

CD44 mediates polymorphonuclear leukocyte motility on hyaluronan

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ABSTRACT

Objectives: To investigate the behavior of polymorphonuclear (PMN) leukocytes on the extracellular matrix carbohydrate component, hyaluronan (HA), in the presence and absence of the chemokine, interleukin-8 (IL-8).

Methods: The present study was conducted at the Department of Hematology, University of Liverpool, United Kingdom, between the period 2000 to 2001. Polymorphonuclear cells were isolated from whole venous blood using Mono-Poly-Resolving Medium. Purified PMN were added alone or with IL-8 to HA-coated plates and the behavior of these cells monitored by time-lapse video microscopy over a period of 40 minutes. For the identification of surface receptor(s) mediating PMN migration on HA, PMN were incubated with blocking and non-blocking antibodies against cluster of differentiation 44 (CD44) and Receptor for Hyaluronan Mediated Motility (RHAMM) prior to addition to HA-coated surfaces.

Results: Approximately 55% of PMN were found to interact

and migrate on HA-coated plates with a mean speed of $6.4 \pm 0.7 \mu\text{m}/\text{min}$. Addition of IL-8 reduced both the percentage moving cells (7.5%) and the average speed of the remaining moving cells ($2.0 \pm 0.3 \mu\text{m}/\text{min}$). The inhibitory effect of IL-8 on PMN migration was associated with reorganization of the cytoplasmic fibrillar form of actin. Anti-CD44 blocking antibody substantially reduced the speed of PMN ($2.5 \pm 0.9 \mu\text{m}/\text{min}$), while non-blocking anti-CD44 and anti-RHAMM antibodies had no effect.

Conclusions: The present study demonstrates for the first time that PMN are able to interact and migrate on the widely distributed extracellular matrix component, HA, using the cell surface receptor, CD44. Such interaction is modified by the chemokine, IL-8, in a way that optimizes the host defense against invading pathogens.

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Polymorphonuclear leukocytes (PMN,) play a central role in the innate branch of the immune response against pathogens. The cells are found normally in a quiescent state in circulation. However, in response to inflammatory stimuli, PMN respond by adhering to and transmigrating across the endothelium and then migrating across the interstitial tissues to arrive at the area of infection, where they are involved in the phagocytosis and killing of invading microorganisms.¹⁻³ These different stages occur sequentially, appropriately and are achieved by cell associated adhesion receptors

located on the cell membranes of PMN and the endothelium.⁴⁻⁷ Although the process of PMN adhesion to and transmigration across the endothelium has been studied extensively, little data exist on the process involved in PMN migration across interstitial tissues. Limited studies have shown that PMN are able to interact with the extracellular matrix components, fibronectin and vitronectin.^{8,9} However, the role of other components remains largely unknown. Moreover, effect of chemokines on PMN motility on the interstitial components has not been widely explored. Recently,

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lymphocytes were shown to interact and move on HA, a major carbohydrate component of the interstitial tissue matrix, using the cell surface receptor cluster of differentiation 44 (CD44).^{10,11} In the present study, the possible role of HA in PMN migration was investigated. Since chemokines play a central role in PMN function, the effect of IL-8 on PMN behavior on HA and vitronectin was investigated.

Methods. Polymorphonuclear leukocytes were isolated from blood as previously described.¹² Briefly, freshly citrated normal venous blood (12.9 mM tri-sodium citrate) was centrifuged for 10 minutes at 200g. After removal of platelet-rich plasma (PRP) the residue of blood was reconstituted to the original volume with 6% solution of Dextran T500 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in phosphate-buffered saline (PBS) pH 7.3, and then left at 37°C for 30 min to sediment the red blood cells. The cell suspension above the sedimented red cells was collected and placed on top of a discontinuous Ficoll-Hypaque density gradient. The gradient was prepared from neat and 15% diluted Mono-Poly Resolving Medium (ICN Biomedicals, Bucks.). After centrifugation for 30 min at 500g at room temperature, >98% pure PMN were collected from the interface between the gradient solutions and washed once in Hanks balanced salt solution (HBSS) (Flow Laboratories, Scotland), supplemented by 12.9 mM tri-sodium citrate. The cells were then resuspended first in 0.5 ml citrated HBSS counted and diluted to a concentration of 10^7 cells/ml with RPMI medium (Northumbria Biologicals Ltd).

Cell viability. Polymorphonuclear viability after isolation was determined by trypan blue exclusion and by fluorescence activated cell sorter analysis of cells stained with propidium iodide (PI)¹³ (dead cells become permeable to PI and bright red). Cell viability was routinely >98%.

