Original Article

Agonists of proteinase-activated receptor-2 affect transendothelial migration and apoptosis of human neutrophils

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Abstract: Skin is the first barrier preventing microorganism invasion in host. Wounds destroy this defense barrier and, without an appropriate care, may lead to sepsis. Neutrophil activation and immigration plays an important role at the inflammatory stage of wound healing. Neutrophils are known to express proteinase-activated receptors (PARs), which can be activated by serine proteases, also by enzymes involved in wound healing. We previously reported that PAR_2 agonists up-regulate cell adhesion molecule expression and cytokine production by human neutrophils. Here, we demonstrate that PAR_2 agonists (serine proteases as well as synthetic peptides) reduce transendothelial migration of neutrophils and prolong their life *in vitro*. Synthetic PAR_2 agonist also enhanced protective interferon (IFN)γ-induced FcγRI expression at neutrophil cell surface. Of note, IFNγ is a cytokine, which was used in clinical trials to reactivate human neutrophil functions during sepsis. Moreover, we observed a significant increase of PAR_2 expression on cell surface of neutrophils from septic patients as compared with healthy volunteers. Together, our results indicate that PAR_2 may be involved in the pathophysiology of neutrophil-endothelial interactions during wound healing or later during sepsis in humans, potentially by affecting neutrophil apoptosis, transendothelial migration and Fcγ receptor-mediated phagocytosis.

Key words: inflammation – innate immunity – leukocyte – proteinase-activated receptor – skin

Introduction

Proteinase-activated receptor-2 (PAR_2) is the second member of a novel G protein-coupled receptor subfamily with a unique mechanism of receptor activation. Accessible serine proteases cleave the receptor to expose a new, previously cryptic, N-terminal sequence, which serves further as a 'tethered' receptor-activating ligand. To date, four PARs are known [reviewed in Refs. (1,2)]. A variety of serine proteinases, including members of the trypsin family, mast cell tryptase, immune cell-derived or microorganism-derived enzymes activate PAR_2 (3–8). Functional PAR_2 is expressed by cells involved in inflammatory and allergic responses such as mast cells, dendritic cells, eosinophils, macrophages and neutrophils (9–14). However, the ability of PAR_2 agonists to act on key neutrophil features such as phagocytosis, apoptosis or transmigration through the

Abbreviations: FCS, fetal calf serum; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; HMEC-1, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; IFNγ, interferon gamma; PAR_2, proteinase-activated receptor-2; PMN, polymorphonuclear neutrophil; tcAP, tc-activating peptide; tcRP, tc-reverse peptide.

endothelium is still uninvestigated. Nonetheless, PAR$_2$ immunoreactivity can be found on neutrophils during inflammation or delayed wound healing, for example. Thus, PAR$_2$ may be involved in wound healing (inflammatory stage) or in further progression of inflammation of untreated wounds, which in very serious cases leads to sepsis.

Recent studies indicated an important role for PAR$_2$ activation in the development of arthritis, acute pancreatitis, airway inflammation, atopic dermatitis, and encephalomyelitis (2,15,16). In some cases, PAR$_2$ activation led to pro-inflammatory effects (17–19), however, under certain conditions to anti-inflammatory protective effects (20,21). Changes in main neutrophil functions are known to play a role in bacteria-induced pathologies (wound infection, sepsis or septic shock) as well as in bacteria-induced complications of skin diseases (22,23). However, the role of PAR$_2$ activation in modulation of neutrophil-endothelial interaction and neutrophil apoptosis during such diseases remains elusive.

Interferons (IFNγ, IFNα) are known to activate protective functions of human neutrophils and monocytes (phagocytosis, generation of reactive oxygen species, bacterial killing) (24–27). IFNγ was also used in clinical trials in sepsis for reactivation of monocyte and neutrophil immune functions (28). However, the ability of PAR$_2$ agonists to enhance (during combined application) IFNγ-induced protective effects at human neutrophils is still unclear.

Previously (29), we demonstrated that PAR$_2$ agonists up-regulate the expression of $\alpha_4\beta_1$ and Mac-1 integrins on neutrophil cell surface, and also enhance the production of IL-1β, IL-6, IL-8 by human neutrophils (29). These integrins and cytokines are known to affect neutrophil transendothelial migration, apoptosis and phagocytic activity [reviewed in Refs. (30,31)]. Therefore, we hypothesized that stimulation of human neutrophils with PAR$_2$ agonists may act on such key neutrophil features *in vitro*. To explore this, we examined whether PAR$_2$ agonists affect: (i) human neutrophil apoptosis and (ii) neutrophil transendothelial migration. Further, we investigated the ability of PAR$_2$ agonist to enhance IFNγ-induced protective effect at human neutrophils (expression of Fcγ-receptors, which are known to play crucial role in phagocytic activity of these cells). Moreover, we determined whether neutrophils of septic patients show altered PAR$_2$ cell surface expression as compared with cells from healthy volunteers.

