Extracellular ATP and P2 receptors are required for IL-8 to induce neutrophil migration

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Abstract

The chemokine interleukin 8 (IL-8) is a major chemoattractant for human neutrophils. Here, we demonstrate novel evidence that IL-8-induced neutrophil chemotaxis requires a concurrent activation of P2 receptors, most likely the P2Y₂ which is dominantly expressed in these cells. Indeed, the migration of human neutrophils towards IL-8 was significantly inhibited by the P2Y receptor antagonists, suramin and reactive blue 2 (RB-2) and potentiated by a P2Y₂ ligand, ATP, but insensitive to specific antagonists of P2Y₁, P2Y₆ and P2Y₁₁ receptors. Adenosine had no effect on neutrophil migration towards IL-8 which contrasted with the stimulatory effect of this molecule on neutrophil chemotaxis caused by formyl-Met-Leu-Phe (fMLP or fMLF). Taken together, these data suggest that extracellular ATP is necessary for IL-8 to exert its chemotactic effect on neutrophils.

Keywords

Chemokine; Chemotaxis; Inflammation; fMLP; Extracellular nucleotides

1. Introduction

Chemotaxis plays a key role in neutrophil recruitment at infected tissue which is a hallmark of the acute immune response. Neutrophil recruitment is activated either by pathogenic determinants per se (e.g., formyl-Met-Leu-Phe – fMLP or fMLF) or via pathogen- induced secretion of chemoattractants by affected tissue [1,2]. These chemoattractants are represented primarily by chemokine interleukin 8 (IL-8, CXCL-8), leukotriene B₄ (LTB₄) and complement protein 5a (C5a). Chemotaxis serves to induce a rapid accumulation of neutrophils at affected site in order to eliminate the invading pathogens by means of respiratory burst, degranulation, and phagocytosis [1,3].

Extracellular nucleotides are signaling molecules that often serve as danger signals which are rapidly produced during the onset of immune responses. These molecules activate P2 nucleotide receptors that include the ionotropic P2X receptors (P2X₁₋₇) and metabotropic (G-protein-coupled) P2Y receptors (P2Y_{1,2,4,6,11-14}) [4]. Neutrophils express all P2Y

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receptors except P2Y₁₂. Among them, P2Y₂ is dominantly expressed at the mRNA level and was also functionally demonstrated at the protein level [5–7]. P2Y₁ and P2Y₁₃ are activated by ADP, P2Y₂ by ATP and UTP, P2Y₄ by UTP, P2Y₆ by UDP, P2Y₁₁ by ATP and P2Y₁₄ by UDP-glucose [4]. Extracellular nucleotides, primarily ATP, trigger various proinflammatory responses of neutrophils including arachidonic acid release, oxidative burst and phagocytosis [8]. ATP and UTP serve as neutrophil chemoattractants, however, markedly less potent than for example IL-8 and fMLP [9]. Nucleotides can also induce degranulation of neutrophils but only in the presence of a co-stimulus such as fMLP [8].

IL-8 is a major chemokine for neutrophils which is largely responsible for the recruitment of these cells at sites of inflammation/infection in human. We have previously shown that P2 receptors regulate IL-8 secretion from human monocytes and thus control neutrophil migration in vitro [10]. Here we report that, interestingly, P2 receptor activation is also required for IL-8 to induce neutrophil chemotaxis.

2. Materials and methods

2.1. Materials

Human recombinant IL-8 was purchased from R&D Systems (Minneapolis, MN). Nucleotides (ATP, ADP, UTP, UDP, ATP γ S and β -NAD), adenosine, pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonate (PPADS) and suramin were purchased from Sigma (St. Louis, MO). MRS2179, MRS2578 and NF157 were obtained from Tocris Bioscience (Bristol, UK). Reactive blue 2 (RB-2) was bought from ICN Biochemicals Inc. (Aurora, OH). Adenosine deaminase (ADA) was provided by Roche Diagnostics (Indianapolis, IN).

IL-8, nucleotides and P2 receptor antagonists (suramin, RB-2, PPADS, MRS2179 and NF157) were prepared in endotoxin-free water from Sigma. MRS2578 (10 mM) was prepared in 100% DMSO and diluted with RPMI-5% FBS medium to 10 μ M for neutrophil migration assays. The experiments performed with this molecule included controls containing 0.01% DMSO. Before use, adenosine deaminase was dialyzed against sterile 0.9% NaCl and 10 mM Hepes, pH 7.4, in a Slide-A-Lyser[®] cassette (MWCO 3500; Pierce, Rockford, IL) to remove ammonium sulphate present in the commercial preparation that induces calcium mobilization in human neutrophils.