Monoclonal antibodies. The following monoclonal antibodies (MoAbs) were used: anti-CD44 (50B4, a blocking MoAb and a gift from Dr. M. Letarte, Hospital for Sick Children, Toronto, Canada, and 2C5 non-blocking reagents from R & D Systems, Abingdon, United Kingdom. Anti-Receptor for (RHAMM) (3T3.5, a blocking antibody which was a gift from Dr E. Turley, Hospital for Sick Children, Toronto). All these MoAbs are of the immunoglobulin (Ig) G1 isotype. To control for non-specific effects of the MoAb, IgG1 control isotype of non-immune Igs was used.

Time-lapse video microscopy. Petri dishes were coated overnight with hyaluronan (HA, Pharmacia, Uppsala, Sweden) or, in certain experiments, with vitronectin (VN; 20 µg/ml). Dishes were washed and PMN added (2×10^6 /ml) with or without IL-8 (10 ng/ml). In preliminary experiments, a range of concentrations of IL-8 (1, 10 and 100 ng/ml) were tested; the effect of IL-8 was maximal at 10 ng/ml. The dishes were then placed on a heated (37°C) microscope stage

and filmed for 40 minutes using TLVM. Cell movement was determined by sequential tracing on the video screen. A cell was considered to have moved when its position had changed by more than one diameter. This methodology allows the percentage and speed of motile cells to be calculated. For the calculations of speed, PMN were assumed to be 12 µm in diameter. In order to establish the receptor responsible for cell motility, PMN were incubated with MoAbs to CD44 (20 µg/ml) and RHAMM (10 µg/ml and an IgG1 control (40 µg/ml) for 30 min on ice prior to addition to petri dishes.

Confocal microscopy. Microscope slides were coated with HA overnight. Following washing, PMN were added to these slides with or without IL-8 (10 ng/ml) and incubated at 37°C for 40 min. Slides were then washed and the adherent cells fixed with 3.8% paraformaldehyde, washed again, and allowed to dry. Cells were then stained with anti-CD44 MoAb, followed by GAM-FITC as a second layer. For detection of fibrillar form of actin (F-actin), cells were stained with rhodamine-phalloidin (Molecular Probes, Leiden, Holland). Fluorescence was analyzed using a Microradiance confocal microscope (Biorad, Hemel Hempstead, United Kingdom).¹⁴

Results. Polymorphonuclear leukocyte motility on hyaluronan. When PMN were added to HA-coated plates, the majority of cells (~55%) adhered and assumed a motile morphology with a clear antrograde (lamellipodia) and retrograde (uropodia) protrusion. These cells migrated with an average speed of 6.4 µm/min (Table 1). The remaining cells adhered and spread with a 'fried egg' appearance and remained motionless for the rest of the observational period. Hyaluronan supported PMN motility in a dose dependent manner up to 200 µg/ml. Increasing the concentration above this concentration had no further effect on PMN motility (Table 2). The morphology of PMN on VN was similar to that observed on HA, but cells moved with a slightly reduced rate (Table 1). These results clearly demonstrate, for the first time that HA can support PMN migration and is thus, likely to play a role in-vivo. Polymorphonuclear leukocyte migration on vitronectin has been demonstrated in the past and shown to be mediated by the cell surface receptor, $\alpha 1$.⁹ In the present study, the possible receptor mediating PMN migration on HA was next investigated.

Cluster of differentiation 44 mediates polymorphonuclear leukocyte motility on hyaluronan. In order to explore the receptor(s) mediating PMN motility on HA, PMN were incubated with saturating amount of monoclonal antibodies against receptors known to bind to HA. A blocking anti-RHAMM monoclonal antibody had no effect on PMN motility on HA, and the results were similar to that observed in the presence of a control monoclonal antibody (Table 3). In contrast, a blocking anti-CD44 monoclonal antibody substantially reduced PMN

Table 1 - Polymorphonuclear leukocyte migration on HA and VN-coated plates.

Surface and treatment	Speed of moving cells ($\mu\text{m}/\text{min}$)	Moving cells (%)
HA alone	6.4 ± 0.7	55 ± 9
HA + IL-8	2.0 ± 0.3	7 ± 5
VN alone	5.5 ± 2.2	30 ± 8
VN + IL-8	14.5 ± 1.4	100

Polymorphonuclear (PMN) alone or with Interleukin (IL)-8 (10 ng/ml) were added to plates pre-coated with hyaluronan (HA) (200 $\mu\text{g}/\text{ml}$), or vitronectin (VN) (40 $\mu\text{g}/\text{ml}$) and their behavior recorded by Time Lapse Video Microscopy (TLVM) over a period of 40 minutes. The speed of PMN was calculated as described in the material and methods section. Results are means \pm SEM of 4 different experiments.