Materials and methods

Reagents

Human recombinant interferon-γ (IFNγ) was obtained from TebuBio (Offenbach, Germany). Trypsin was purchased from Sigma (Deisenhofen, Germany). Human β-tryptase was

expressed in *Pichia pastoris* and used as described (32). Human PAR$_2$ activating peptide with the sequence *trans-cinnamoyl-LIGRLO-NH$_2$* (tcAP) and reverse peptide with sequence *trans-cinnamoyl-OLRGIL-NH$_2$* (tcRP) (Dr. McMaster, University of Calgary, Canada) was used as described previously (29,33). Primary antibodies were: unconjugated or fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-human PAR$_2$ (clone SAM-11) (Santa Cruz Biotechnology, Heidelberg, Germany); monoclonal mouse anti-human CD64 (DakoCytomation, Hamburg, Germany) and secondary antibodies were PE-conjugated goat anti-mouse from Jackson Immuno-Research (Hamburg, Germany). The FITC-conjugated mouse IgG$_1$ isotype control antibodies were from PharMingen (Hamburg, Germany). Cell culture reagents were from BioWhittaker (Aachen, Germany), PromoCell (Heidelberg, Germany), Gibco-Life Technologies (Karlsruhe, Germany), Sigma, Beckton-Dickenson (Heidelberg, Germany), Falcon (via Beckton-Dickenson, Heidelberg, Germany), Corning (Schiphool, the Netherlands).

Isolation and culture of human neutrophils

For sepsis studies, the blood from patients who fulfilled the clinical criteria for septic shock, was used (34). The study of patients with septic shock ($n = 19$) and healthy volunteers ($n = 18$, sex and aged matched) was performed with the permission of the Independent Ethics Committee of the University of Ulm (approval No. 82/2002). Exclusion criteria were age <18 years, pregnancy, rapidly progressing underlying disease, HIV/AIDS, cardiogenic shock as the primary underlying disease, underlying haematological disease, or cytotoxic therapy given within the previous 6 months.

Blood for *in vitro* experiments with normal human neutrophils was obtained from healthy adult human volunteers in heparinized tubes or in buffy-coats (Deutsches Rotes Kreuz, Muenster, Germany). Polymorphonuclear neutrophils (PMNs) were isolated using lymphocyte separation medium (equivalent of Percoll produced by PAA Laboratories, Cölbe, Germany) followed by hypotonic stress to remove erythrocytes, as described (29,35). Neutrophils were cultured in RPMI 1640 medium supplied with: 1% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% L-Glutamine and 1% non-essential amino acids. After isolation, cells were cultured (37°C, 5%CO$_2$) during 2 h for recovery.

For human neutrophil stimulation, IFNγ was used at a concentration of 200 U/ml. The same IFNγ concentration was used in all co-stimulation experiments (together with the PAR$_2$ agonist). For PAR$_2$ stimulation, PAR$_2$-tcAP was used in a concentration of 1×10^{-4} M. This peptide has been previously documented to be an effective and specific PAR$_2$ agonist in PAR$_2$-expressing cells (33), causing effects identical to a 'tethered' ligand agonist like SLIGRL-NH$_2$

or SLIGKV-NH$_2$. The corresponding reverse peptide (PAR$_2$-tcRP) also was used in concentration 1×10^{-4} M and served as negative control. Trypsin and tryptase [tryptase was dissolved in heparin solution, with concentration of heparin 30 μg/ml of phosphate-buffered saline (PBS)] were used in concentration of 1×10^{-7} M. Stimulation with heparin solution alone (30 μg heparin per millilitre of PBS) also was used as an additional control.

Transendothelial migration assay

Transmigration assays were performed as described in details elsewhere (36). Briefly, 2.2×10^5 human microvascular endothelial cells (HMEC)-1 cells were seeded on fibronectin-coated 6.5-mm Transwell filters (Costar, Bodenheim, Germany) with a 5-μm pore size, and were grown at 37°C and 5%CO$_2$ to confluence. The integrity of the endothelial monolayer was confirmed by Filipin III (Sigma, cat. 4767) staining followed by fluorescent microscopic investigation, and, in parallel by the measurement of transendothelial electric resistance. After 48 h, medium and non-adherent cells were removed. Immediately, 600 μl of assay medium together with chemo-attractant IL-8 (25 ng/ml) was added to the lower compartment of a two-chamber system separated by the Transwell filters. Assay medium consisted of Dulbecco's modified Eagle's medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% FCS, 25 mM HEPES, 2% L-glutamine and 100 U/ml penicillin/streptomycin. Always the constant number of neutrophils was taken for assay. Neutrophils (2×10^6 cells) in 100-μl assay medium together with stimuli were added to the upper chamber, and cells were subsequently incubated for 2 h at 37°C and 5% CO$_2$. Among stimuli were either N-formylmethionyl-leucyl-phenylalanine (fMLP) (a well-known granulocyte activator, used as a positive control, concentration 5 nM) or PAR$_2$ agonists (mast cell tryptase 10^{-7} M; trypsin 10^{-7} M; PAR$_2$-tcAP 1×10^{-4} M) or PAR$_2$ reverse peptide (PAR$_2$-tcRP 1×10^{-4} M). Experiments were routinely carried out in triplicates.

At the end of the assay, the upper chamber was removed and the migrated neutrophils in the lower compartment were collected and quantified in a Casy TT (Schärfe Instruments, Reutlingen, Germany) or Coulter Counter Z2 (Coulter, Krefeld, Germany). Each sample was made in triplicate. Four independent experiments were performed.