2.2. Purification of human neutrophils

Human neutrophils were isolated as described [11], with some modifications. Briefly, venous blood of healthy volunteers was collected on isocitrate anticoagulant solution, centrifuged (250g, 10 min, 24 °C), and the resulting platelet-rich plasma discarded. Leukocytes were obtained following erythrocyte sedimentation in 2% Dextran T-500 and centrifuged (525g, 20 min, 24 °C) through a 10 ml Ficoll-Paque cushion (Wisent, St.-Bruno, Canada). The neutrophil-enriched pellet was subjected to a 15 s hypotonic lysis to remove the remaining erythrocytes and centrifuged (1000g, 5 min, 24 °C). The purified neutrophils were resuspended in RPMI 1640-5% FBS medium and used freshly for migration assays.

2.3. Neutrophil chemotaxis assay

Neutrophil migration towards human recombinant IL-8 was carried out in a trans-well system. Briefly, cell culture inserts (3 µm pore size) were used to form dual compartments (upper and bottom chamber) in a Falcon™ 24-well culture plate (Becton Dickinson, Franklin Lakes, NJ). Freshly purified human peripheral blood neutrophils were loaded into the upper chamber (cell culture insert) and their migration was initiated by the addition of human recombinant IL-8 (0.5 or 0.1 ng/ml, unless indicated otherwise) to the medium of the bottom chamber in a 24-well plate. Where indicated, P2 receptor antagonists or nucleotides were added to the media of the bottom or upper chambers 15 min before the addition of neutrophils and IL-8. Neutrophil migration was carried out for 1 h at 37 °C and 5% CO₂. The migrated neutrophils were collected from the bottom chambers and counted with a hemacytometer.

2.4. Statistical analysis

Student's *t*-test was performed using Excel software (Microsoft® Office OneNote™ 2003).

3. Results

3.1. P2 receptor antagonists inhibit IL-8-induced neutrophil migration

Neutrophil migration was investigated in a trans-well system where freshly purified human neutrophils were loaded into the upper chamber and human recombinant IL-8 to the bottom chamber. Neutrophil migration was assessed from the number of neutrophils that passed through the polyethylene membrane to the bottom chamber within 1 h from the addition of IL-8. Firstly, we determined IL-8 concentrations that induced optimal and suboptimal neutrophil migration required to perform the subsequent experiments (0.5 and 0.1 ng/ml, respectively; Fig. 1). To determine whether the autocrine activation of P2 receptors is required for neutrophil migration towards IL-8, chemotaxis assays were carried out with 0.5 ng/ml IL-8 added to the bottom chamber in the presence or absence of the non-selective P2 receptor antagonists (suramin, RB-2 and PPADS; 100 µM) added either directly in contact with neutrophils in the upper chamber, or in the bottom chamber, as indicated. The antagonists added to the upper chamber were also preincubated for 15 min with neutrophils before migration. The inhibition of neutrophil migration by P2 receptor antagonists in the upper chamber would suggest that the activation of these receptors is involved in IL-8-induced neutrophil migration. In contrast, the effect of these inhibitors in the bottom chamber would indicate that nucleotides are not required for IL-8 to induce neutrophil migration but potentiate this migration by inducing chemotaxis themselves and/or by activating already migrated cells to release substances (e.g., chemoattractant(s)) attracting the cells present in the upper chamber. Fig. 2A shows that neutrophil migration was inhibited once suramin and RB-2 were present in the upper chamber in direct contact with neutrophils (>40% inhibition; Fig. 2A, empty bars) whereas these antagonists had no effect in the bottom chamber (Fig. 2A, solid bars). These results suggest that the autocrine activation of P2 receptors is necessary for IL-8 to induce neutrophil migration. The P2X receptors are generally highly sensitive to PPADS [12]. As the presence of PPADS (100 µM) in the upper chamber did not affect neutrophil chemotaxis towards IL-8 (Fig. 2A) the most

probable mechanism underlying this effect would be that IL-8-induced neutrophil chemotaxis requires the concomitant activation of P2Y receptor(s).

