) and syndecan ligands (*25*, *26*). The mechanisms by which non-G protein-coupled receptors induce migration may involve trans-activation of sphingosine 1-phosphate receptors after induction of sphingosine kinase. Inhibition of sphingosine kinase with DMS abrogates such migratory responses (*57*). Inhibition of naloxone-dependent effects on leukocyte migration by DMS, as reported here, may indicate the involvement of this chemotaxis pathway in this study. DMS is considered a

specific inhibitor of sphingosine kinase (*64*) even though some inhibitory action for protein kinase C was also proposed (*65*). As leukocyte migration toward IL-8 which can be hindered by inhibitors of protein kinase C (*30*) was not affected by DMS, potential inhibition of protein kinase C by DMS may not be relevant for leukocyte migration here.

In summary, exposure of untouched, isolated lymphocytes or neutrophils to nanomolar concentrations of naloxone alters cell migration by involving HSPG and probably a sphingosine kinase-dependent pathway. More detailed mechanisms for the activation of leukocyte migration by the potent opiate receptor antagonist remain speculative. Interestingly, the migration inhibiting effects of naloxone clustered around chemotactic ligands of the δ-opiate receptor family. This receptor family has recently been used to clone ORL-1, a novel opiate chemotaxis receptor of neutrophils (*9*). Whether naloxone exerts its effect via ORL-1 or other ligand binding sites that are shared among members of the δ-opiate receptor family, as well as the precise character of the proposed coreceptor function of syndecan-4, requires further study.

REFERENCES

1. Ruff, M. R., Wahl, S. M., Mergenhagen, S., and Pert, C. B. (1985) Opiate receptor-mediated chemotaxis of human monocytes, *Neuropeptides 5*, 363−366.
2. Heagy, W., Laurance, M., Cohen, E., and Finberg, R. (1990) Neurohormones regulate T cell function, *J. Exp. Med. 171*, 1625−1633.
3. Van Epps, D. E., and Saland, L. (1984) β-Endorphin and met-enkephalin stimulate human peripheral blood mononuclear cell chemotaxis, *J. Immunol. 132*, 3046−3053.
4. Szabo, I., Chen, X. H., Xin, L., Adler, M. W., Howard, O. M., Oppenheim, J. J., and Rogers, T. J. (2002) Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain, *Proc. Natl. Acad. Sci. U.S.A. 99*, 10276−10281.
5. Stefano, G. B., Leung, M. K., Zhao, X. H., and Scharrer, B. (1989) Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes, *Proc. Natl. Acad. Sci. U.S.A. 86*, 626−630.
6. Stefano, G. B., Goumon, Y., Casares, F., Cadet, P., Fricchione, G. L., Rialas, C., Peter, D., Sonetti, D., Guarna, M., Welters, I. D., and Bianchi, E. (2000) Endogenous morphine, *Trends Neurosci. 23*, 436−442.
7. Rogers, T. J., Steele, A. D., Howard, O. M., and Oppenheim, J. J. (2000) Bidirectional heterologous desensitization of opioid and chemokine receptors, *Ann. N.Y. Acad. Sci. 917*, 19−28.
8. Esche, C., Makarenkova, V. P., Kost, N. V., Lotze, M. T., Zozulya, A. A., and Shurin, M. R. (1999) Murine dendritic cells express functional delta-type opioid receptors, *Ann. N.Y. Acad. Sci. 885*, 387−390.
9. Serhan, C. N., Fierro, I. M., Chiang, N., and Pouliot, M. (2001) Cutting edge: nociceptin stimulates neutrophil chemotaxis and recruitment: inhibition by aspirin-triggered-15-epi-lipoxin A4, *J. Immunol. 166*, 3650−3654.
10. Mollereau, C., Parmentier, M., Mailleux, P., Butour, J. L., Moisand, C., Chalon, P., Caput, D., Vassart, G., and Meunier, J. C. (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization, *FEBS Lett. 341*, 33−38.
11. Calvo, C. F., Cesselin, F., Gelman, M., and Glowinski, J. (2000) Identification of an opioid peptide secreted by rat embryonic mixed brain cells as a promoter of macrophage migration, *Eur. J. Neurosci. 12*, 2676−2684.
12. Zhang, L., and Rogers, T. J. (2000) κ-Opioid regulation of thymocyte IL-7 receptor and C-C chemokine receptor 2 expression, *J. Immunol. 164*, 5088−5093.