Flow cytometric analysis of CD64 and PAR$_2$ cell surface expression on neutrophils from healthy volunteers and septic patients

Briefly, 1×10^6 cells from healthy donors were used for each analysis. Cells were washed twice in PBS (1%FCS) and subsequently incubated with primary mouse anti-human CD64 antibody or with anti-human PAR$_2$ antibody (in both cases 1 μg/ml) for 30 min on ice. Then, cells were washed and further incubated with PE-conjugated goat anti-mouse (1:50) secondary antibody in PBS (1%FCS) for additional 30 min on ice. Cells, which were incubated with secondary antibody alone served as a negative control. At least 25 000 stained cells were analysed with the FACScalibur and Cell Quest Pro Software (Becton Dickinson, Heidelberg, Germany).

For septic patient studies, whole blood was collected in an EDTA-containing syringe and erythrocytes were lysed using $1 \times$ flow cytometric analysis (FACS) lysing solution (BD PharMingen, San Diego, CA, USA). A total of 10 μl of FITC-labelled anti-human PAR$_2$ was used for receptor staining at neutrophil surface (20 min at RT). FITC-labelled IgG was added for isotype control (negative control) samples. After washing, the leukocytes were reconstituted in a fixing solution (1% paraformaldehyde in PBS). The purity of granulocytes was estimated with anti-CD45/anti-CD14 (to exclude monocyte contamination among analysed cells). At least 20 000 stained neutrophils from each donor in both groups were analysed with the FACScalibur and Cell Quest Pro Software (Becton Dickinson).

Annexin-V staining assay

Labelling of apoptotic neutrophils with annexin-V-FITC (37) and counterstaining with propidium iodide for necrotic cells (Annexin V-FITC Apoptosis Detection kit, BD PharMingen) were performed according to manufacturer's instructions. Labelled neutrophils were analysed by flow cytometry using FACScalibur and Cell Quest Pro software (Becton Dickinson).

Statistical analysis

Results are expressed as mean ± SEM. At least three independent experiments have been performed ($n > 3$). Statistical evaluation was performed by an analysis of variance and Student's t-test. Significance was assigned where $P < 0.05$.
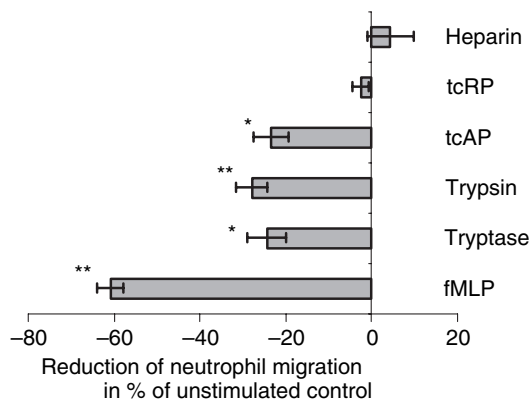
Results

Proteinase-activated receptor-2 agonist stimulation reduces the transendothelial migration of human neutrophils *in vitro*

Neutrophil-endothelial interactions play a crucial role in the pathophysiology of various skin diseases such as infection, neutrophilc vascular disorders (certain subtypes of vasculitis) or wound healing, for example. We demonstrated previously that PAR$_2$ agonist treatment affects surface expression of β_1 and β_2 integrins by human neutrophils (29). Thus, we hypothesized that PAR$_2$ agonist stimulation of endothelial cells as well as granulocytes may influence migratory ability of the latter. To test this

hypothesis, we used an experimental system where both cell types (granulocytes and HMEC-1 endothelial cells) were exposed to PAR_2 agonists during leukocyte migration. We revealed that application of PAR_2 activating serine proteases (trypsin or mast cell tryptase) as well as a synthetic PAR_2 activating peptide (tcAP) leads to significant reduction in the number of neutrophils migrated via endothelial monolayer in comparison with untreated control (Fig. 1). The magnitude of this effect was very similar for all PAR_2 agonists used in study – about $25 \pm 4\%$ of reduction (the application of fMLP, which was used as positive control, led to $60 \pm 4\%$ reduction) (Fig. 1). Neither treatment with heparin nor PAR_2 reverse peptide (tcRP) resulted in significant changes of neutrophil transendothelial migration (Fig. 1).

Proteinase-activated receptor-2 agonist stimulation prolongs human neutrophil survival *in vitro*

Human neutrophils are known to have a relatively short half-life in the circulation [(reviewed in Ref. (30)]. The life span of mature neutrophils may be extended *in vitro* by incubation with either pro-inflammatory cytokines or with bacterial products (38). Therefore, we investigated whether treatment of isolated human neutrophils with PAR_2 agonists might extend neutrophil survival *in vitro*. Indeed, PAR_2-tcAP stimulation of human neutrophils from healthy donors prolonged cell survival (estimated using population

of early apoptotic cells in Annexin V/propidium-iodide assay) (Fig. 2). At 6 h after PAR_2-tcAP application, the amount of early apoptotic neutrophils was $47 \pm 4\%$ lower as compared with untreated control cells. However, this trend was still detectable up to 12 h after agonist treatment. Moreover, application of trypsin in a concentration of 10^{-7} M also resulted in extended neutrophil survival *in vitro*. At 6 h after trypsin stimulation, the amount of early apoptotic cells becomes $21 \pm 4\%$ lower as compared with the amount of early apoptotic cells in untreated control samples. Application of trypsin or PAR_2-tcAP did not change significantly the number of neutrophils in the population of late apoptotic and necrotic cells (data not shown). Stimulation of cultured neutrophils with scrambled PAR_2-tcRP (negative control) did not affect neutrophil apoptosis *in vitro* (Fig. 2).

