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Front page: Two undifferentiated HL60 cells labelled with anti-CD13 antibody by fluorescence microscopy.

ABSTRACT

Background and aim

Neutrophil granulocytes express well known adhesion molecules on their cell membranes that during inflammation participate in neutrophil adhesion to endothelial cells. Earlier experiments have shown that neutrophils adhere to artificial surfaces and biomaterials when known adhesion molecules are inactive, indicating that also other adhesion molecules are involved. Certain proteoglycans expressed on neutrophils are thought to participate in neutrophil adhesion. Neutrophils are short lived cells and cannot be cultured for experimental use. A promyelocytic HL60 cell line can differentiate towards neutrophils and be used as a model to study neutrophil adhesion. The aim of this study was to investigate the expression of certain proteoglycans on HL60 cells and their ability to adhere to artificial surfaces compared to isolated neutrophils.

Methods

HL60 cells were differentiated towards mature neutrophils with stimulation of all-trans-retinoic-acid. Differentiated HL60 cells were labelled with antibodies against two different proteoglycans, syndecan-4 and CD44, and measured by flow cytometry. Adhesion experiments were performed on differentiated HL60 cells pre-treated with anti-CD44 antibodies and quantified with an enzymatic method based on the level of myeloperoxidase in the cells. Neutrophils were isolated from whole blood from blood donor samples and compared to HL60 cells in the adhesion experiments.

Conclusions

The experiments from flow cytometry showed that differentiated HL60 cells express CD44 on their cell membrane, but there was no expression of syndecan-4 on differentiated HL60. Using antibody-mediated blockage of CD44, adhesion of differentiated HL60 cells and isolated neutrophils to a polystyrene surface was reduced. This indicated that CD44 acted as a proadhesive molecule involved in HL60 cell and neutrophil adhesion. The differentiated HL60 cells can function as a model of CD44 proteoglycan mediated adhesion of neutrophils to polystyrene surfaces, but they probably cannot be used as a model to study syndecan-4 mediated adhesion of neutrophils.

ABBREVATIONS

PSGL-1 – P-selectin glycoprotein ligand-1

ESL-1 – E-selectin ligand-1

GlyCAM-1 – Glycosylation-dependent cell adhesion molecule-1

MadCAM-1 – Mucosal vascular addressin cell adhesion molecule-1

LFA-1 (CD11a/CD18) – Lymphocyte function-assosiated antigen-1

Mac-1 (CD11b/CD18) – Macrophage-1 antigen

ICAM-1 – Intercellular adhesion molecule-1

ICAM-2 – Intercellular adhesion molecule-2

JAM – Junctional adhesion molecule

PECAM-1 – Platelet endothelial cell adhesion molecule-1

NADPH – Nicotinamide adenine dinucleotide phosphate

MPO – Myeloperoxidase

GAGs – Glycosaminoglycans

DMSO – Dimethyl sulfoxid

ATRA – All-trans-retinoic-acid

siRNA – Small interfering ribo nucleic acid

EDTA – Ethylenediaminetetraacetate

REC Central – Regional Committee for Medical and Health Research Ethics, Central Norway

g – Gravitational constant

PBS – Phosphate buffered saline

IMDM – Iscove’s Modified Dulbecco’s Medium

RPMI – Roswell Park Memorial Institute

BSA – Bovine serum albumine

PFA – Paraformaldehyde

HDTM – hexadecetyltrimethyl

TMB – tetrametylbenzidin

ELISA – Enzyme linked immunosorbent assay

p – p-value

OD – Optical density

CV – coefficient of variation

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1 INTRODUCTION

1.1 The immune system

The immune system consists of cells and molecules that protect the body against pathogenic microorganisms and tissue damage (1). It discriminates between self and non-self molecules and mutated cells causing cancer (1, 2). One type of cells that is important for the body to counter invading agents are the leukocytes (3, 4). The immune system neutralizes or eliminates foreign substances. The innate immune system consists of anatomic barriers like the skin and mucosal membranes and phagocytes, in addition to several protein cascade systems (1). The innate response comes immediately but does not give long-lasting protection like the adaptive immune system, which mainly consists of specific cells and antibodies. Signal molecules are important for both the innate- and adaptive immune response. When pathogenic microorganisms (bacteria, virus, fungi or parasites) breach the physiological barriers an infection occurs with the innate immune system as the first line of defence (5), and the adaptive immune system as a second line of defence (1). In this study we are focusing on the innate immune system.

1.1.1 Inflammation

Inflammation can be induced by infection, injury of the tissue or hypersensitivity reactions like allergy (5, 6). The five cardinal signs of acute inflammation are pain (dolor), heat (calor), redness (rubor), swelling (tumor) and loss of function (functio laesa) (7). Inflammation is a cascade of well coordinated cellular and molecular responses from the immune system to repair the damage (1, 5). It is important to keep this response in balance to limit over-stimulation and avoid more destruction of tissue than needed. Leukocytes are rapidly recruited to the site of inflammation, and they migrate from the blood vessel to the tissue to combat infection and repair damaged tissue (Figure 1-1, section 1.2.1) (3, 8-10).

1.2 Neutrophils

Neutrophil granulocytes (neutrophils) are leukocytes which are the first cells of the innate immune system to respond during an inflammation (11, 12). They mature in the bone marrow from myeloblasts to promyelocytes, myelocytes, metamyelocytes and to the last step in the bone marrow, which is banded neutrophils (13). The banded neutrophils are released into the

vasculature where they continue to differentiate into mature neutrophils with multilobed nuclei. The bone marrow has a large reserve of hematopoetic stem cells, and if needed neutrophils can be produced at a rate of $1 – 2 \times 10^{11}$ per day (8, 14). Granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor and cytokines, a type of signalling molecules, regulate the number of circulating neutrophils by rapidly inducing maturation and differentiation of neutrophils in the bone marrow if an inflammation occurs. 50 % to 70 % of the circulating leukocytes in the blood are neutrophils at a concentration of approximately 4.5×10^{9}/L (15). They migrate into tissues after 4 to 10 hours, where they live for only a few days (14).

Polymorphonuclear neutrophils have granulated cytoplasm (14, 15). Lysosomal enzymes are stored in azurophilic granules of the cytoplasm (Figure 1-1) (16). Granula like specific granules and gelatinase granules contain antimicrobial enzymes, and neutrophils have secretory vesicles that contain various receptors (14, 17). Other organelles like golgi apparatus, mitochondria and glycogen as well as phagosomes, that are vesicles produced after phagocytosis, are also present in the cytoplasm of neutrophils (18).

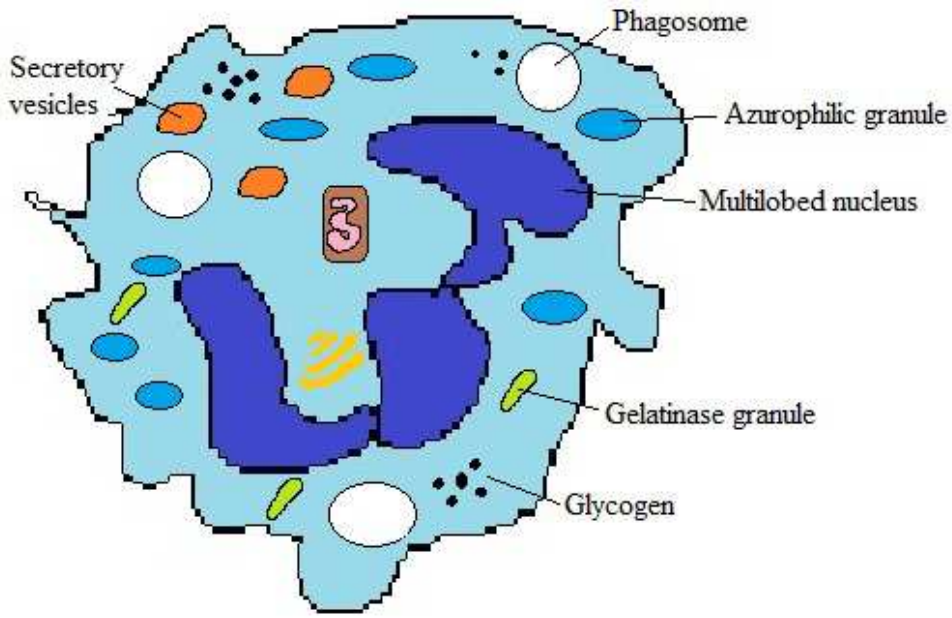


Figure 1-1: *Neutrophil granulocyte. The figure is modified after Kuby et al. 2007 (18).*

Under normal circumstances neutrophils remains unactivated in the blood vessel and capillaries and if they are not recruited into tissue during inflammation they die because of aging from apoptosis, programmed cell death (8, 14, 19). In the vasculature a homeostasis of neutrophil levels is maintained and following an inflammation the concentration returns to a normal level.

1.2.1. Neutrophil extravasation

Following an infection or tissue injury the local permeability of the endothelium and blood volume increases, and neutrophils are rapidly recruited in the blood vessel near the site of inflammation (8, 20). The neutrophils have to leave the blood stream to get to the damaged tissue, by so-called extravasation. This is a multistep process, that involves rolling, activation, arrest, firm adhesion, and transmigration of neutrophils trough the endothelium (Figure 1-2) (3, 8-10). Different receptors and ligands on the endothelium and the cells themselves and signalling molecules are responsible for the migration of neutrophils into tissues (21). The extravasation of neutrophils mostly takes place in the body's postcapillary venules (8).