Table 2 - Hyaluronan support PMN migration in a dose-dependent fashion.

HA concentration ($\mu\text{g}/\text{ml}$)	Speed of moving cells ($\mu\text{m}/\text{min}$)
0	0
100	3.4 ± 0.24
200	6.4 ± 0.77
500	5.4 ± 0.56

Polymorphonuclear leukocytes were added to plates pre-coated with different concentration of hyaluronan (HA) and the speed of the cells monitored by Time Lapse Video Microscopy

Table 3 - Cluster of differentiation 44 (CD44) mediates PMN migration on HA.

Surface and treatment	Speed of moving cells ($\mu\text{m}/\text{min}$)
HA alone	8.02 ± 0.2
HA + IgG1	8.4 ± 0.5
HA + anti-CD44 (non-blocking)	7.9 ± 0.4
HA + anti CD44 (blocking)	2.5 ± 0.9
HA + anti-RHAMM	7.5 ± 0.4

Polymorphonuclear (PMN) leukocytes were incubated with different monoclonal antibodies for 30 minutes on ice prior to addition to hyaluronan (HA) coated plates. Polymorphonuclear behavior was then recorded by Time Lapse Video Microscopy (TLVM). Results are means \pm SEM from 3 different experiments. RHAMM - receptor for HA mediated motility, IgG -Immunoglobulin G

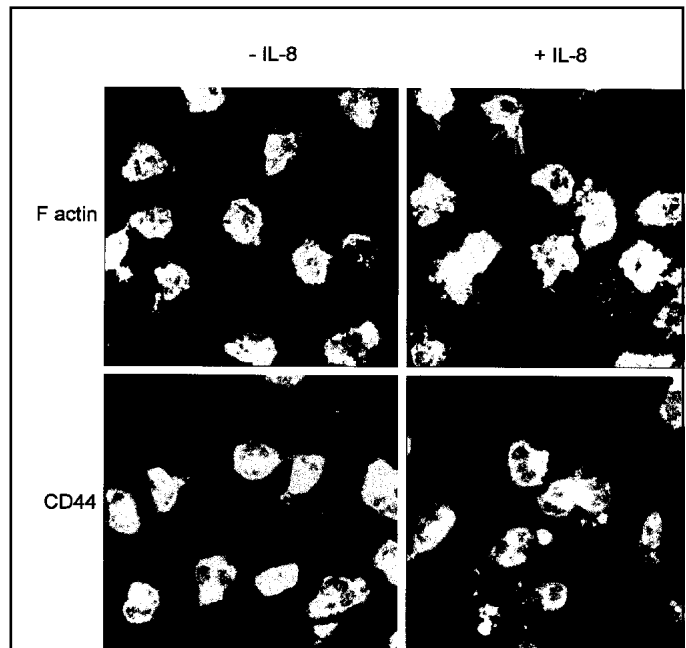


Figure 1 - Polymorphonuclear leukocytes were cultured without (-IL-8), or with (+IL-8) IL-8 (10 ng/ml) for 40 minutes and the distribution of F-actin and cluster of differentiation 44 examined by confocal microscopy as described in the methods section. IL- interleukin.

migration on HA-coated plates (Table 3). Non-blocking anti-CD44 antibody, as expected, had no effect on PMN migration (Table 3). These results clearly show that CD44 is the receptor mediating PMN migration on hyaluronan.

Effect of interleukin-8 on polymorphonuclear leukocyte motility on hyaluronan and vitronectin-coated surfaces. When IL-8 was added with PMN, it was found to inhibit PMN migration on HA, with substantial reduction in both the average speed and the percentage of moving cells (Table 1). In contrast, on vitronectin, IL-8 increased the percentage and the average speed of moving PMN (Table 1). Since the inhibitory effect of IL-8 on PMN motility on HA was unexpected, further investigation into this phenomenon was conducted.

Effect of interleukin-8 on polymorphonuclear leukocyte F-actin and cluster of differentiation 44. In the absence of IL-8, PMN on HA showed a motile morphology with F-actin concentrated mainly at the front and the back of cells (Figure 1). The distribution of F-actin was paralleled by the distribution of CD44 (Figure 1) (-IL-8). In contrast, in the presence of IL-8 (+IL-8), PMN spread extensively over the surface and numerous villi projecting from the cell, particularly from the tail region, were observed. In these cells, F-actin was organized mainly into concentrated plaques, which presumably constitute the main attachment sites of PMN to HA-coated surfaces (Figure 1). Cluster of differentiation 44 paralleled the distribution of F-actin

and was also found concentrated in plaques at the attachment sites (**Figure 1**) (+IL-8). These results indicate that IL-8 exerts its inhibitory effect on PMN migration through its effect on the mechanism responsible for F-actin redistribution.