To further support this view we designed experiments to define the P2Y subtype involved in IL-8-induced neutrophil chemotaxis where we tested the effect of available selective antagonists for P2 receptors expressed in neutrophils on IL-8-induced neutrophil migration. These antagonists, namely MRS2179, MRS2578 and NF157 (for P2Y₁, P2Y₆ and P2Y₁₁, respectively), were present with neutrophils in the upper chamber during migration and had also been preincubated with these cells for 15 min before the migration assay. IL-8-induced neutrophil migration was unaffected in the presence of these antagonists (Fig. 2B) suggesting that P2Y₁, P2Y₆ and P2Y₁₁ receptors were not involved in this process. As suramin does not inhibit P2Y₄, the latter could also be excluded from the effect observed with this antagonist.

The above data suggest that IL-8-induced neutrophil migration is dependent on nucleotide release (basal or IL-8-induced) to stimulate P2Y₂ receptor that can be strongly inhibited by suramin and RB-2. However, there is no selective antagonist for this receptor. To further document whether P2Y₂ is involved in IL-8-induced neutrophil migration we performed the additional experiments described below.

3.2. ATP synergizes with IL-8 to induce neutrophil chemotaxis

As P2Y receptor subtypes have different specificity towards nucleotides, the identification of nucleotides that synergize with IL-8 to activate neutrophil migration would help define the P2 subtype involved. Therefore, we next investigated which of the following nucleotides (P2Y agonists), ATP, ADP, UTP, UDP, ATP γ S and/or β -NAD, would increase neutrophil migration due to IL-8. The above experiments with P2 receptor antagonists suggested that IL-8 may cause an endogenous nucleotide release. To limit this potential nucleotide release, a suboptimal concentration of IL-8 (0.1 ng/ml) was used in these assays that was sufficient to modestly activate IL-8 receptors, as determined in Fig. 1. Exogenous nucleotides (all at 100 μ M) were added together with neutrophils in the upper chamber, or in the bottom chamber (as controls), while IL-8 (0.1 ng/ml) was administered to the bottom chamber. For nucleotides added to the upper chamber, neutrophil migration was strongly increased by ATP (~5-fold) and weakly by ATP γ S, UTP and ADP (~2-fold but not statistically significant, $n \geq 3$) whereas UDP and β -NAD had no effect (Fig. 3A). Importantly, the increase of IL-8-induced neutrophil migration by ATP was synergistic, as ATP alone in the upper chamber did not induce significant neutrophil migration (Fig. 3A). In comparison to these effects, the migration of neutrophils in the presence of ATP and IL-8 in the bottom chamber was increased by only ~1.7-fold compared to the migration of these cells towards IL-8 alone (Fig. 3B). This modest increase in neutrophil migration was caused by an additive effect of ATP and IL-8. These data are in agreement with the inhibition of neutrophil migration by the P2 receptor antagonists in the upper chamber.

Collectively with the results obtained with suramin and RB-2, these data suggest that ATP synergizes with IL-8 to induce neutrophil migration but is insufficient by itself to induce a potent chemotaxis. Neutrophils express two P2Y receptors that are activated by ATP, the P2Y₂ and P2Y₁₁. As the involvement of the P2Y₁₁ was already excluded above with a

selective antagonist (Fig. 2B), P2Y₂ receptor appears as the only remaining candidate to mediate IL-8-induced neutrophil chemotaxis.

3.3. Adenosine is not involved in IL-8-induced neutrophil migration

Previous work by Chen et al. [7] demonstrated that extracellular adenosine originating from extracellular ATP hydrolysis markedly potentiates fMLP-induced neutrophil chemotaxis as this migration is significantly inhibited by adenosine deaminase (ADA) that hydrolyzes adenosine to inosine [7,13]. Therefore, we next aimed to determine the role of adenosine in neutrophil migration towards IL-8. In contrast to fMLP, IL-8-induced neutrophil migration was not affected by ADA (5 U/ml; Fig. 4A) suggesting that endogenous adenosine was not involved in this migration. In addition, exogenous adenosine (1 and 10 µM) added either to the upper chamber or bottom chamber did not potentiate neutrophil migration either in the presence or absence of suboptimal IL-8 concentration (0.1 ng/ml; Fig. 4B and data not shown, respectively) showing that adenosine could not act on neutrophil migration towards IL-8 in this model. In contrast, adenosine increased fMLP-induced neutrophil migration used as a positive control (Fig. 4B).

Importantly, the above results also confirmed that suramin and RB-2 diminished neutrophil migration exclusively by blocking P2Y receptors and not by inhibiting extracellular adenosine formation. Indeed, suramin and RB-2 also potently inhibit NTPDase1 [14] which is responsible for the production of extracellular adenosine via hydrolysis of ATP and ADP to AMP that can be then further degraded to adenosine by other enzymes [13,15,16]. This possibility, however, was excluded by the data presented in Fig. 4.