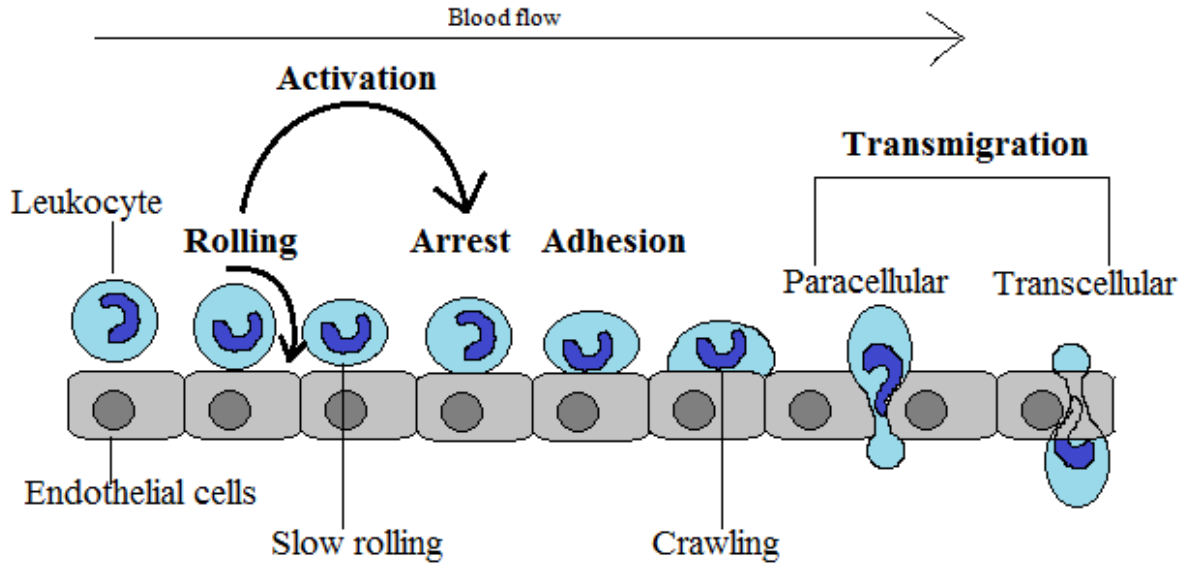


Figure 1-2: *Extravasation of leukocytes. This figure is modified from Ley et. al. 2007 (3).*

During an inflammatory response, the endothelial cells are stimulated by signal molecules to initiate attachment of neutrophils. Cytokines are signal molecules which can induce changes in both endothelial cells and neutrophils during inflammation (3, 8, 9). Chemokines are a group of small polypeptide cytokines which generate direct chemotaxis, meaning that

leukocytes are attracted towards them along an increasing concentration gradient (3, 8, 9, 12, 22). Interferons are larger proteins that can be released from infected cells (23). Chemokines and interferons are examples of molecules that can guide leukocytes to a site of inflammation.

Selectins are a family of transmembrane single-chained cell adhesion molecules on endothelial cells and neutrophils which are up-regulated by stimuli from cytokines during inflammation (3, 8, 24-27). Selectins have lectins on the N-terminal domain which bind to siasylated glycoprotein ligands, and the most common is sialyl Lewis^{x} (28-31). The siasylated glycoprotein ligands consist of carbohydrates with sialin acid which bind to selectins in a calcium dependent reaction. There are three types of selectins, P- and E-selectin (CD62P and CD62E) expressed on inflamed endothelial cells and L-selectin (CD62L), which is constitutively expressed on neutrophils (3, 8, 20, 26, 27, 29, 31-34).

Interleukin-1, interleukin-17 (chemokines), tumor necrosis factor-α (interferon) and lipopolysaccharides from pathogens are examples of mediators responsible for inducing P- and E-selectins on endothelial cells (8, 27). P-selectin is preformed stored in granules of endothelial cells and can therefore rapidly be up-regulated, unlike E-selectin that is produced by protein synthesis before expression on the endothelial cells (27). Selectins mediate rolling and tethering of neutrophils to the endothelium by binding to ligands on neutrophils (3, 8, 24-29, 31, 34-38). P-selectin glycoprotein ligand (PSGL-1) is expressed on microvilli on the surface of neutrophils and serves as receptors for E- and P-selectins (26, 27, 31, 33, 34). E-selectin can bind to E-selectin ligand-1 (ESL-1) and CD44, and both ligands are expressed on neutrophils (3).

L-selectin (CD62L) expressed on neutrophil microvilli binds to different glycoprotein receptors on endothelial cell, including glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) , mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1) and CD34 (3, 30). PSGL-1 can also bind to L-selectin on neutrophils and form leukocyte-leukocyte interactions which in turn can alter the adhesion of neutrophils to endothelial cells (3). Binding of L-selectin and PSGL-1 to their respective receptors together with stimuli from chemoattractants up-regulate the expression of integrins on neutrophils (26, 30).

Neutrophils have G-protein-coupled receptors for chemokines on their surface. When these receptors are stimulated signal transduction is activated, which means that intracellular molecules are altered to give a response (12, 31, 39). L-selectin and PSGL-1 and their counter-reseptors also transduce signals in leukocytes or endothelial cells (29).

As a result of increased binding of selectins to their ligands and decreased blood flow because of vasodilation of the blood vessel, the neutrophils roll slowly along the endothelium (3, 14, 40). P- and L-selectins are enzymatically removed when the neutrophil rolling has stopped, and this creates better affinity for binding of other adhesion molecules which are important for firm adhesion of neutrophils.

After chemokines like interleukin-8 or platelet activating factor have stimulated the neutrophils, conformational changes are induced of the transmembrane resceptors integrins on the surface of neutrophils (3, 8, 9, 20, 21, 34). The neutrophils have to be in close contact with the endothelial cells so that the integrins can bind to their ligands. Integrins consist of an α- and β-unit (27, 34). Conformal changes and clustering of integrins are responsible for the activation and arrest of leukocytes (3, 8, 26, 27, 34), by increasing the affinity and avidity of integrins to their ligands (12). β_2-integrins have the common β-unit CD18. They are important adhesion molecules on neutrophils, and they form heterodimeric receptors with the α-unit consisting of CD11a, -b, -c or –d (20, 24, 34, 41-44). CD11a/CD18 is called lymphocyte function-assosiated antigen-1 (LFA-1) and CD11b/CD18 is called macrophage-1 antigen (Mac-1). An important ligand for LFA-1 and Mac-1 is the intercellular adhesion molecule-1 (ICAM-1 (CD54), a member of the Ig superfamily (24)) on the surface of endothelial cells (27, 42). ICAM-1 is up-regulated by stimulation of interleukin-1, tumor necrosis factor-α, interferon-γ or lipopolysaccharides (45). Figure 1-3 shows the most common molecules involved in rolling, tethering and adhesion of neutrophils.

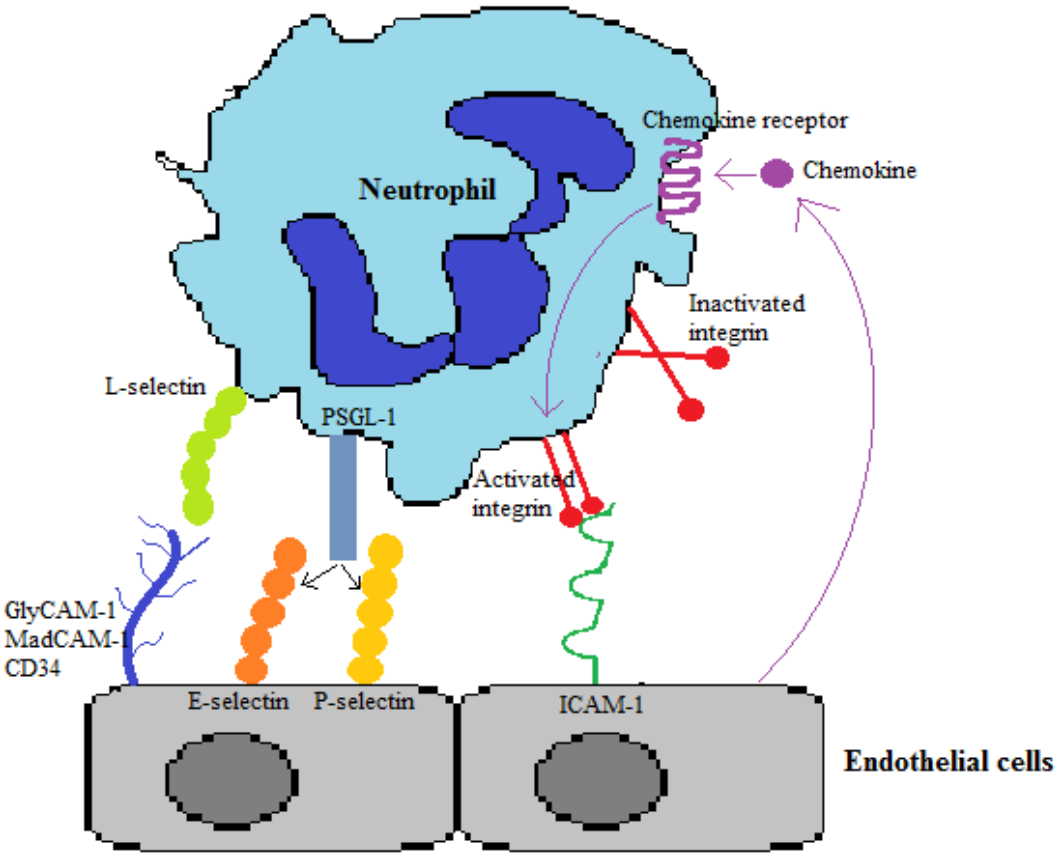


Figure 1-3: *Adhesion molecules which mediate rolling and firm adhesion of neutrophils during extravasation. GlyCAM: glycosylation-dependent cell adhesion molecule-1, MadCAM-1: mucosal vascular addressin cell adhesion molecule-1, PSGL-1: P-selectin glycoprotein ligand-1, ICAM-1: intercellular adhesion molecule-1. This figure is modified from Kuby et al. 2007 (18).*

Following the adhesion step, neutrophils have to migrate through the blood vessel wall (3, 46). Neutrophils can transmigrate in a paracellular or transcellular way, between the endothelial cells or penetrating an endothelial cell, respectively (3, 8, 46). Both routes involve binding of LFA-1 and Mac-1 (favors the paracellular route) to ICAM-1 and ICAM-2 (3, 8). Binding of ICAMs reduce the contact of cell junctions between endothelial cells and make the junctions more permeable to facilitate migration. Paracellular migration is dependent on members of the junctional adhesion molecule (JAM) family and platelet endothelial cell adhesion molecule-1 (PECAM-1). These molecules are expressed both on neutrophils and endothelial cells. Stimulation of interleukin-1β mediates also migration through cellular junctions (3, 8, 46). JAMs are receptors for integrins and PECAM -1 binds to PECAM-1 on neutrophils. Migration of neutrophils by the transcellular route is thought to depend on vesico-vacuolar organneles, which passage the neutrophils through a gateway through the endothelial cells (3).

Table 1-1 gives an overview of the most relevant adhesion molecules on neutrophils and endothelial cells.

Table 1-1: *Receptors and ligands on neutrophils and endothelial cells involved in extravasation of neutrophils (3, 8, 20, 30, 39, 46).*

Receptor/ligand on neutrophil	Receptor/ligand on endothelial cells	Extravasation step
PSGL-1	P-selectin (CD62P)	Rolling/tethering
PSGL-1	E-selectin (CD62E)	Rolling/tethering
ESL-1		
CD44		
L-selectin (CD62L)	GlyCAM-1	Rolling/tethering
	MadCAM-1	
	CD34	
LFA-1 (CD11a/CD18)	ICAM-1 (CD54)	Adhesion/ transmigration
Mac-1 (CD11b/CD18)		
LFA-1 (CD11a/CD18)	ICAM-2 (CD102)	Adhesion/ transmigration
PECAM-1 (CD31)	PECAM-1 (CD31)	Transmigration
LFA-1 (CD11a/CD18)	JAM	Transmigration
Mac-1 (CD11b/CD18)		

Abbrevations in table: PSGL-1: P-selectin glycoprotein ligand-1, ESL-1: E-selectin ligand-1, GlyCAM: glycosylation-dependent cell adhesion molecule-1, MadCAM-1: mucosal vascular addressin cell adhesion molecule-1, LFA-1: lymphocyte function-assosiated antigen-1, Mac-1: macrophage-1 antigen, PECAM-1: platelet endothelial cell adhesion molecule-1, ICAM-1: intercellular adhesion molecule-1, ICAM-2: intercellular adhesion molecule-2, JAM: junctional adhesion molecule.