Effects of IFNγ stimulation on PAR_2 expression on the human neutrophil cell surface

Previously, Nystedt et al. (39) reported that stimulation of cultured human umbilical vein endothelial cells (HUVEC) with such pro-inflammatory cytokines as TNFα or IL-1α elevated the expression of PAR_2 mRNA in these cells. IFNγ is an immune cell produced cytokine with dual properties (pro- and anti-inflammatory) and has an ability to modulate the level of certain pro-inflammatory cytokines (TNFα, IL-1α) (40). Therefore, we were interested whether IFNγ stimulation may also affect PAR_2 expression on human neutrophil cell surface. At 6 h after IFNγ application, the amount of PAR_2 positive cells increases by $45 \pm 4\%$ as
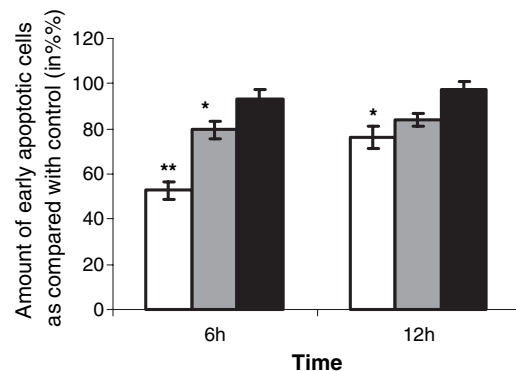
Figure 1. Proteinase-activated receptor-2 (PAR_2) agonists reduce transendothelial migration of human neutrophils from healthy volunteers. The number of granulocytes used for assay (input) was always constant in all experiments and for each sample (2×10^6 cells). The average number of neutrophils migrated in control sample was $5.96 \times 10^5 \pm 0.8 \times 10^5$ cells. In our experimental system, both cell types [human neutrophils and human microvascular endothelial cells (HMEC-1)] were stimulated with PAR_2 agonists (see 'Materials and Methods' for details). Stimuli were used in concentrations: tryptase and trypsin 10^{-7} M; PAR_2-tcAP 10^{-4} M; PAR_2-tcRP 10^{-4} M; heparin 30 μg/ml; N-formylmethionyl-leucyl-phenylalanine (fMLP) 5 nM. Four independent experiments were performed, each in triplicates. Results are presented as an average effect of agent application at neutrophil migration (expressed in percents) ±SEM. *$P < 0.05$, **$P < 0.01$.
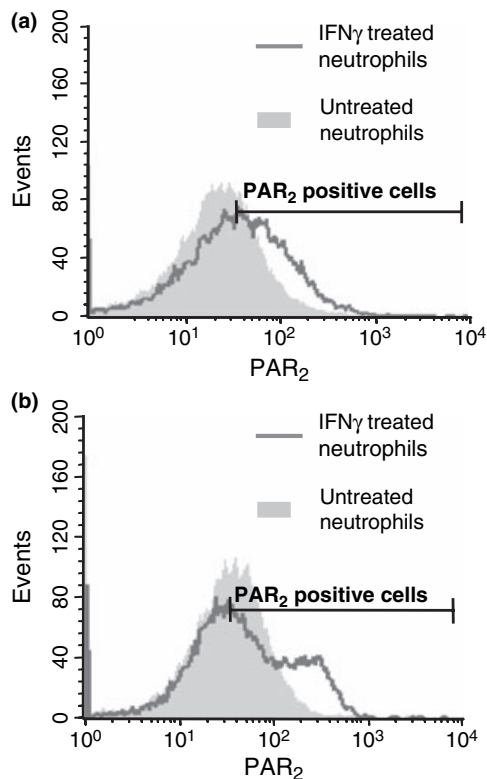
Figure 2. Agonists of proteinase-activated receptor-2 (PAR_2) prolong survival of cultured human neutrophils from healthy volunteers. No induction of apoptosis was performed. Presented data are relative where as 100% is considered the number of early apoptotic cells in untreated control samples. Application of stimuli did not change significantly the number of neutrophils in populations of late apoptotic and necrotic cells. Results are presented as an average amount of early apoptotic cells relatively to untreated control sample in per cents ± SEM. White filled (☐) boxes represent the data of PAR_2-tcAP (10^{-4} M) treated; grey filled (▨) boxes – trypsin (10^{-7} M) treated; black filled (■) boxes – PAR_2-tcRP (10^{-4} M) treated samples. *$P < 0.05$, **$P < 0.01$. Six independent experiments were performed.