4. Discussion

This work demonstrates that neutrophil migration induced by IL-8 is associated with an autocrine activation of P2 receptors, most likely P2Y₂. Indeed, the P2 receptor antagonists, suramin and RB-2, strongly inhibited neutrophil migration towards IL-8 while the antagonists for P2 receptors other than P2Y₂ had no effect, namely PPADS, MRS2179, MRS2578 and NF157. Moreover, ATP, a ligand of the P2Y₂ receptor, but not other nucleotides tested, synergized with IL-8 in suboptimal concentration to activate neutrophil migration. Although P2 receptor antagonists used in this work did not inhibit neutrophil migration completely, the addition of exogenous ATP (100 µM) to suboptimal IL-8 (0.1 ng/ml) raised the migration due to IL-8 alone by 5-fold which corresponded to the migration level obtained with optimal IL-8 concentration further supporting the view that extracellular ATP is necessary for IL-8-induced neutrophil migration. Interestingly, a previous report showed a cross-talk between P2Y₂ and CXC chemokine receptor 2 in human embryonic kidney cells that results in markedly increased phospholipase C activity and intracellular Ca²⁺ level compared to these signaling events activated by each of these receptors alone [17]. Such a P2Y₂-CXC cross-talk also appears to drive neutrophil migration towards IL-8 in the model used here.

The data presented here together with previous works showing that P2Y₂ receptor is involved in neutrophil migration towards fMLP [7,13] suggest that this nucleotide receptor may play a general role in neutrophil chemotaxis. The importance of the P2Y₂ receptor in

neutrophil migration has also been confirmed in vivo. Indeed, P2Y₂ deficient mice exhibit an impaired neutrophil infiltration in different models: intraperitoneal injection of Gram-positive bacteria *Staphylococcus aureus* and cecal ligation and puncture sepsis [7,18]. In agreement, P2Y₂ KO neutrophils isolated from these mice had an impaired chemotaxis in vitro [7].

Interestingly, in addition to P2Y₂, fMLP-induced neutrophil migration also involved adenosine A₃ receptor [7]. These receptors are responsible for gradient sensing and migration speed, respectively. A₃ receptor is activated by extracellular adenosine produced by the hydrolysis of extracellular ATP that is secreted by fMLP-activated neutrophils. Indeed, human neutrophils express the ectonucleotidase NTPDase1 that efficiently degrades ATP and ADP to AMP [13]. The latter molecule is then further hydrolyzed to adenosine by other enzymes, e.g., ecto-5'-nucleotidase and alkaline phosphatase, but none of these two enzymes is present at the cell surface of resting neutrophils [13]. Alkaline phosphatase, however, is present in neutrophil granules and can be secreted as a result of neutrophil degranulation due to fMLP stimulation to subsequently generate adenosine for A₃ activation [13,19]. We know that IL-8 does not induce neutrophil degranulation unless these cells have been pre-treated with cytochalasin B to disrupt the motility response. Therefore, in our migration assays, IL-8-treated neutrophils might not have had the capacity to generate adenosine from extracellular AMP. In any case, adenosine had no effect on neutrophil migration induced by IL-8 in the model used in this work, even when it was added exogenously. This contrasting effect of adenosine on IL-8- vs. fMLP-induced neutrophil migration is intriguing. Taken together, this work demonstrates a novel function of nucleotide receptors, most likely P2Y₂, in neutrophil migration towards IL-8. It also demonstrates a part of the mechanism of IL-8 to exert neutrophil chemotaxis. As IL-8 is largely responsible for neutrophil recruitment at sites of inflammation, this nucleotide-dependent mechanism may potentially reveal useful for treatments against inflammatory diseases.

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Fig. 1.

IL-8 concentration dependent migration of human neutrophils. Chemotaxis of freshly isolated human neutrophils (10^6 in 0.2 ml of RPMI 1640-5% FBS) towards human recombinant IL-8 (0.1–1 ng/ml) was investigated in a trans-well system. Neutrophils were allowed to migrate for 1 h at 37 °C and 5% CO₂. The migrated cells were collected from the bottom chamber and counted with a hemacytometer. These data represent the mean $\pm$ SD of three assays with neutrophils from different donors. The 100% set for neutrophil migration induced by 1 ng/ml IL-8 corresponded to $0.75 \pm 0.23 \times 10^6$ cells.

Fig. 2.