Neutrophils recruited into the tissue during inflammation have an anti-inflammatory function by phagocytosing and killing microorganisms and debris (8, 14, 15). For example, bacteria are captured by the neutrophil cell membrane into a phagosome and merged with azurophilic- and specific granula, to create a phagolysosome (14). In the phaglysosome NADPH oxidase catalyse reactions to produce reactive oxygen species like superoxide and hydrogen peroxide. Together with lysosomal enzymes, for example myeloperoxidase (MPO), and proteases they destroy the bacteria (14, 16, 47, 48). Lysosomal enzymes are able to digest and break down waste material from microorganisms and damaged tissue, and the granules make the environment in the phagolysosome toxic to the bacteria (14). The neutrophils have a proinflammatory role by degranulation (release of granules to the cell environment) and release from secretory vesicles, which result in recruitment of other cells to the site of inflammation (11, 17). Neutrophils also link the innate and adaptive immune system together

by releasing proinflammatory cytokines and chemokines which attract and stimulate cells of the adaptive immune system (11).

1.3 Proteoglycans

During inflammation, proteoglycans are responsible for some of the cell-cell interactions between leukocytes and the endothelium (25). They consist of a protein core and polysaccharides (long carbohydrate molecules) called glycosaminoglycans (GAGs) (49-51). Different chains of GAGs are coupled to the protein core of proteoglycans, and the GAGs attached to a certain protein core varies (49). The GAGs are negatively charged because of attached sulphate groups, and they bind water and attracts cations (49, 50). The different groups of GAG chains that covalently bind to the protein core are heparan sulphate, keratan sulphate, chondroitin sulphate and dermatan sulphate and hyaluronic acid (49). These different groups of GAGs are characterized by the composition of disaccharides. Proteoglycans are stored in secretory vesicles and granula of neutrophils and can be expressed on the cell surface. They are able to capture and inactivate other molecules and can also act as an adhesion molecule (25, 49, 50).

1.3.1 CD44 as an adhesion molecule on neutrophils

A proteoglycan involved in neutrophil adhesion is CD44 (25, 35). CD44 is a member of the hyaluronan-binding transmembrane glycoproteins and contains both chondroitin sulphate and heparan sulphate GAGs (24, 51, 52). CD44 is expressed in erythroid and myeloid cells, epithelium, endothelium and neurons, and it is located on the planar cell body on neutrophils (24, 43, 51). There are many different isoforms of CD44 because of alternative splicing and post-translational modifications, and the most abundant form is CD44s (standard) which is found on hematopoetic cells (24, 53). All forms of CD44 have diversity in the glycosylation of N- and O-glycans on the extracellular domain (figure 1-4) (24, 51, 53). E-selectin on endothelial cells interact with the N- and O-glycosylation sites on CD44 molecules, and thereby CD44 mediates neutrophil rolling to the endothelium (24, 25, 35, 36, 39). Following binding to E-selectin, CD44 activates signal transduction of L-selectin and PSGL-1 so they can be redistributed to the surface of neutrophils (39).

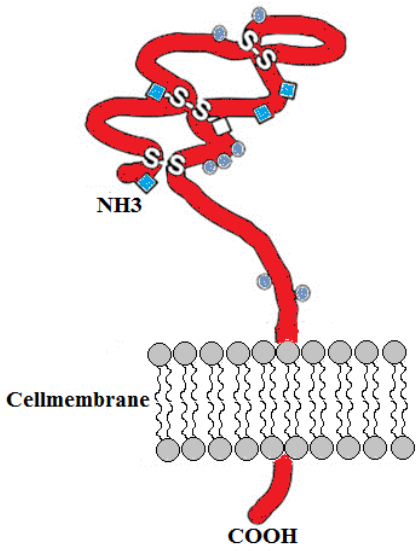


Figure 1-4: *Structure of the transmembrane CD44 molecule. O- and N-glycosylation sites on the extracellular domain are coloured in blue (on the molecule) (25). The figure is modified from: Katayama Y et al. 2005(25)*

1.3.2 Syndecan-4 as an adhesion molecule on neutrophils

Syndecan-4 is a proteoglycan with heparan sulphates GAG chains and expressed on neutrophils (54-57). They are thought to mediate cell adhesion by its heparan sulphate chains that interact with P-selectin (54, 56, 57). Syndecan-4 can also be a potential cell adhesion molecule by mediating integrin activation. Another important effect is that the heparan sulphates GAG chains function as ligands to antithrombin III in blood plasma (56). Antithrombin III contributes to an anticoagulatory effect in plasma, and it binds to neutrophils via the syndecan-4 molecule. Migration of neutrophils is inhibited when antithrombin III is bound to them. By blocking the protein core on syndecan-4, neutrophil adhesion was increased, indicating that syndecan-4 acts as an inhibitor of neutrophil adhesion (58). The findings in the previous master theses indicate that the protein core of syndecan-4 is involved in negative regulation of neutrophil adhesion, which *in vivo* can be compared with the blockage of syndecan-4 from antithrombin III (56, 59, 60).

1.4 HL60 cells

Promyelocytic cells can be kept in culture and differentiated to mature cells, like neutrophils, and be used as a model to study mechanisms of neutrophil adhesion (61-63). One myeloid cell line that grows well in culture is HL60 cells. (61, 62, 64) They were originally isolated from peripheral blood from a patient with acute promyelocytic leukemia, and the cells are

predominantly promyelocytes. All HL60 cells have myeloid-specific markers like for example myeloperoxidase (61).

HL60 cells differentiate towards myelocytes, metamyelocytes, banded and mature neutrophils with multilobulated nuclei (61, 62). Approximately 10 % spontaneously differentiate to neutrophils, but addition of dimethyl sulfoxid (DMSO), all-trans-retinoic-acid (ATRA) or other polar compounds to the media rapidly enhances the differentaion rate towards neutrophils (61, 64-69). When the HL60-cells are terminally differentiated, they are able to phagocytose and perform chemotaxis like normal neutrophils (61, 62, 64). No eosinophil or basophil granulocytes are detected in the culture, but a few monocytes with folded nuclei are present.

ATRA is a vitamin A derivate playing a role in hematopoiesis, and affects differentiation of neutrophils (66). ATRA binds to the retinoic acid receptor on the cell which forms a heterodimer with another retinoic receptor (66, 69). The complex binds to the promoter region of the genes responsive of retinoic acid and induces transcription that results in terminal differention to neutrophils. After 5 days with ATRA stimulation the HL60-cells are thought to be completely differentiated (64). However, a lot of cells die during the differentiation process and the cells vary in number of divisions after stimulation with a differentative agent (67).

1.5 Hypothesis and aims

The mechanisms of neutrophil adhesion to endothelial cells which involve the most common adhesion molecules *in vivo* (explained earlier) are well characterized (3, 8). Neutrophils can also adhere to artificial surfaces and biomaterials (70-72). Circulating neutrophils in patients who for example are treated with hemodialysis or use of heart-lung machines can spontaneously be activated and adhere to the biomaterials in the extracorporal circulations (70). The neutrophils can then release proinflammatory mediators which can lead to a systematic inflammatory response, and the consequences can be damage to the organs and organ failure. Earlier experiments have been performed in the lab using methods preventing normal function of selectins and integrins, and neutrophils still adhered strongly to artificial surfaces (59, 60, 70). These findings indicate that other adhesion molecules participate in

neutrophil adhesion at least under certain conditions. Neutrophils express several proteoglycans and two of them that are thought to participate in neutrophil adhesion are CD44 and syndecan-4 (24, 25, 35, 36, 54, 56, 57).

Neutrophils have a short lifetime and cannot be kept in culture, and after isolation from whole blood they only live for a few hours (14, 62). This makes methods that involve transfection or siRNA impossible to use. Such methods can for example over-express relevant adhesion molecules to better investigate their function. It is therefore interesting to investigate if the HL60 cell line, which can differentiate towards mature neutrophils, can be used as a model for neutrophils. The hypothesis for this study is that HL60 cells express the proteoglycans CD44 and syndecan-4 and function as a model of proteoglycan-mediated adhesion of neutrophils to artificial surfaces.

The purpose of this study was therefore to characterize the expression and function of relevant proteoglycan adhesion molecules on HL60 cells compared to neutrophils.
The specific aims were to:

1) Characterize the expression of CD44 and syndecan-4 on HL60 cells.
2) Compare whether CD44 and syndecan-4 has parallel functions as adhesion molecules on HL60 cells and isolated neutrophils using antibody-mediated blockage.

2 MATERIALS AND METHODS

2.1 Reagents, buffers and cell culture medium

Detailed information about commercial solutions and chemicals, prepared reagents and solutions and antibodies used in this study is given in Appendixes 1-3.

2.2 HL60 cells in culture

HL60 cells (ATCC, Manassas, Virginia, USA) were kept in BD Falcon culture flasks (BD Biosciences, San Jose, California, USA) with Iscove's Modified Dulbecco's Medium (IMDM, ATCC) supplemented with 20 % fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). The flasks were incubated at 37 °C with 95 % air and 5 % carbon dioxide (CO_2) (73). During this study the concentration of the HL60 cells in the culture flasks was approximately 0.5 – 8.0 x 10^6/mL. Monitoring of cell density and cell appearance were performed almost daily using an inverted microscope (Nikon Corporation, Tokyo, Japan). The cells were quantified using a hemocytometer (Sigma-Aldrich, Oslo, Norway), with manual counting in a light microscope (Carl Zeiss AS, Skårer, Norway).

2.2.1 Differentiation of HL60 cells

Stimulation with ATRA (Sigma-Aldrich) of the HL60 cells was performed by adding ATRA to a final concentration of 10^{-6} M. Stimulation was continued for five days with renewal of the ATRA-containing media every second or third day. Non-stimulated HL60 cells and cells stimulated with ATRA for five days were applied to SP-slides (Sysmex Europe GmbH, Norderstedt, Germany) and stained by the May-Grünwald-Giemsa method in an automated staining machine (Sysmex SP1000i, Sysmex Corporation, Kobe, Japan). The May-Grünwald and Giemsa solutions contain eosin methylene blue and azur blue, respectively, which at a neutral pH stain the nuclei of the cells purple and give the cytoplasm a transparent blue/pink color (74). The stained HL60 cells were visualized by light microscopy.