compared with unstimulated neutrophils. However, the up-regulation of the mean fluorescence intensity (MFI) remains less pronounceable (increased by $33 \pm 3\%$ as compared with the level of untreated control) (Fig. 3a). The situation changes at 12 h after IFNγ application (Fig. 3b). The number of PAR₂-positive neutrophils remains very similar to the one of untreated control cells (increased by only $18 \pm 5\%$ as compared with untreated cells). Nonetheless, the up-regulation of MFI was significant (MFI increases by $98 \pm 9\%$ as compared with untreated control) (Fig. 3b).
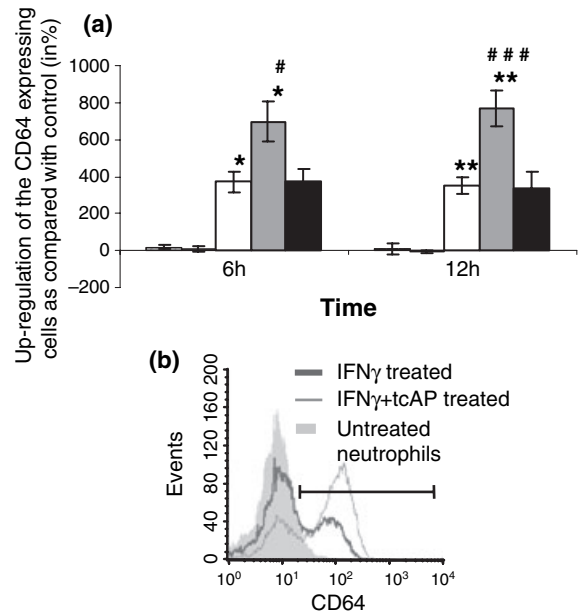
Simultaneous application of PAR₂ agonist and interferon γ enhances IFNγ-induced expression of FcγRI (CD64) at neutrophil cell surface

Despite granulocytes from healthy individuals are known to express CD64 at very low level, its display is induced by stimulation of neutrophils with IFNγ (31). Therefore, we investigated whether co-administration of PAR₂ agonist and IFNγ might enhance the known IFNγ-induced effect at CD64 display on neutrophil cell surface. Indeed, our results suggest that the treatment of human neutrophils with both agents (PAR₂-tcAP and IFNγ) leads to stronger up-regulation of CD64 on neutrophil cell surface than stimulation with IFNγ alone (Fig. 4a,b). The effect of PAR₂-tcAP and IFNγ co-application reaches its maximum at 12 h, but it already becomes significant at 6 h after agonist treatment (Fig. 4a). At 12 h after agonist application, this effect is characterized by up-regulation of MFI (increased by $41 \pm 9\%$ as compared with cells treated with IFNγ alone) as well as of the number of positive neutrophils (up-regulated at $90 \pm 8\%$ as compared with neutrophils stimulated by IFNγ alone) (Fig. 4b). Of note, co-stimulation of cultured neutrophils with IFNγ and the scrambled PAR₂-tcRP

Figure 3. Interferon (IFN)γ stimulation of human neutrophils from healthy volunteers up-regulates proteinase-activated receptor-2 (PAR₂) expression on the cell surface. Neutrophils were treated with IFNγ (200 U/ml) and analysed at 6 h (a) or 12 h (b) after stimulation. PAR₂ positive cells are gated. Left border of the gate was always set the way that not more than 1% cells of secondary antibody control sample were included inside the gate region. The percent of PAR₂-positive cells in untreated control samples was $44.9 \pm 8\%$ for 6 h, and $52.8 \pm 6\%$ for 12 h. The mean fluorescence intensity (MFI) absolute value of PAR₂ positive cells in untreated control was 52.2 ± 11 for 6-h samples, and 68 ± 6 for 12-h samples. Results one of three independent experiments are shown.

Figure 4. Simultaneous application of proteinase-activated receptor-2 (PAR₂) agonist and interferon (IFN)γ enhances IFNγ-induced expression of CD64 expression at human neutrophil cell surface. Neutrophils were isolated from healthy volunteers. The graph (a) represents changes in CD64 cell surface expression after stimulation of cells with PAR₂-tcAP 10^{-4} M ▨; PAR₂-tcRP 10^{-4} M ▧; IFNγ 200 U/ml □; IFNγ (200 U/ml) + PAR₂-tcAP(10^{-4} M) ▦; IFNγ (200 U/ml) + PAR₂-tcRP(10^{-4} M) ■. There were no significant changes in CD64 expression after stimulation of neutrophils with either PAR₂-tcAP or PAR₂-tcRP (the respective columns at the graph have very small value). Results are presented as an average effect of agent application at the number of CD64-positive neutrophils as compared with untreated control samples (expressed in percents) ±SEM. *$P < 0.05$, **$P < 0.01$. Additionally, the difference in average effects between samples stimulated with IFNγ alone and co-stimulated with PAR₂-tcAP and IFNγ was analysed. Statistical significance of such difference is marked #$P < 0.05$, ###$P < 0.001$. Four independent experiments were performed. Histogram shows CD64 surface expression at 12 h after stimuli application (b). Gated cells are CD64-positive cells.

(negative control) did not affect neutrophil CD64 expression as compared with cells stimulated by IFNγ alone (Fig. 4a). Stimulation of human neutrophils either by PAR$_2$-tcAP or PAR$_2$-tcRP alone also did not affect CD64 cell surface display (Fig. 4a).