Discussion. Hyaluronan, a major carbohydrate component of the extracellular matrix tissues, has been shown previously to support adhesion and migration of lymphocytes.^{11,15} Moreover, interaction of HA with CD44 on the surface of monocytes and PMN has been shown to enhance their functions including cell killing and phagocytosis.^{16,17} However, the role of HA in PMN adhesion and migration has not been explored previously. In the present study, the behavior of PMN on HA was investigated in the presence and absence of the chemokine, IL-8. The study clearly shows that PMN are able to interact and move on HA using the cell surface receptor, CD44. This receptor is normally found in an active form on the cell surface.¹⁷ The fact that CD44 mediates PMN motility on HA suggests that these molecules are in the active form. Activation of CD44 could have resulted from the partial PMN activation known to occur during cell preparation. Alternately, PMN could pose an isoform of CD44 (V3), which can interact directly with HA without the need for cellular activation.¹¹

Other cell types influence PMN functions, both directly and indirectly through production of chemokines and growth factors.^{18,19} Interleukin-8 is the most active chemokine on PMN function and influences their migration during inflammatory reaction.¹⁹ It is produced by a variety of cell types including macrophages, endothelial cells, fibroblasts, lymphocytes and PMN themselves.¹⁹⁻²¹ When IL-8 was added along with PMN to HA, it was found unexpectedly to inhibit PMN migration. However, addition of IL-8 with PMN to VN was found, as expected, to enhance cell migration. Since the effect of IL-8 on PMN migration on HA was unexpected, the possible mechanism of inhibition was further investigated. Cell motility is associated with increased F-actin polymerization and redistribution to the front and the back of the cell. During cell migration, polymerized F-actin binds adhesion receptor located on the cell membrane to the cytoskeleton and cause their recycling from the front to the back of the cell and hence the distribution of adhesion receptors parallel that of F-actin.^{22,23} In the present study, as would be expected from moving cells, PMN on HA showed a clear polarization of F-actin and CD44, mainly, to the back and front of cells. However, in the presence of IL-8, such polarization was not observed, instead, F-actin and CD44 were mainly concentrated into discrete areas throughout the spread cell. Concentration of CD44 in this manner would be expected to enhance PMN adhesion to HA and consequently, counteract the contractile force generated by the cell leading to cell arrest.

Vitronectin, a protein component of the extracellular matrix, has been shown to promote PMN migration

through interaction with cell surface receptor $\alpha 5 \beta 1$.⁸ Unlike its effect on PMN migration on HA, IL-8 increased the percentage moving cells and enhanced their average speed on vitronectin. These results suggest that signals generated from an adhesion molecule on the cell surface determine the overall response of PMN. Thus, signals generated from CD44 and IL-8 lead to inhibition of PMN migration, while signals from IL-8 and $\alpha 5 \beta 1$ lead to enhancement of PMN motility. It would be interesting to examine the intracellular signaling pathways generated by these 2 receptors. The above observations are important and relevant to the overall general inflammatory reaction, in which PMN plays a central role. Hyaluronan and vitronectin are found differently distributed in tissues. Vitronectin distribution is restricted to areas adjacent to blood vessels, while HA is strongly distributed throughout the interstitial tissues.^{10,24} It is anticipated that during infection, PMN would migrate from blood vessels across the endothelium into interstitial tissues guided by chemokines such as IL-8. In the interstitial tissues, PMN would initially interact with vitronectin. Inhibition of PMN at this stage would be undesirable, as it would inhibit cells from reaching the area of infection. Therefore, PMN would interact and move on vitronectin and such movement would be enhanced in the presence of chemokines and cytokines. Once PMN move deeper into tissues, they would interact with HA and in the presence of IL-8 or other chemokines; their movement would be inhibited. Such effect would be desirable, as it would keep PMN in the vicinity of infection where they would participate in killing of invading pathogens. In addition to localization of PMN close to infection sites, HA would simultaneously enhance the processes of phagocytosis and killing of pathogens^{16,17} and thus lead to effective elimination of such pathogens. Phagocytosis of pathogens would activate PMN maximally and result in the down-regulation of CD44 from the cell surface, as we and others have demonstrated previously.^{25,26} Down-regulation of CD44 would allow PMN to move to other areas for further participation in the elimination of invading pathogens.

In summary, the present study has shown for the first time that PMN are able to interact and move on HA using the cell surface receptor, CD44 and such interaction is modulated by the chemokine, IL-8, in a way that is physiologically beneficial.

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