In the following, the term "undifferentiated HL60 cells" refers to cells that were not stimulated with ATRA and "differentiated HL60 cells" refers to cells stimulated with ATRA for five days.

2.2.2 Viability of HL60 cells

Viability of the HL60 cells before and after stimulation with ATRA was determined by incubating the cells with tryptan blue (0,4 % solution, Sigma-Aldrich) and visualization by light microscopy.(62) In one experiment a cell culture was incubated with ATRA for a period of 11 days and cell viability was measured every day. The ATRA-containing media was changed as indicated above. An equal amount of cell suspension and tryptan blue solution were mixed and placed on a slide and the number of dead cells was counted by using a light microscope. The percent of living cells was registered. Tryptan blue immediately leaks into dead cells and give them a blue colour (75). Living cells are maintained colourless because no dye is absorbed by them.

2.3 Neutrophils

Fresh neutrophils were isolated from whole blood samples collected with the anticoagulant ethylenediaminetetraacetate (EDTA). These samples were from informed volunteer blood donors at the Blood Bank at St. Olavs Hospital, Trondheim, who had signed an agreement for donating their blood to research. The part of this study that includes use of blood samples from donors was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (REC Central).

2.3.1 Isolation procedure of neutrophils

Neutrophils were separated from the other blood cells with an isolation technique based on gradient centrifugation (76). Every step in the procedure for isolating neutrophils from whole blood was performed at a temperature of 4°C, to avoid neutrophil activation. At the first step platelets are removed. The sample was centrifugated for 10 min, 170 g (Sorvall RT6000D centrifuge CE, Du Pont, Dordrecht, Netherlands), and plasma was carefully removed and substituted by 1 x phosphate buffered saline (PBS). After careful mixing, the blood was layered on top of Lymhoprep (Axis-Shield, Oslo, Norway) and centrifugated for 20 minutes, 540 g. The layers containing plasma, PBS, mononuclear cells and Lymphoprep were carefully removed. After a washing step for the remaining neutrophils and erythrocytes with 1 x PBS, the erythrocytes were lysed by a 60 seconds incubation with Aqua dest (Braun Melsungen AG, Melsungen, Germany), before re-establishing isotonic conditions in 1x PBS and centrifugation for 10 minutes, 240 g. The step of erythrocyte lysis could be repeated if required. The isolated neutrophils were resuspended in 1 x PBS and counted with use of the

hemocytometer before resuspension at a given concentration in buffer (used in adhesion experiments).

2.4 Principle of flow cytometry

Flow cytometry uses fluorescence emission (Figure 2-1) and light scatter from single cells to rapidly quantify the amount of cells in a sample and their phenotypes, and is therefore a quantitative and qualitative method (77-80). A flow cytometer gives information about several functions and characteristics of a given cell type and only a small amount of sample is needed. The principle of a flow cytometer is shown in Figure 2-2. Hydrodynamic focusing is a tool used in flow cytometry, where a fluid (sheath fluid) is added at a higher speed than the flow of the cell suspension resulting in single cell passage through a laser beam (78-81). Forward light scatter indicates the size of the cell and side light scatter indicates the internal complexity of the cell (78, 80).

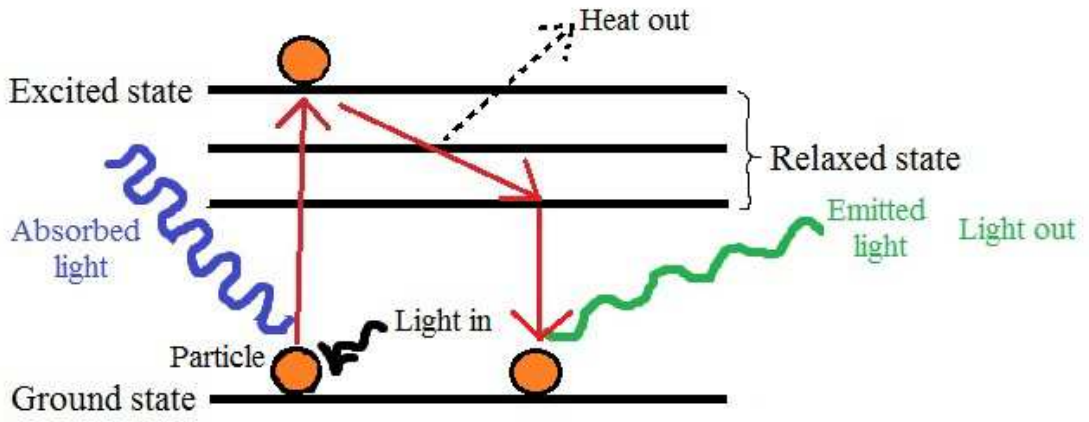


Figure 2-1: Energy state diagram of fluorescence. *A particle in the ground energy state is excited by light (80, 82). Some energy is lost as heat when it returns to a relaxed state, and the remaining energy is emitted as fluorescence before the particle returns to the ground energy state again. This figure is modified from: Hemmila (82).*

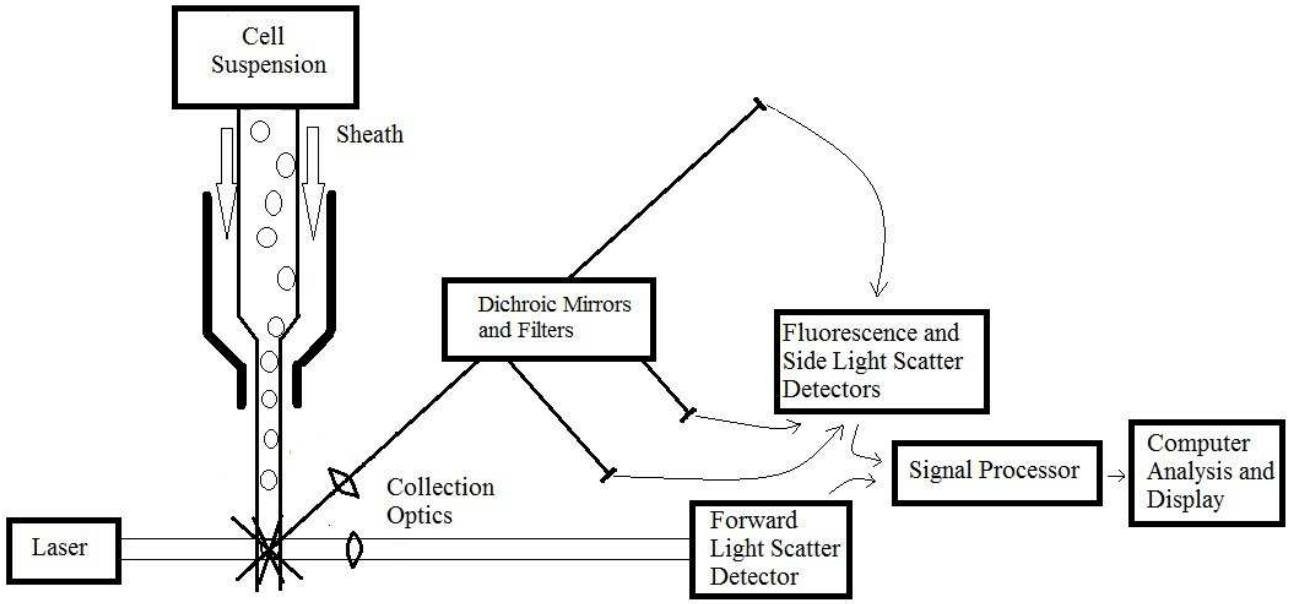


Figure 2-2: Principle of a flow cytometer. *Sheath fluid is added to the cell suspension and the cells pass the laser beam one by one (78-81). Signals are collected with a forward light scatter detector and side light scatter, and fluorescence emission is collected by appropriate detectors with the help of dichroic mirrors and filters (79, 80, 83). The signals are further electronically converted to a digital form and visualized on a computer (79, 80). This figure is modified from: Brown et.al. (80).*

The cells are labelled with antibodies that are conjugated to fluorescent dyes before analysis by flow cytometry (78, 80). For example, the antibodies can be against proteins on the cell membrane or inside permeabilized cells (80). In this study we used fluorescent antibodies that bound to adhesion molecules. The employed fluorescent dyes are shown in Table 2-1.

Table 2-1: Fluorescent dyes used in flow cytometry (84).

Fluorescent dyes	**Abbrevation**	**Laser excitation line (nm)**	**Maximal excitation (nm)**	**Maximal emission (nm)**	**Fluorescence color**
Allophycocyanin	APC	595, 633, 635, 647	650	660	red
Fluorescein isothiocyanate	FITC	488	494	519	green
Phycoerythrin	PE	488, 532	496, 546	578	yellow

Emitted fluorescence has a higher wavelength than the excitation wavelength (80), as shown in figure 2-1. The difference between the excitation and emission wavelength is called Stokes shift (82). Because of the Stokes shift, emitted light has a different colour than the excitation light. By using fluorochromes with the same excitation wavelength and different emission wavelengths several fluorescent dyes can be used in the same measurement, simultaneously allowing multiple characteristics of a single cell to be analyzed (77, 80). When two or more antibodies with different fluorescent dyes are used in the same experiment it is necessary to perform electronic compensation because some of the fluorochromes can have a common range of emission wavelengths (79). Emission that overlaps from different fluorochromes is then mathematically corrected.

Data from flow cytometry can be treated and presented in many different ways (80). For example, forward light scatter and side light scatter can indicate the distribution of different cell populations in a sample. Cells with determined properties can be discriminated from others using gating as a tool, where a PC is used to plot cells that were stained with antibodies with different fluorochromes. The amount of a given cell type can also be shown in histograms. From these histograms the median fluorescence intensity can be calculated.

The flow cytometry instrument used in this study is BD FACS Canto (BD Biosciences). It is equipped with two lasers (wavelength 488 nm and 633 nm).

2.5 Flow cytomertry performed on HL60 cells

2.5.1 Antibodies

The level of differentiation of the HL60 cells was measured by flow cytometry and two different antibodies, CD13 APC and CD33 PE (both from eBioscience, AH Diagnostics, Oslo, Norway). These two antibodies were chosen because they have a varying expression during the differentiation process. Maturing of myeloblasts is initiated by an increase of CD13 expression followed by expression of CD33 (85). The density of CD13 is high in promyelocytes and further differentiation of the cells result in lower density of CD13 before the expression increases to mature neutrophils. Blasts do not express much of CD33, but the density of CD33 increases to promyelocytes and slowly decreases when promyelocytes

mature to granulocytes. Based on pilot experiments anti-CD13 was used at a concentration of 0.6 µg/mL and anti CD33 was used at concentrations of 5.0 µg/mL and 6.3 µg/mL.