Changes of PAR$_2$ cell surface display on neutrophils of septic patients

Our *in vitro* data indicate that PAR$_2$ agonists reduce migration of neutrophils via endothelium and also prolong neutrophil survival, therefore, potentially may promote an increase of neutrophil count in circulation. We also demonstrated that, PAR$_2$ agonists acting together with IFNγ, significantly enhance the effect of latter at CD64 expression on neutrophils, and these results indicate the involvement of PAR$_2$ activation in phagocytosis. Together, our data suggest that neutrophil PAR$_2$ could be involved in the pathogenesis of human diseases, where phagocytes are in contact with pathogens in the blood flow. One of such diseases is sepsis. Therefore, we tested whether the level of neutrophil PAR$_2$ cell surface expression might change in septic patients. Indeed, our data clearly demonstrate an increased (about 82% of up-regulation as compared with healthy volunteers) PAR$_2$ expression on neutrophils of septic patients (Fig. 5). Thus, PAR$_2$ may contribute to the modulation of neutrophil function during human sepsis.

Discussion

In a recent study (29), we demonstrated the ability of PAR$_2$ agonists to up-regulate neutrophil cytokine production and motility in 3-D collagen lattices. The main goal of the present study was to further explore the role of PAR$_2$ on neutrophils with special regard to its capability to modulate neutrophil-endothelial interactions.
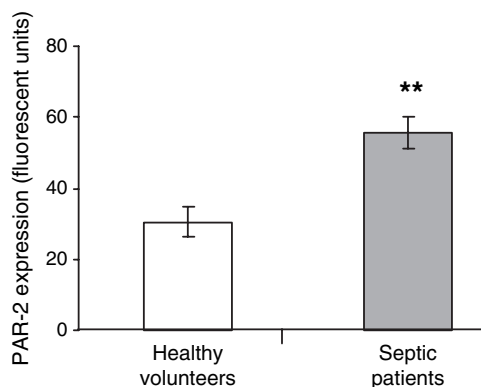
Figure 5. An increased expression of PAR$_2$ at the cell surface of human neutrophils from septic patients. PAR$_2$ display on human neutrophil cell surface is up-regulated in septic patients (19 cases) as compared with healthy volunteers (18 cases). The results are presented as average in mean fluorescence intensity units ±SEM. **$P < 0.01$.

Our previous results were predominantly associated with a scenario in which the activation of neutrophil PAR$_2$ took place in the extracellular matrix, i.e. after cell migration into the extravascular compartment. However, PAR$_2$ activation on neutrophils in the vascular compartment (before transendothelial migration) cannot be completely excluded, as various natural PAR$_2$ agonists are released within blood vessel. Among such agonists are endothelial-derived trypsin and factor VII/Xa, for example (41–43). Therefore, in the current study, we focused on the effects of PAR$_2$ agonists, which are especially important for leukocyte biology in the blood flow. We revealed that PAR$_2$ agonists down-regulate trans-endothelial migration of human granulocytes *in vitro*. Moreover, PAR$_2$ agonists extended neutrophil life span and enhanced the IFNγ-induced FcγRI (CD64) receptor expression on human neutrophils. The increase in CD64 expression is known to be associated with an enhancement of antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (31,44). Consequently, this ability of PAR$_2$ agonists to enhance IFNγ effects strongly indicates that co-activation of PAR$_2$ and cellular cytokine receptors may affect the progress of acute inflammatory diseases. This idea is linked to our first-time observations that PAR$_2$ and its proteases may be involved in human diseases *in vivo*, as demonstrated in septic patients. Our results clearly suggest the involvement of PAR$_2$ in neutrophil-endothelial interactions by modulating transmigration and apoptosis of human neutrophils.

Transendothelial migration of granulocytes is considered as a key step in immune response to pathogens (45). However, uncontrolled adhesion to endothelium and recruitment of neutrophils towards the site of inflammation may lead to significant tissue damage and, therefore, to poor prognosis for patient (46,47). Thus, only the accurate regulation of granulocyte recruitment in extravascular compartment promotes resolution of inflammation without serious complications. Our *in vitro* data indicate the potential role of serine proteases and their receptors in the regulation of leukocyte transendothelial migration. Indeed, proteases (trypsin and tryptase) as well as a PAR$_2$ activating peptide reduced the migration of human neutrophils via endothelial monolayer. Therefore, at an early stage after stimulation, PAR$_2$ agonists and their receptor may represent an additional system which controls leukocyte recruitment and consequently modulates the intensity of immune response.

Delayed apoptosis of neutrophils migrated in extravascular space can affect the progression of inflammatory responses during infection. This effect is reached via a reduced clearance of neutrophils from the inflamed tissues and therefore via a risk of tissue injury by neutrophil granule contents (48). On the other hand, extended life of neutrophils in vascular compartment may be beneficial as it secures additional time for the development of adaptive

immune response (31). Therefore, it was of importance to note that PAR₂ agonists (trypsin as well as activating peptide) down-regulate human neutrophil apoptosis. Such an effect of PAR₂ agonists on neutrophil apoptosis together with reduction of transendothelial migration may modulate the amount of human nuetrophils in circulation at early stages after stimulation.

CD64 is a high-affinity Fcγ receptor, which is known to be expressed only at a very low level by neutrophils from healthy adults (31,49). However, IFNγ stimulation or bacterial infections are reported to induce the expression of CD64 on neutrophils (50,51). The increase in CD64 display appears to be associated with an enhancement of ADCC and phagocytic activity of neutrophils (31,44). As we showed in the current study, PAR₂-tcAP stimulation alone did not markedly affect CD64 expression on neutrophil cell surface. However, co-application of PAR₂ agonists together with IFNγ enhanced the effect of latter. Our data also indicate that IFNγ stimulation up-regulates PAR₂ expression on the cell surface of isolated human neutrophils. Therefore, coordinated interactions between IFNγ and PAR₂ agonists may represent a mechanism by which IFNγ-induced protective effects could be enhanced during bacterial infection.