13. Chao, C. C., Hu, S., Shark, K. B., Sheng, W. S., Gekker, G., and Peterson, P. K. (1997) Activation of mu opioid receptors inhibits microglial cell chemotaxis, *J. Pharmacol. Exp. Ther. 281*, 998−1004.
14. Zagon, I. S., Sassani, J. W., and McLaughlin, P. J. (1995) Opioid growth factor modulates corneal epithelial outgrowth in tissue culture, *Am. J. Physiol. 268*, R942−R950.
15. Wiedermann, C. J., Schratzberger, P., and Kahler, C. M. (1994) Migration of neutrophils across endothelial monolayers is stimulated by treatment of the monolayers with β-endorphin, *Brain, Behav., Immun. 8*, 270−277.
16. Singhal, P. C., Sharma, P., Gibbons, N., Franki, N., Kapasi, A., and Wagner, J. D. (1997) Effect of morphine on renomedullary interstitial cell proliferation and matrix accumulation, *Nephron 77*, 225−234.
17. Liu, Y., Blackbourn, D. J., Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. (1992) Effects of in vivo and in vitro administration of morphine sulfate upon rhesus macaque polymorphonuclear cell phagocytosis and chemotaxis, *J. Pharmacol. Exp. Ther. 263*, 533−539.
18. Grimm, M. C., Ben Baruch, A., Taub, D. D., Howard, O. M., Resau, J. H., Wang, J. M., Ali, H., Richardson, R., Snyderman, R., and Oppenheim, J. J. (1998) Opiates transdeactivate chemokine receptors: delta and mu opiate receptor-mediated heterologous desensitization, *J. Exp. Med. 188*, 317−325.
19. Manfreda, S. E., Dunzendorfer, S., Schratzberger, P., Buratti, T., Reinisch, N., Kahler, C. M., List, W. F., and Wiedermann, C. J. (1998) The chemotaxis of human peripheral blood B lymphocytes by β-endorphin is reversible by naloxone, *Anesth. Analg. 86*, 670−672.
20. Simpkins, C. O., Dickey, C. A., and Fink, M. P. (1984) Human neutrophil migration is enhanced by beta-endorphin, *Life Sci. 34*, 2251−2255.
21. Marotti, T., and Haberstok, H. (1992) Naloxone is an inappropriate antagonist of met-enkephalin-modulated superoxide anion release, *Brain, Behav., Immun. 6*, 223−233.
22. Horvath, K., Graf, L., Walcz, E., Bodanszky, H., and Schuler, D. (1985) Naloxone antagonises effect of alpha-gliadin on leucocyte migration in patients with coeliac disease, *Lancet 2*, 184−185.
23. Pasotti, D., Mazzone, A., and Ricevuti, G. (1992) The nervous system and the immune system: the role of morphine and opioid peptides in the function of neutrophilic granulocytes, *Minerva Med. 83*, 433−438.
24. Marcoli, M., Ricevuti, G., Mazzone, A., Pasotti, D., Lecchini, S., and Frigo, G. M. (1989) A stereoselective blockade by naloxone of opioid and non-opioid-induced granulocyte activation, *Int. J. Immunopharmacol. 11*, 57−61.
25. Kaneider, N. C., Reinisch, C. M., Dunzendorfer, S., Romisch, J., and Wiederman, C. J. (2002) Syndecan-4 mediates antithrombin-induced chemotaxis of human peripheral blood lymphocytes and monocytes, *J. Cell Sci. 115*, 227−236.
26. Kaneider, N. C., Egger, P., Dunzendorfer, S., and Wiedermann, C. J. (2001) Syndecan-4 as antithrombin receptor of human neutrophils, *Biochem. Biophys. Res. Commun. 287*, 42−46.
27. Echtermeyer, F., Streit, M., Wilcox-Adelman, S., Saoncella, S., Denhez, F., Detmar, M., and Goetinck, P. (2001) Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4, *J. Clin. Invest. 107*, R9−R14.
28. Bass, M. D., and Humphries, M. J. (2002) Cytoplasmic interactions of syndecan-4 orchestrate adhesion receptor and growth factor receptor signalling, *Biochem. J. 368*, 1−15.
29. Martin-Kleiner, I. (2002) The effect of opioid agonists of delta-class DSLET, mu-class DAMGO, kappa-class U-69593 and an opioid antagonist, naloxone, on MTT activity of NALM-1 leukemic cells, *Biomed. Pharmacother. 56*, 458−462.
30. Dunzendorfer, S., Kaneider, N., Rabensteiner, A., Meierhofer, C., Reinisch, C., Romisch, J., and Wiedermann, C. J. (2001) Cell-surface heparan sulfate proteoglycan-mediated regulation of human neutrophil migration by the serpin antithrombin III, *Blood 97*, 1079−1085.