Differentiated HL60 cells were also characterized by flow cytometry with antibodies against the adhesion molecules CD44 and syndecan-4 (25, 57). These antibodies were chosen based on previous experiments on human neutrophils (59, 60). Two different antibodies against CD44 were used in these experiments, sc-7297 and sc-65265 (Santa Cruz Biotechnologies, AH Diagnostics, Oslo, Norway), both mouse monoclonal IgG_1 antibodies. The antibodies were used at different concentrations to find the most optimal concentration. For the anti-CD44 antibody sc-7297 different concentrations from 3.75 to 25 µg/mL were examined, and for the anti-CD44 antibody sc-65265 concentrations of 15, 20, 25, 50 and 100 µg/mL were used.

Four different antibodies against syndecan-4, sc-12766, sc-15350, sc-33912 and sc-9497 (all from Santa Cruz Biotechnologies) were analyzed at concentrations of 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 µg/mL and used on differentiated HL60 cells.

Goat anti-mouse IgG-FITC sc-2010 (Santa Cruz Biotechnologies) was used as secondary antibody for the experiments with CD44 and syndecan-4 antibodies on differentiated HL60 cells.

Isotype control antibodies (sc-2025, sc-12766, sc-15350, sc-33912 and sc-9497, Santa Cruz Biotechnologies) were used for the flow cytometry experiments regarding expression of adhesion molecules CD44 and syndecan-4 on differentiated HL60 cells. Control antibodies were used in the same concentration as the primary antibodies.

Detailed information about the antibodies is given in Appendix 3.

2.5.2 Procedure

The cells were washed in 1 x PBS with 0.2 % bovine serum albumin (BSA), and then resuspended in the same buffer to an amount of 0.5 x 10^6 cells in each tube used for flow cytometry. This procedure was performed at room temperature and every centrifugation was performed at 240 g for 7 minutes. The samples were preincubated with polyclonal human

antibodies (Octagam 50 mg/mL, Octapharma AG, Lachen, Switzerland) for 5 minutes to block Fc-receptor mediated binding of the primary antibodies. The primary antibodies were added and incubated in the dark for 20 minutes, centrifuged, washed and resuspended with PBS before the secondary antibody was added to the samples and incubated in the dark for 15 minutes. (No secondary antibody was added to the cells stained with CD13 and CD33 antibodies because the fluorochromes are attached to the antibodies.) Finally, the samples were centrifuged and washed with PBS, before they were resuspended in PBS with 1% paraformaldehyd (PFA) and analyzed in the flow cytometer.

2.6 Adhesion experiments of HL60 cells and isolated neutrophils

Adhesion experiments were performed with differentiated HL60 cells and isolated neutrophils on tissue culture plates. Costar 96 well flat bottom polystyrene plates treated for optimal cell attachment to promote cell adhesion (Sigma-Aldrich) were used. Two different CD44 antibodies (sc-7297 and sc-65265) and isotype control antibody (sc-2025) was used.

Pilot experiments of cell concentration (1.5, 3.0, 5.0 and 10.0 $x10^6$ cells/mL) and time of incubation in the tissue culture wells (12, 15, 20 and 30 minutes) were previously analyzed for differentiated HL60 cells. From these experiments it was further decided that a cell concentration of 10.0 $x10^6$ cells/mL and 12 minutes incubation time shold be used. The concentrations of the two different CD44 antibodies were 10.0 µg/mL and 7.5 µg/mL. These concentrations were chosen based on the result from flow cytometry.

2.6.1 Procedure for adhesion arrays

Differentiated HL60 cells were resuspended in RPMI+ (Appendix 2) at the given cell concentration. Isolated neutrophils were resuspended at a cell concentration of 1.5 x 10^6 cells/mL. The antibodies were added to the samples and incubated for 10 minutes at room temperature. The samples were pipetted into tissue culture wells in parallels after gentle resuspension. Further the tissue culture plate was incubated in CO_2 incubator. After the end of incubation non-adherent cells were carefully suctioned off, and the adherent cells were washed with preheated (37°C) 1 x PBS.

Adherent differentiated HL60 cells and isolated neutrophils were quantified using the Myeloperoxidase-Tetramethylbenzidine method (MPO-TMB-method).

2.6.2 Principles of the Myeloperoxidase-Tetramethylbenzidine method

With the MPO-TMB method, adhesion of neutrophils or HL60 cells is quantified based on the MPO content of the adherent cells (48). MPO is released from the adherent cells with a buffer that solubilises them, and then MPO in the supernatants catalyzes the reaction where hydrogen peroxide and TMB as substrates give a coloured product that can be measured with a spectrophotometer (Figure 2-3).

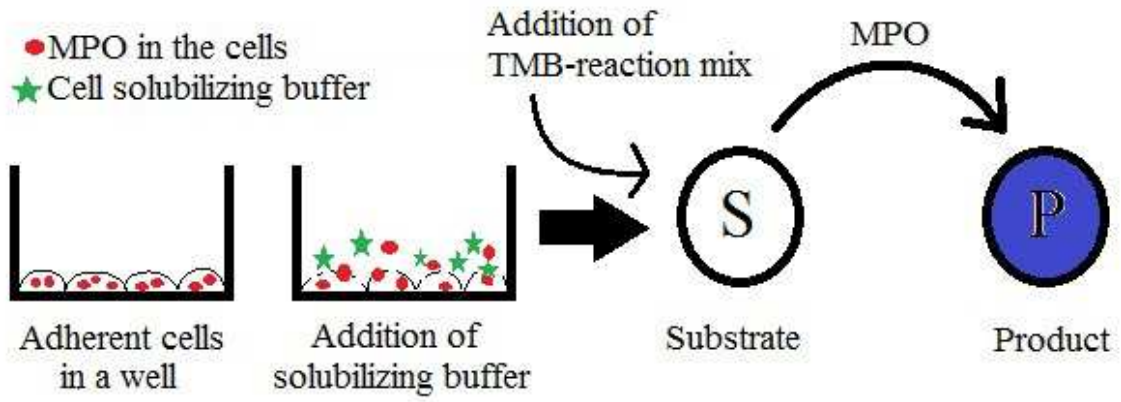


Figure 2-3: *The principle of MPO-TMB method. This figure is modified from: Suzuki et. al. 1983 (48).*

2.6.3 Procedure for the Myeloperoxidase-Tetramethylbenzidine-method

The adhered cells in tissue culture plates were solubilized with 100 µL 0.5 % hexadecetyltrimethyl (HDTM) ammonium bromide buffer and incubated for 20 minutes at room temperature. The supernatant from each parallel well from the adhesion experiments was pipetted in triplicate (20 µL/well) into ELISA plates (Nunc Maxisorp, eBioscience). 80 µL TMB-reaction solutions was added to each well, and after exactly three minutes the plate was placed on ice and 100 µL ice cold 200 mM sodium acetate buffer was added to stop the reaction. Optical density (OD) in each well was immediately measured at 620 nm in a Sunrise Microtiter Photometer (Tecan Austria GmbH, Grödig, Austria).

2.7 Statistics

A small number of experiments gave a dataset that was not normal distributed, and this was the case for both flow cytometric and adhesion experiments. Data are given as median with 95 % confidence interval ($n \geq 6$) or range ($n < 6$). Non-parametric statistics were chosen, with use of Friedman's test (86). P-values > 0.05 were considered statistically significant. The statistical programs used were SPSS (SPSS Inc., Chicago, Illinois, USA) and Minitab 15 (Minitab Inc., Lead Technologies, Pennsylvania, USA).

3 RESULTS

3.1 HL60 cells

The HL60 cells remained as single cells in suspension without adhering to the plastic of the flask (Figure 3-1). Their appearance was small and round.

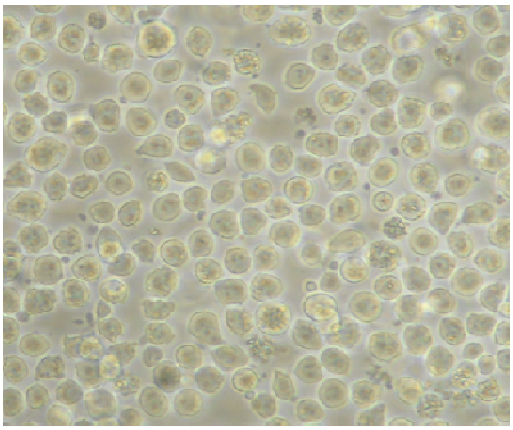


Figure 3-1: *Example of HL60 cells grown in a culture flask, visualized with an inverted light microscope (magnification: 40x). This picture was taken at the edge of the culture flask, where cells have a tendency to grow at a higher density than in the middle of the flask.*

3.1.1 Differentiation of HL60-cells

Examples of smears stained by the May-Grünwald-Giemsa method from undifferentiated HL60 cells before addition of ATRA and HL60 cells that were stimulated with ATRA for five days and are shown in Figures 3-2 and 3-3.

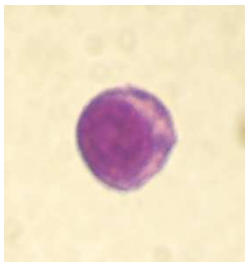


Figure 3-2: *Undifferentiated HL60 cell, May-Grünwald-Giemsa stain (magnification: 100x).*

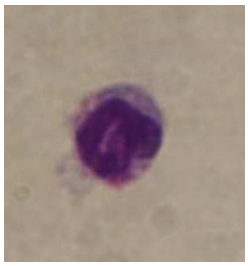
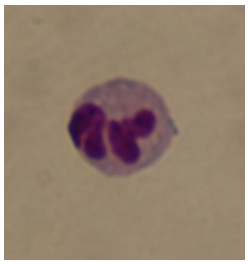
A:  B: 

Figure 3-3: *HL60 cells after 5 days of stimulation with ATRA, May-Grünwald-Giemsa stain (magnification: 100x). A: This cell resembles a banded neutrophil. B: This cell more closely resembles a mature neutrophil with a multilobulated nucleus.*

As expected, undifferentiated HL60 cells appeared like promyelocytes (Figure 3-2). After ATRA stimulation the cells had differentiated to a varying degree and resembled immature neutrophils (not shown here), banded neutrophils (Figure 3-3A) and mature neutrophils (Figure 3-3B).

Undifferentiated HL60 cells expressed CD13 on their surface but were negative for expression of CD33 (Figure 3-4, upper panels). Differentiated HL60 cells expressed CD13 on their cell membrane, as well as CD33 to a lower extent ($p = 0.001$ for each antibody compared to negative control, Figure 3-5). With differentiation, the expression of CD13 decreased whereas the expression of CD33 increased slightly (Figure 3-4, lower panels).