Sepsis in humans is known to be characterized by a state of immunosuppression associated with shutdown of neutrophil phagocytic activity and some other neutrophil functions (22). In attempt to reactivate monocyte and neutrophil immune functions, IFNγ-intravenous injections were used in clinical trials in sepsis (28). Considering the ability of PAR₂ agonists to enhance one of IFNγ-induced protective effects on human neutrophils *in vitro*, we investigated whether cell surface expression of neutrophil PAR₂ may be affected during sepsis in human patients. Our study clearly demonstrates the up-regulation of PAR₂ on the neutrophil cell surface in septic patients. In accordance with these findings, the potential role of the combined action of PAR₂ agonist and IFNγ during human sepsis represents an innovative field for further investigations.

Neutrophils are the most numerous cells of blood leukocytes and represent the first line of the innate immune defense against infection. Consequently, neutrophils need to be recruited to the site of inflammation very rapidly. During disease state, molecules exist which may support or counteract this capability (52). Thus, proteases released by endothelial cells, mast cells or neutrophils themselves may be involved in the pathophysiology of cutaneous inflammation and sepsis in humans by affecting neutrophil apoptosis, transendothelial migration and Fcγ receptor-mediated phagocytosis. This may be of importance for the regulation of neutrophil activity during infection including septic conditions, inflammation as well as wound healing.

In conclusion, our data demonstrated that PAR₂ agonists are able to enhance the protective IFNγ-induced effect on neutrophil function. Moreover, PAR₂ agonists (activating serine proteases or peptides) and their receptor represent an additional system which controls neutrophil transendothelial migration and apoptosis *in vitro*. Thus, such system may be involved in the changes of neutrophil-endothelial interactions as well as in the enhancement of cytokine-induced effects at neutrophil functions during inflammatory or acute bacteria-induced pathologies in humans.

Acknowledgements

References

1 Hollenberg M D, Compton S J. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol Rev 2002: **54**: 203–217.

2 Steinhoff M, Buddenkotte J, Shpacovitch V *et al.* Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocr Rev 2005: **26**: 1–43.

3 Molino M, Barnathan E S, Numerof R *et al.* Interactions of mast cell tryptase with thrombin receptors and PAR-2. J Biol Chem 1997: **272**: 4043–4049.

4 Nystedt S, Emilsson K, Larsson A K, Strombeck B, Sundelin J. Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. Eur J Biochem 1995: **232**: 84–89.

5 Lourbakos A, Chinni C, Thompson P *et al.* Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from Porphyromonas gingivalis. FEBS Lett 1998: **435**: 45–48.

6 Sun G, Stacey M A, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. J Immunol 2001: **167**: 1014–1021.

7 Uehara A, Muramoto K, Takada H, Sugawara S. Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. J Immunol 2003: **170**: 5690–5696.

8 Steinhoff M, Corvera C U, Thoma M S *et al.* Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. Exp Dermatol 1999: **8**: 282–294.

9 Miike S, McWilliam A S, Kita H. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. J Immunol 2001: **167**: 6615–6622.

10 Colognato R, Slupsky J R, Jendrach M, Burysek L, Syrovets T, Simmet T. Differential expression and regulation of protease-activated receptors in human peripheral monocytes and monocyte-derived antigen-presenting cells. Blood 2003: **102**: 2645–2652.

11 Howells G L, Macey M G, Chinni C *et al.* Proteinase-activated receptor-2: expression by human neutrophils. J Cell Sci 1997: **110**: 881–887.

12 Moormann C, Artuc M, Pohl E *et al.* Functional characterization and expression analysis of the proteinase-activated receptor-2 in

human cutaneous mast cells. J Invest Dermatol 2006: **126**: 746–755.

13 Maurer M, Metz M. The status quo and quo vadis of mast cells. Exp Dermatol 2005: **14**: 923–929.

14 Stander S, Steinhoff M. Pathophysiology of pruritus in atopic dermatitis: an overview. Exp Dermatol 2002: **11**: 12–24.

15 Ossovskaya V S, Bunnett N W. Protease-activated receptors: contribution to physiology and disease. Physiol Rev 2004: **84**: 579–621.

16 Noorbakhsh F, Tsutsui S, Vergnolle N et al. Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. J Exp Med 2006: **203**: 425–435.

17 Seeliger S, Derian C K, Vergnolle N et al. Proinflammatory role of proteinase-activated receptor-2 in humans and mice during cutaneous inflammation in vivo. FASEB J 2003: **17**: 1871–1885.

18 Schmidlin F, Amadesi S, Dabbagh K et al. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J Immunol 2002: **169**: 5315–5321.

19 Ferrell W R, Lockhart J C, Kelso E B et al. Essential role for proteinase-activated receptor-2 in arthritis. J Clin Invest 2003: **111**: 35–41.

20 Fiorucci S, Mencarelli A, Palazzetti B et al. Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. Proc Natl Acad Sci U S A 2001: **98**: 13936–13941.