31. Schratzberger, P., Reinisch, N., Prodinger, W. M., Kahler, C. M., Sitte, B. A., Bellmann, R., Fischer-Colbrie, R., Winkler, H., and Wiedermann, C. J. (1997) Differential chemotactic activities of sensory neuropeptides for human peripheral blood mononuclear cells, *J. Immunol. 158*, 3895−3901.
32. Patel, L., et al. (2001) Expression and functional analysis of chemokine receptors in human peripheral blood leukocyte populations, *Cytokine 14*, 27−36.
33. Cockwell, P., et al. (2002) Chemoattraction of T cells expressing CCR5, CXCR3 and CX3CR1 by proximal tubular epithelial cell chemokines, *Nephrol., Dial., Transplant. 17*, 734−744.

34. Pace, E., et al. (1999) Interleukin-8 induces lymphocyte chemotaxis into the pleural space. Role of pleural macrophages, *Am. J. Respir. Crit. Care Med. 159*, 1592−1599.
35. Schratzberger, P., Geiseler, A., Dunzendorfer, S., Reinisch, N., Kahler, C. M., and Wiedermann, C. J. (1998) Similar involvement of VIP receptor type I and type II in lymphocyte chemotaxis, *J. Neuroimmunol. 87*, 73−81.
36. Leonard, E. J., Skeel, A., Yoshimura, T., Noer, K., Kutvirt, S., and Van Epps, D. (1990) Leukocyte specificity and binding of human neutrophil attractant/activation protein-1, *J. Immunol. 144*, 1323−1330.
37. Schratzberger, P., Dunzendorfer, S., Reinisch, N., Kahler, C. M., and Wiedermann, C. J. (1997) Interleukin-8-induced human peripheral blood B-lymphocyte chemotaxis in vitro, *Immunol. Lett. 58*, 167−170.
38. Wiedermann, C. J., Reinisch, N., Niedermuhlbichler, M., and Braunsteiner, H. (1993) Inhibition of recombinant human growth hormone-induced and prolactin-induced activation of neutrophils by octreotide, *Naunyn-Schmiedeberg's Arch. Pharmacol. 347*, 336−341.
39. Goetzl, E. J., Klickstein, L. B., Watt, K. W., and Wintroub, B. U. (1980) The preferential human mononuclear leukocyte chemotactic activity of the substituent tetrapeptides of angiotensin II, *Biochem. Biophys. Res. Commun. 97*, 1097−1102.
40. Weinstock, J. V., Blum, A. M., and Kassab, J. T. (1987) Angiotensin II is chemotactic for a T-cell subset which can express migration inhibition factor activity in murine schistosomiasis mansoni, *Cell. Immunol. 107*, 180−187.
41. Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) Cloning of a delta opioid receptor by functional expression, *Science 258*, 1952−1955.
42. Hammond, M. E., Lapointe, G. R., Feucht, P. H., Hilt, S., Gallegos, C. A., Gordon, C. A., Giedlin, M. A., Mullenbach, G., and Tekamp-Olson, P. (1995) IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors, *J. Immunol. 155*, 1428−1433.
43. Baciu, P. C., and Goetinck, P. F. (1995) Protein kinase C regulates the recruitment of syndecan-4 into focal contacts, *Mol. Biol. Cell 6*, 1503−1513.
44. Mertens, G., Cassiman, J. J., Van den, B. H., Vermylen, J., and David, G. (1992) Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties, *J. Biol. Chem. 267*, 20435−20443.
45. Kaneider, N., Djanani, A., Fischer-Colbrie, R., and Wiedermann, C. (2002) Sphingosine kinase-dependent directional migration of leukocytes in response to phorbol ester, *Biochem. Biophys. Res. Commun. 297*, 806.
46. Olivera, A., and Spiegel, S. (2001) Sphingosine kinase: a mediator of vital cellular functions, *Prostaglandins Other Lipid Mediators 64*, 123−134.
47. Vaday, G. G., Franitza, S., Schor, H., Hecht, I., Brill, A., Cahalon, L., Hershkoviz, R., and Lider, O. (2001) Combinatorial signals by inflammatory cytokines and chemokines mediate leukocyte interactions with extracellular matrix, *J. Leukocyte Biol. 69*, 885−892.