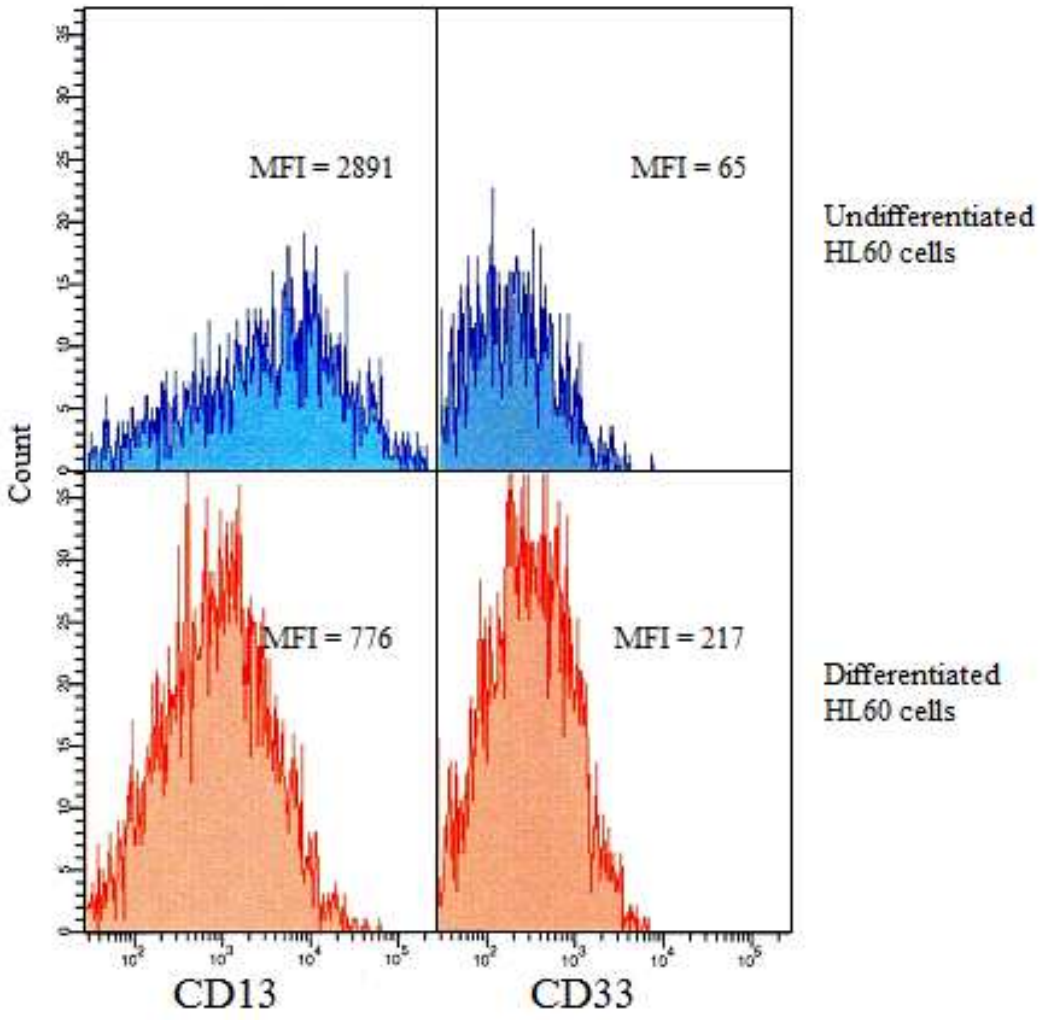


Figure 3-4: *Examples of expression of CD13 and CD33 on differentiated and undifferentiated HL60 cells by flow cytometry (MFI = median fluorescence intensity).*

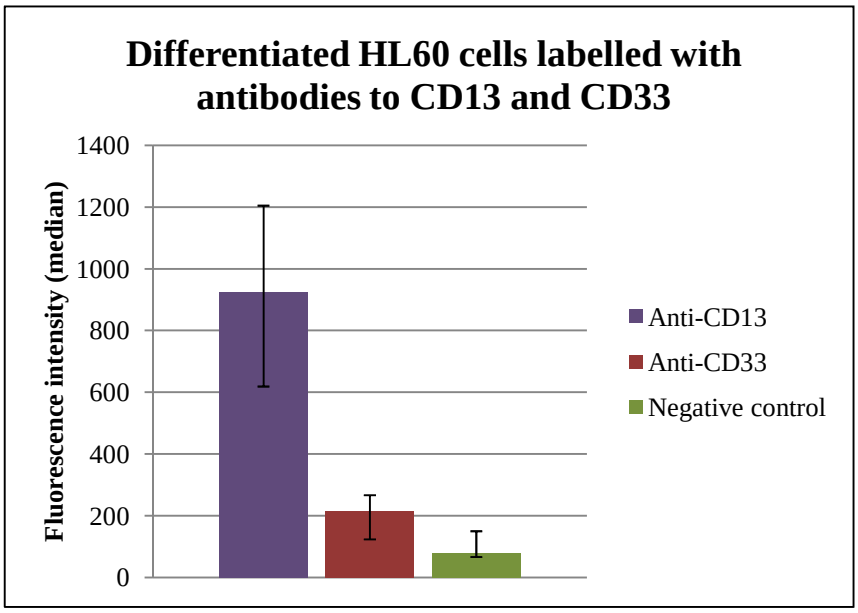


Figure 3-5: *Differentiated HL60 cells labelled with anti-CD13 and anti-CD33 antibodies. Median fluorescence intensity with range by flow cytometry, CD13 (0.6 µg/mL, n=3), CD33 (5.0 µg/mL, n=4) and negative control (2.0 µg/mL, n=4).*

3.1.2 Viability of the HL60-cells

The viability of HL60 cells before stimulation with ATRA was 94 % (95 % confidence interval: 91 % - 97 %, n = 10) and after five days with ATRA stimulation the viability was 72 % (range: 70 % - 74 %, n = 2).

The viability of a single HL60 culture during ATRA stimulation was followed for 11 days (Figure 3-6). The viability was relatively stable around 80 – 90 % during the first 4 days of stimulation with ATRA, on day 5 the viability was 74 %, but from day 9 and onwards it was below 20 % (figure 4-6). During the first 5 days with ATRA stimulation the concentration of cells in culture was 2 – 3 x10^6 cells/mL. From day 6 it was difficult to count the exact amount of cells due to a lot of dead cells and artifacts in the suspension.

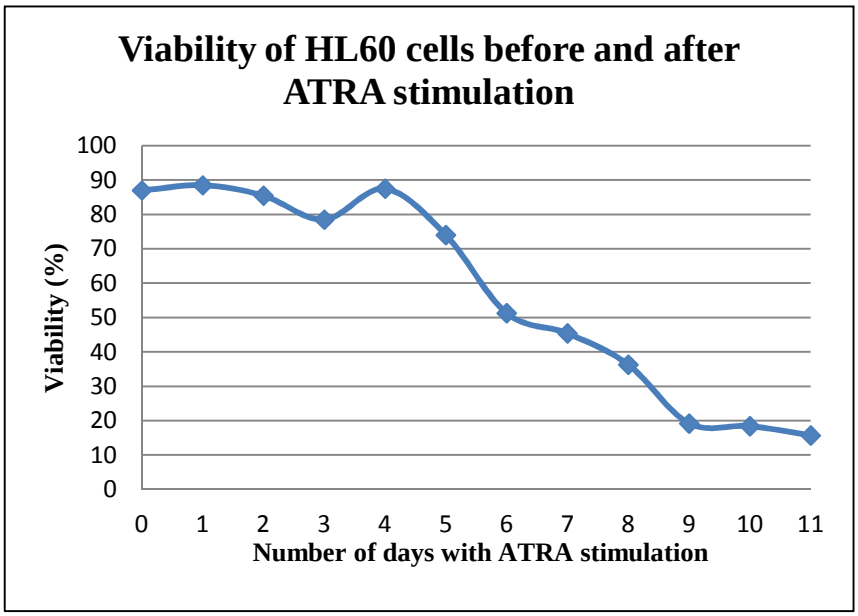


Figure 3-6: *The viability (%) of HL60-cells by number of days with ATRA stimulation. Day 0 denotes unstimulated cells. Data from one representative experiment are shown.*

3.2 Adhesion molecules on differentiated HL60 cells by flow cytometry

3.2.1 CD44 expression on the cell membrane of HL60 cells

Using the sc-65265 antibody, surface staining for CD44 on the HL60 cells was found, indicating that HL60 cells express CD44 on the cell membrane (Figure 3-7). The sc-7297 antibody was tested at a lower concentration ranging from 3.75 to 15 µg/mL. Staining with the sc-7297 antibody was only slightly stronger than with the negative control. There was a small increase with higher antibody concentrations, indicating that this antibody may bind to the cells.

Several further experiments were performed with these anti-CD44 antibodies on differentiated HL60 cells with negative results for binding of both. Sc-7297 was tested at concentrations up to 25 µg/mL but did not give any higher fluorescence signal (data not shown).

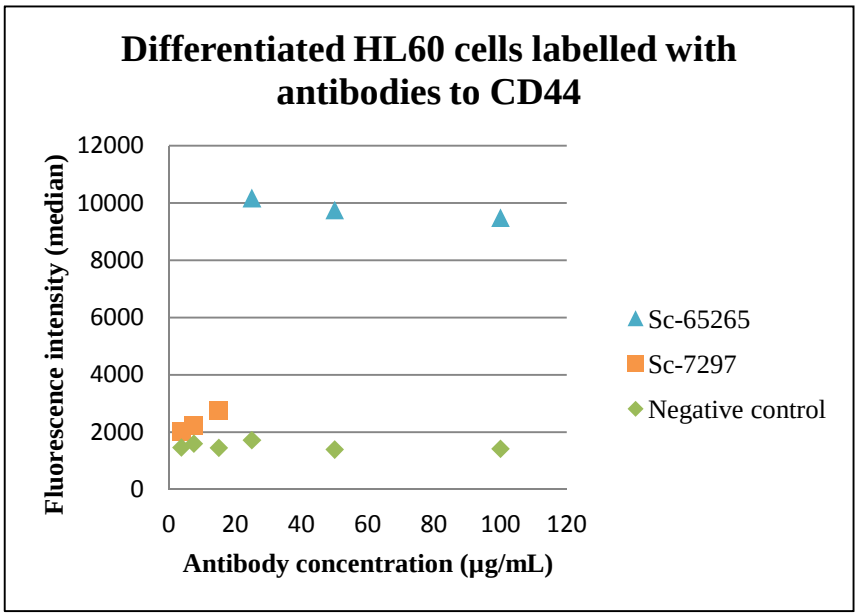


Figure 3-7: *Differentiated HL60-cells labelled with two different CD44 antibodies, sc-65265 and sc-7297 at different antibody concentrations (n=1). Median fluorescence intensity by flow cytometry.*

3.2.2 Syndecan-4 expression on the cell membrane of HL60 cells

There were no consistent differences in fluorescence intensity of differentiated HL60 cells after staining with four different anti-syndecan-4 antibodies and their isotype controls. Thus, the HL60 cells did not express syndecan-4 on the cell membrane (data not shown).