21 Namkung W, Han W, Luo X et al. Protease-activated receptor 2 exerts local protection and mediates some systemic complications in acute pancreatitis. Gastroenterology 2004: **126**: 1844–1859.

22 Riedemann N C, Guo R F, Ward P A. The enigma of sepsis. J Clin Invest 2003: **112**: 460–467.

23 McGirt L Y, Beck L A. Innate immune defects in atopic dermatitis. J Allergy Clin Immunol 2006: **118**: 202–208.

24 Ellis T N, Beaman B L. Interferon-gamma activation of polymorphonuclear neutrophil function. Immunology 2004: **112**: 2–12.

25 Cassatella M A, Huber V, Calzetti F et al. Interferon-activated neutrophils store a TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) intracellular pool that is readily mobilizable following exposure to proinflammatory mediators. J Leukoc Biol 2006: **79**: 123–132.

26 Schroder K, Hertzog P J, Ravasi T, Hume D A. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 2004: **75**: 163–189.

27 Schiller M, Metze D, Luger T A, Grabbe S, Gunzer M. Immune response modifiers–mode of action. Exp Dermatol 2006: **15**: 331–341.

28 Vincent J L, Sun Q, Dubois M J. Clinical trials of immunomodulatory therapies in severe sepsis and septic shock. Clin Infect Dis 2002: **34**: 1084–1093.

29 Shpacovitch V M, Varga G, Strey A et al. Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices. J Leukoc Biol 2004: **76**: 388–398.

30 Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 2000: **80**: 617–653.

31 Edwards S. Biochemistry and Physiology of the Neutrophil. New York: Cambridge University Press, 1994.

32 Shpacovitch V M, Brzoska T, Buddenkotte J et al. Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells. J Invest Dermatol 2002: **118**: 380–385.

33 Vergnolle N, Macnaughton W K, Al-Ani B, Saifeddine M, Wallace J L, Hollenberg M D. Proteinase-activated receptor 2 (PAR2)-activating peptides: identification of a receptor distinct from PAR2 that regulates intestinal transport. Proc Natl Acad Sci U S A 1998: **95**: 7766–7771.

34 Bone R C, Balk R A, Cerra F B et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 1992: **101**: 1644–1655.

35 Feige U, Overwien B, Sorg C. Purification of human blood monocytes by hypotonic density gradient centrifugation in Percoll. J Immunol Methods 1982: **54**: 309–315.

36 Rohnelt R K, Hoch G, Reiss Y, Engelhardt B. Immunosurveillance modelled in vitro: naive and memory T cells spontaneously migrate across unstimulated microvascular endothelium. Int Immunol 1997: **9**: 435–450.

37 Homburg C H, de Haas M, von dem Borne A E, Verhoeven A J, Reutelingsperger C P, Roos D. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. Blood 1995: **85**: 532–540.

38 Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 1992: **80**: 2012–2020.

39 Nystedt S, Ramakrishnan V, Sundelin J. The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. J Biol Chem 1996: **271**: 14910–14915.

40 Muhl H, Pfeilschifter J. Anti-inflammatory properties of pro-inflammatory interferon-gamma. Int Immunopharmacol 2003: **3**: 1247–1255.

41 Camerer E, Huang W, Coughlin S R. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci U S A 2000: **97**: 5255–5260.

42 Camerer E, Kataoka H, Kahn M, Lease K, Coughlin S R. Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells. J Biol Chem 2002: **277**: 16081–16087.

43 Jin E, Fujiwara M, Pan X et al. Protease-activated receptor (PAR)-1 and PAR-2 participate in the cell growth of alveolar capillary endothelium in primary lung adenocarcinomas. Cancer 2003: **97**: 703–713.

44 de Haas M, Vossebeld P J, von dem Borne A E, Roos D. Fc gamma receptors of phagocytes. J Lab Clin Med 1995: **126**: 330–341.

45 Wagner J G, Roth R A. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol Rev 2000: **52**: 349–374.

46 Varani J, Ward P A. Mechanisms of neutrophil-dependent and neutrophil-independent endothelial cell injury. Biol Signals 1994: **3**: 1–14.

47 Lentsch A B, Ward P A. Regulation of inflammatory vascular damage. J Pathol 2000: **190**: 343–348.

48 Savill J. Apoptosis in resolution of inflammation. J Leukoc Biol 1997: **61**: 375–380.

49 McKenzie S E, Schreiber A D. Fc gamma receptors in phagocytes. Curr Opin Hematol 1998: **5**: 16–21.

50 Goulding N J, Knight S M, Godolphin J L, Guyre P M. Increase in neutrophil Fc gamma receptor I expression following interferon gamma treatment in rheumatoid arthritis. Ann Rheum Dis 1992: **51**: 465–468.

51 Davis B H, Olsen S H, Ahmad E, Bigelow N C. Neutrophil CD64 is an improved indicator of infection or sepsis in emergency department patients. Arch Pathol Lab Med 2006: **130**: 654–661.

52 Steinhoff M, Griffiths C M E, Church M K, Luger T A. Inflammation. In: Burns T, Breathnach S, Cox N, Griffiths C, eds. Rook's Textbook of Dermatology, 7th edn. Oxford: Blackwell Publishing Ltd., 2004.