48. Csaba, G., Kovacs, P., and Pallinger, E. (2002) β-Endorphin in granulocytes, *Cell Biol. Int. 26*, 741−743.
49. Vindrola, O., Padros, M., Sterin-Prync, A., Ase, A., Finkielman, S., and Nahmod, V. (1990) Proenkephalin system in human polymorphonuclear cells. Production and release of a novel 1.0-kD peptide derived from synenkephalin, *J. Clin. Invest. 86*, 531−537.
50. Wang, D., Raehal, K. M., Bilsky, E. J., and Sadee, W. (2001) Inverse agonists and neutral antagonists at mu opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence, *J. Neurochem. 77*, 1590−1600.
51. Ventayol, P., Busquets, X., and Garcia-Sevilla, J. A. (1997) Modulation of immunoreactive protein kinase C-alpha and beta isoforms and G proteins by acute and chronic treatments with morphine and other opiate drugs in rat brain, *Naunyn-Schmiedeberg's Arch. Pharmacol. 355*, 491−500.
52. Sacerdote, P., Manfredi, B., Gaspani, L., and Panerai, A. E. (2000) The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in BALB/cJ mice, *Blood 95*, 2031−2036.
53. Wiedermann, C. J., Sacerdote, P., Propst, A., Propst, T., Judmaier, G., Kathrein, H., Vogel, W., and Panerai, A. E. (1994) Decreased beta-endorphin content in peripheral blood mononuclear leukocytes from patients with Crohn's disease, *Brain, Behav., Immun. 8*, 261−269.
54. Buratti, T., Schratzberger, P., Dunzendorfer, S., Manfreda, S. E., Pechlaner, C., Joannidis, M., Sacerdote, P., Panerai, A. E., and Wiedermann, C. J. (1998) Decreased levels of beta-endorphin in circulating mononuclear leukocytes from patients with acute myocardial infarction, *Cardiology 90*, 43−47.
55. Sacerdote, P., Breda, M., Barcellini, W., Meroni, P. L., and Panerai, A. E. (1991) Age-related changes of beta-endorphin and cholecystokinin in human and rat mononuclear cells, *Peptides 12*, 1353−1356.
56. Kuschert, G. S., Coulin, F., Power, C. A., Proudfoot, A. E., Hubbard, R. E., Hoogewerf, A. J., and Wells, T. N. (1999) Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses, *Biochemistry 38*, 12959−12968.
57. Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S., and Spiegel, S. (2001) Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility, *Science 291*, 1800−1803.
58. Oh, E. S., Woods, A., and Couchman, J. R. (1997) Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C, *J. Biol. Chem. 272*, 8133−8136.
59. Yu, G., LeBrun, L., Gunay, N. S., Hoppensteadt, D., Walenga, J. M., Fareed, J., and Linhardt, R. J. (2000) Heparinase I acts on a synthetic heparin pentasaccharide corresponding to the antithrombin III binding site, *Thromb. Res. 100*, 549−556.
60. Yamashita, Y., Oritani, K., Miyoshi, E. K., Wall, R., Bernfield, M., and Kincade, P. W. (1999) Syndecan-4 is expressed by B lineage lymphocytes and can transmit a signal for formation of dendritic processes, *J. Immunol. 162*, 5940−5948.
61. Kveberg, L., Bryceson, Y., Inngjerdingen, M., Rolstad, B., and Maghazachi, A. A. (2002) Sphingosine 1-phosphate induces the chemotaxis of human natural killer cells. Role for heterotrimeric G proteins and phosphoinositide 3 kinases, *Eur. J. Immunol. 32*, 1856−1864.
62. Idzko, M., Panther, E., Corinti, S., Morelli, A., Ferrari, D., Herouy, Y., Dichmann, S., Mockenhaupt, M., Gebicke-Haerter, P., Di Virgilio, F., Girolomoni, G., and Norgauer, J. (2002) Sphingosine 1-phosphate induces chemotaxis of immature dendritic cells and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses, *FASEB J. 16*, 625−7.
63. Brandes, M. E., Mai, U. E., Ohura, K., and Wahl, S. M. (1991) Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta, *J. Immunol. 147*, 1600−1606.
64. Buehrer, B. M., and Bell, R. M. (1992) Inhibition of sphingosine kinase in vitro and in platelets. Implications for signal transduction pathways, *J. Biol. Chem. 267*, 3154−3159.
65. Yung, B. Y., Hsiao, T. F., Wei, L. L., and Hui, E. K. (1994) Sphinganine potentiation of dimethyl sulfoxide-induced granulocyte differentiation, increase of alkaline phosphatase activity and decrease of protein kinase C activity in a human leukemia cell line (HL-60), *Biochem. Biophys. Res. Commun. 199*, 888−896.

BI035295I