3.3 Adhesion experiments with HL60 cells and fresh isolated neutrophils

Pre-treatment with the anti-CD44 antibody sc-65265 at 10 µg/mL (Figure 3-8, panel A) or 7.5 µg/mL (Figure 3-8, panel B) significantly reduced adhesion of HL60 cells to tissue culture plates compared to the control antibody (10 µg/mL: $p<0.01$, 7.5 µg/mL: $p<0.05$). Pre-treatment with the anti-CD44 antibody sc-7297 had no effect on adhesion.

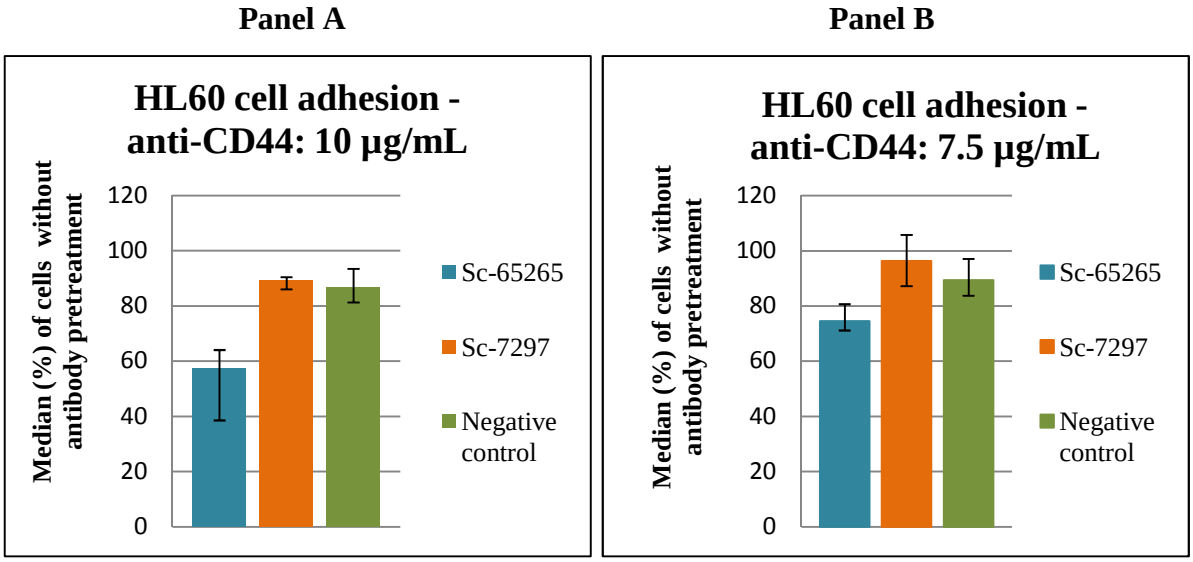


Figure 3-8: *HL60 cell adhesion to tissue culture plates following anti-CD44 antibody pre-treatment with two different antibodies (sc-65265 and sc-7297) at a concentration of 10 µg/mL (panel A, n=3) and 7.5 µg/mL (panel B, n=3). The median (%) is given as percentages compared to number of adhered cells without antibody pre-treatment (median and range).*

Pre-treatment with the anti-CD44 antibody sc-65265 at 10 µg/mL reduced adhesion (Figure 3-9, panel A) and 7.5 µg/mL (Figure 3-9, panel B) significantly reduced adhesion of fresh isolated neutrophils to tissue culture plates ($p<0.001$) compared to the control antibody. Pre-treatment with the anti-CD44 antibody sc-7297 had no effect on adhesion.

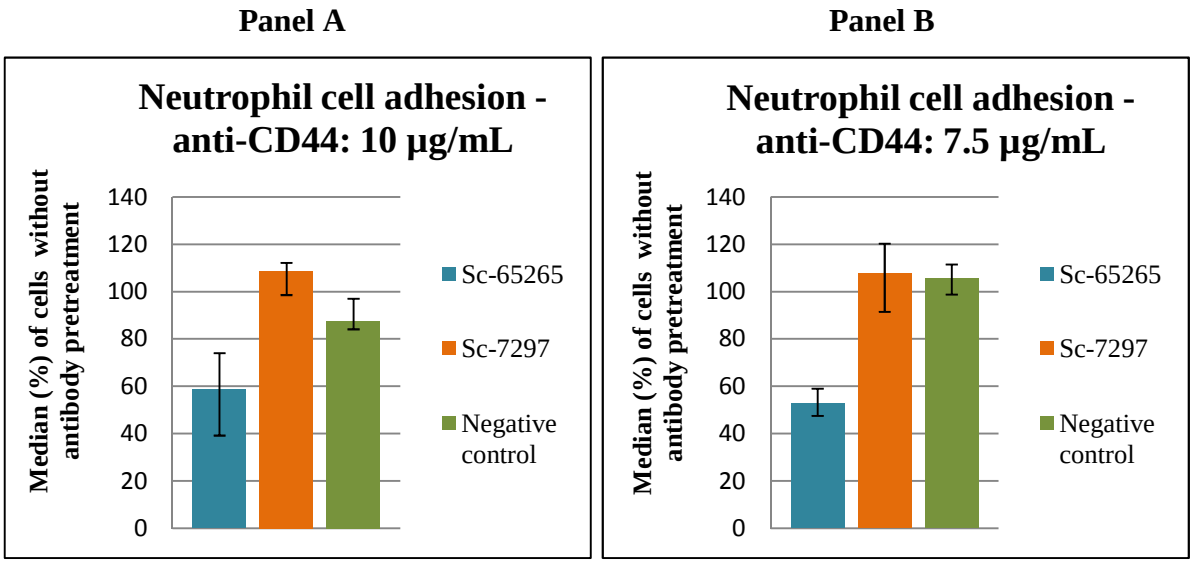


Figure 3-9: *Fresh isolated neutrophil cell adhesion to tissue culture plates following anti-CD44 antibody pre-treatment with two different antibodies (sc-65265 and sc-7297) at a concentration of 10 µg/mL (panel A, n=3, negative control: n=2) and 7.5 µg/mL (panel B, n=3). The median (%) is given as percentages compared to numbers of adhered cells without antibody pre-treatment (median and range, n=3).*

Data including OD-values from adhesion experiments on differentiated HL60 cells and isolated neutrophils are given in Appendix 4.

4 DISCUSSION

As expected the HL60 cells differentiated towards mature neutrophils with ATRA stimulation (61, 64-69). The adhesion molecule CD44 was present on the cell membrane of the HL60 cells as shown by flow cytometry with the antibody sc-65265. Adhesion of HL60 cells to tissue culture plates was reduced following pre-treatment with antibody against CD44 (sc-65265) and the result were similar for fresh isolated neutrophils. By flow cytometry, there was no evidence of syndecan-4 expression on HL60 cells.

4.1 Methodological considerations

4.1.1 HL60 cells

Unstimulated cells consisted of promyelocytes and stimulated cells mostly of banded and mature neutrophils. Banded and mature neutrophils had smaller cell size, decreased nuclear-cytoplasm ratio and increased segmentation of the nucleus compared to unstimulated cells. The stimulated cells consisted of a more heterogeneous cell population including cells from promyelocytes to mature granulocytes, but the main population consisted of differentiated cells with appearance like mature neutrophils (13, 67). Breitman et al. found that 90 % of the HL60 cells incubated with ATRA for six days appeared like mature neutrophils despite their malignant functions (64). Staining against differentiation markers with anti-CD13 and anti-CD33 to the unstimulated cells and ATRA stimulated cells showed a varying binding of the antibodies to the cells because of their grade of differentiation. Based on light microscopy and staining of the differentiation markers, we conclude that the cells stimulated with ATRA for five days were differentiated towards mature neutrophils and therefore relevant to investigate as a model for neutrophils.

The viability of the HL60 cells decreased during differentiation from 94 % to 72 % following ATRA stimulation for five days, indicating that some cells died during the differentiation process. This result agrees well with the findings of Breitman et al. where HL60 cells at the same concentration had a viability of 70 % at day 5 of ATRA stimulation (64). The amount of living cells in the culture was sufficiently high to perform experiments on the cells, but remaining dead cells and debris could also interact in the experiments. A disadvantage of the HL60 cells is that they are non-adherent cells growing in suspension, and therefore dead cells

and cell debris cannot be removed during renewal of the medium (62). The viability of HL60 cells decreased rapidly after 5 – 11 days of ATRA stimulation, but cells stimulated for more than 5 days were never included in the experiments.

To maintain cells in culture was time consuming, and a consequence of the limited time available for the study was that few experiments could be performed. Ideally the experiments should have been repeated at least three times making the results more reliable. The datasets were not normally distributed and therefore the median values were chosen to visualize the results, with the range of lowest and highest values to indicate the variation. In a small dataset with few parallels there is a risk of false negatives, especially if the variation is large, so-called type II errors (86).

4.1.2 Flow cytometry experiments on HL60 cells

One important thing to consider is that during the flow cytometry analysis no threshold was set to exclude the smallest cells. The consequence was that too many small cells, indicating "smudge cells" or cell debris and dead cells were counted instead of the cells of interests. This probably explains why most of the experiments with anti-CD44 antibodies were negative. It was difficult to determine what the threshold should have been to avoid excluding the cells of interest. By increasing the total number of analyzed cells, more of the larger cells may have been included but also more smudge cells would have been there. The results shown in Figure 3-7 had a threshold of 50 which excluded a lot of smudge cells and gave a more reliable result, although this conclusion is only based on one experiment.

The anti-CD44 antibody sc-7297 gave a slightly stronger signal than the negative control. However, it is difficult to tell whether the antibody bound to the cells, because higher antibody concentration should also have been tested with the same threshold parameter. Antibody concentrations up to 25 µg/mL of the antibody sc-7297 were used without a size threshold, but did not increase the fluorescence intensity. Four different antibodies against syndecan-4 were tested on differentiated HL60 cells by flow cytometry, but none of them seemed to bind to the cells. The missing threshold was probably the reason for the negative results of anti-CD44 antibody sc-7297 and perhaps also the anti-syndecan-4 antibodies.

The dead cells could have been excluded by staining the cells with propidium iodide, which is a fluorescent molecule that binds to DNA (87). Living cells remain unstained because propidium iodide does not manage to penetrate their cell membranes to stain the DNA. By adding propidium iodide together with the cells and the antibody of interest the dead cells could have been gated away. A disadvantage is that propidium iodide is a carcinogenic molecule, but an alternative non-carcinogenic fluorescent molecule could have been used instead (88).

The distribution of differentiated cells in the dot plots with forward light scatter (size) versus side light scatter (complexity) gave cells over the whole area in the dot plot (data not shown), and the fluorescence histograms showed no single peak but were widely spread. These results may be due to the ATRA stimulated cells having a varying grade of differentiation as confirmed by microscopy. The dot plots were gated in different ways but the different interpretations of the results led to the same conclusion, i.e. that most of the cells except for smudge cells were of interests. A disadvantage of flow cytometry is that gating of cell populations is highly subjective, and to manage correct gating of them requires experience from flow cytometry analysis (80).