P2 receptor antagonists, suramin and RB-2, inhibit neutrophil migration towards IL-8. Chemotaxis of freshly isolated human neutrophils (10^6 in 0.2 ml of RPMI 1640-5% FBS) towards human recombinant IL-8 (0.5 ng/ml) was investigated in a trans-well system. Indicated P2 receptor antagonists, (100 μ M suramin, RB-2 or PPADS, 10 μ M MRS2179 (P2Y₁), 10 μ M MRS2578 (P2Y₆) or 1 μ M NF157 (P2Y₁₁)) were added to the upper chamber in direct contact with neutrophils (empty bars) or to the bottom chamber (solid bars). Neutrophils were allowed to migrate for 1 h at 37 °C and 5% CO₂. The migrated cells were collected from the bottom chamber and counted with a hemacytometer. These data represent the mean $\pm$ SD of at least three assays with neutrophils from different donors. The 100% set for neutrophil migration towards IL-8 alone corresponded to $0.69 \pm 0.15 \times 10^6$ cells. (A) The general P2 antagonists, suramin and RB-2, significantly inhibited neutrophil migration when present in the upper chamber together with neutrophils but had no effect in the bottom chamber. * $P < 0.02$ vs. IL-8 alone. (B) Selective antagonists of P2Y₁, P2Y₆ and P2Y₁₁ did not affect IL-8-induced neutrophil migration.

Fig. 3.

Synergistic effect of ATP on IL-8-induced neutrophil migration. Chemotaxis of freshly isolated human neutrophils (10^6 in 0.2 ml of RPMI 1640-5% FBS) was investigated in a trans-well system. Suboptimal IL-8 concentration was determined from the dose dependent neutrophil migration shown in Fig. 1. To determine the effect of nucleotides towards 0.1 ng/ml IL-8 added to the bottom chamber, indicated nucleotides (100 μ M) were added to the upper (A) or bottom chamber (B). Neutrophils were allowed to migrate for 1 h at 37 °C and 5% CO₂. The migrated cells were collected from the bottom chamber and counted with a hemacytometer. These data represent the mean $\pm$ SD of at least three assays with neutrophils from different donors. For (A) and (B), 100% was set for neutrophil migration towards IL-8 alone (0.1 ng/ml) which corresponded to $0.08 \pm 0.03 \times 10^6$ cells. ATP added to the upper chamber in direct contact with neutrophils potentially increased (5-fold) neutrophil migration due to suboptimal concentration of IL-8 (0.1 ng/ml; * $P < 0.02$). ATP γ S, UTP and ADP had a weaker effect while UDP and β -NAD did not affect neutrophil migration. In contrast, ATP added to the bottom chamber had a weak effect (additive) on IL-8-induced neutrophil migration which was not statistically significant.

Fig. 4.

Adenosine has no effect on IL-8-induced neutrophil migration. Chemotaxis of freshly isolated human neutrophils (10^6 in 0.2 ml of RPMI 1640-5% FBS) was investigated in a trans-well system. To determine the role of endogenous adenosine in IL-8 induced neutrophil migration (0.5 ng/ml; added to the bottom chamber), adenosine deaminase (ADA; 5 U/ml) was added either to the upper or bottom chamber, as indicated, to deplete this nucleoside (A). To determine whether exogenous adenosine would increase neutrophil migration towards IL-8 (0.1 ng/ml; added to the bottom chamber), this nucleoside (1 and 10 μ M) was added to the upper or bottom chamber (B). Neutrophils were allowed to migrate for 1 h at 37 °C and 5% CO₂. The migrated cells were collected from the bottom chamber and counted with a hemacytometer. These data represent the mean $\pm$ SD of 2–4 assays with neutrophils from different donors. (A) ADA did not affect IL-8-induced neutrophil migration suggesting that endogenously produced adenosine is not involved in this migration. In contrast, ADA markedly diminished neutrophil migration towards 0.1 μ M fMLP used as a positive control. 100% set for neutrophil migration towards IL-8 and fMLP alone corresponded to $0.66 \pm 0.14 \times 10^6$ and $0.51 \pm 0.01 \times 10^6$ cells, respectively. (B) Exogenous adenosine had no effect on IL-8-induced neutrophil migration but markedly increased neutrophil migration towards 0.1 μ M fMLP used as a positive control. 100% set for neutrophil migration towards IL-8 and fMLP alone corresponded to $0.15 \pm 0.04 \times 10^6$ and $0.51 \pm 0.01 \times 10^6$ cells, respectively.