It is also likely that double events have been present because very big cells with high fluorescence intensity were visible in the dot plots of forward light scatter and side light scatter. These cells were excluded by gating, and thereby not included in the results.

Autofluorescence is something to consider when the results from flow cytometry are interpreted because it can give falsely high fluorescence intensity. The HL60 cells also seemed to have considerable autofluorescence in some experiments. Cells with strong autofluorescence were attempted to be avoided by gating, but this proved to be difficult because of overlap with other cells in the area of interest. The NADPH molecules in neutrophils' lysosomes have fluorescent properties and are probably the cause of their autofluorescence (89, 90). Since neutrophils are known to have a lot of autofluorescence it is probable that also HL60 cells had autofluorescence that was NADPH-dependent. Nonspecific fluorescence could also be due to antibody binding to Fc-receptors, but the samples were pre-treated with high-dose human immunoglobulins (Octagam) to block unspecific Fc-binding of antibodies. It is therefore more likely that the HL60 cells had autofluorescence.

4.1.3 Adhesion experiments on HL60 cells and neutrophils

The adhesion experiments with HL60 cells and isolated neutrophils were performed in polystyrene wells optimized to promote cell adhesion. Neutrophils are activated on a polystyrene surface (72), and the HL60 cells also adhered to the wells at a high cell concentration, indicating that they were probably also activated by the polystyrene. Upon activation, the surface expression of several adhesion molecules including the β_2-integrin Mac-1 (CD11b/CD18) increases and activated cells become able to adhere firmly to many surfaces (71).

The MPO-TMB method was used to quantify the adhesion of differentiated HL60 cells and neutrophils. Previous experiments performed in the laboratory have shown that the MPO-TMB method is the most sensitive method with fewer sources of errors compared to other enzymatic methods to quantify neutrophil adhesion (59, 60). An advantage is that this method does not include washing steps and probably very few cells are lost during analysis. Previous investigations have shown that HL60 cells have MPO in their cytoplasmic granula, permitting quantification of adhesion by detection of the enzyme (48, 61). Few adhesion experiments were performed but use of duplicate interventions in the adhesion experiments and triplicate measurements in the MPO-TMB method increased the reliability of the results. The coefficient of variation (CV) was below 10 % for the triplicates in almost every intervention, which is good enough for these methods.

4.2 Discussion of the results

4.2.1 Expression of syndecan-4 on HL60 cells

In this study differentiated HL60 cells gave negative result for expression by four different anti-syndecan-4 antibodies. The negative results may either be due to mentioned threshold problems on the flow cytometer, or that the cells do not express syndecan-4. Syndecan-4 is expressed on normal neutrophils (56), and since we have shown that HL60 cells differentiate towards mature neutrophils we may expect that also HL60 cells express syndecan-4 on their cell membrane. Further experiments with flow cytometry analysis and adhesion experiments should have been performed to confirm or reject this assumption. Little time was available for further experiments and because of the negative result this was not given priority.

The syndecan-4 molecule has a short cytoplasmic domain, a transcellular domain and a longer extracellular domain with GAG chains (57). The anti-syndecan-4 antibody sc-12766 binds to an epitope on the ectodomain of syndecan-4, and this is a monoclonal antibody which binds with high affinity to its epitope (91, 92). Two polyclonal antibodies, sc-15350 and sc-9497 bind to epitopes on the N-terminal part of the syndecan-4 molecule (93, 94). The N-terminal part is on the extracellular domain of syndecan-4 (55). The binding sites of the fourth anti-syndecan-4 polyclonal antibody sc-33912 are not known, but it is raised against the extracellular part of the syndecan-4 molecule (95). A polyclonal antibody binds to several epitopes and they have a broader specificity and sometimes a lower affinity to the epitopes than monoclonal antibodies (91). Since all the tested anti-syndecan-4 antibodies bound to the extracellular part of the syndecan-4 protein core and both monoclonal and polyclonal antibodies were used we would have expected a positive signal by at least one of them, if HL60 cells express the syndecan-4 proteoglycan on their membrane.

Earlier adhesion experiments with the anti-syndecan-4 antibodies sc-12766 and sc-15350 on isolated neutrophils have shown that antibody sc-12766 can inhibit neutrophil adhesion while sc-15350 had no effect on neutrophil adhesion (59, 60). This result cannot be directly compared to the flow cytometry experiments performed in the present study because we did not perform adhesion experiments with anti-syndecan-4 on HL60 cells. However, for the anti-CD44 antibodies, the results from flow cytometry and adhesion experiments were in agreement.

4.2.2 Expression of CD44 on HL60 cells and isolated neutrophils

Using the anti-CD44 antibody sc-65265 on differentiated HL60 cells there was a stronger signal by flow cytometry than with the negative control in one experiment. The same antibody gave significantly reduced adhesion of cells pre-treated with it at antibody concentrations of both 7.5 µg/mL and 10.0 µg/mL. It is expected that pre-treated cells with antibodies against adhesion molecules will reduce adhesion, because the molecules are blocked. The adhesion of neutrophils was also significantly reduced after pre-treatment with anti-CD44 antibody sc-65265, in agreement with earlier experiments with neutrophils (59). Although only one experiment from flow cytometry showed a positive result, the adhesion experiments from differentiated HL60 cells together with the results from neutrophils support the conclusions

that differentiated HL60 cells express CD44 on their cell membrane and that it participates in adhesion.

The anti-CD44 antibody sc-7297 did not seem to bind to the cells by flow cytometry analysis, and there was no effect on adhesion after pre-treatment with the antibody in the adhesion experiments. The same result was found for neutrophil adhesion in this study and earlier experiments in the laboratory (59).

The reason why this specific antibody did not bind to differentiated HL60 cells or show an effect in adhesion experiments can be low affinity of the antibody or that it did not bind to the correct epitope. Since the antibody was monoclonal it should bind with high affinity to the epitope. The manufacturer does not indicate where the binding sites are of either tested anti-CD44 antibody. Because one of the two bound to differentiated HL60 cells we can assume that they are directed to different epitopes on the CD44 molecule. The epitope against the antibody that gave no effect on adhesion could also been located on a part of the molecule that was not involved in adhesion. If the epitope was located on the intracellular part of the CD44 molecule it would have been unavailable for attachment to the antibody. CD44 has a short intracellular domain. Since the antibody was raised against CD44 antigen from lymphocyte membrane, it is perhaps more likely that the antibody binds to an epitope in the extracellular part of the molecule (25, 96). To verify that differentiated HL60 cells expressed CD44 and that the molecule participated in adhesion, another antibody with known binding to the ectodomain of the molecule should preferably have been used instead.

Taken together, our data support that differentiated HL60 cells express CD44 and that they participates in adhesion to biomaterials. Further adhesion experiments of differentiated HL60 cells on an endothelial cell line could have been used to investigate if they can work as a model for adhesion to endothelial cells. During the adhesion experiments performed in this study CD44 appeared like a proadhesive molecule. When the molecule was blocked the cells adhered less to the polystyrene wells, and thereby CD44 promoted adhesion to the wells. We can also assume that this antibody is directed to an epitope on the protein core of the molecule and not the GAG chains, because it is specific for the CD44 molecule. Even if GAG chains show some specificity, similar structures are usually formed on several different molecules.

It is known that the heparan sulphate chains on the GAGs of the CD44 molecule are receptors for hyaluronic acid which is expressed on endothelial cells, and this binding mediates adhesion of neutrophils to the endothelial cells (24, 25). Together with the important adhesion molecules selectins, ICAMs and integrins, hyaluronic acid and CD44 play a role during inflammation. CD44 on neutrophils can also bind to E-selectin on endothelial cells (24, 25, 28, 35, 36). Findings from Katayama et. al. indicate that the binding of CD44 to E-selectin can occur even when hyaluronic acid on endothelial cells is blocked (25). It is unlikely that the polystyrene tissue culture wells used in this study expressed ligands like hyaluronic acid or E-selectin. The protein core of the CD44 molecule seemed to be involved in adhesion of differentiated HL60 cells and isolated neutrophils, indicating that the protein core of CD44 can mediate adhesion to unknown ligands on the polystyrene surface.

Our study indicates that differentiated HL60 cells can work as a model for neutrophil adhesion related to the CD44 molecule, but HL60 cells did not seem to work as a model for syndecan-4 mediated adhesion. Further experiments are needed to clarify the issue for syndecan-4 before a definite conclusion can be drawn. It is not known whether adhesion mechanisms related to CD44 and syndecan-4 may cooperate. Since both CD44 and syndecan-4 are expressed on neutrophils (54, 56, 57) the ideal model to use for neutrophil adhesion is probably a cell line that with certainty expresses both molecules.

5 CONCLUSION

The differentiated HL60 cells can function as a model of CD44 proteoglycan mediated adhesion of neutrophils to polystyrene surfaces, but they probably cannot be used as a model to study syndecan-4 mediated adhesion of neutrophils.

1) Differentiated HL60 cells express CD44 as indicated by one antibody, anti-CD44 sc-65265. There was no expression of syndecan-4 on differentiated HL60 cells when tested with four different antibodies.

2) Using antibody-mediated blockage of CD44, adhesion of differentiated HL60 cells and isolated neutrophils to a polystyrene surface was reduced. This indicated that CD44 acted as a proadhesive molecule involved in HL60 cell and neutrophil adhesion.

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94. Datasheet Syndecan-4 (N-19): sc-9497. Santa Cruz Biotechnology.

95. Datasheet Syndecan-4 (H-17): sc-33912. Santa Cruz Biotechnology.

96. Datasheet HCAM (DF1485): sc-7297. Santa Cruz Biotechnology.

Appendix 1: Commercial solutions and chemicals

Table I: Commercial solutions and chemicals with suppliers

Product	Supplier
HL60 (ATCC® CCL-240™) cells	ATCC, Manassas, USA
Iscove's Modified Dulbecco's Medium (IMDM)	ATCC, Manassas, USA
Fetal Bovine Serum, Standard Quality	PAA Laboratories GmbH, Pasching, Austria
All-trans retinoic acid (ATRA), Retinolsyre Fluka 95152	Sigma-Aldrich, Oslo, Norway
Tryptan blue, 0,4 %	Sigma-Aldrich, Oslo, Norway
Octagam, 50 mg/mL	Octapharma AG, Lachen, Switzerland
Lymphoprep, 1.077 ± 0.001 g/mL	Axis-Shield, Oslo, Norway
Aqua B. Braun (aqua dest.)	Braun Melsungen AG, Melsungen, Germany
RPMI 1640	PAA Laboratories GmbH, Pasching, Austria

Appendix 2: Prepared reagents and solutions

Table II: Prepared reagents and solutions

Buffer/Reagents	Reagents	Amount/Concentration
PBS stock solution 5 x salt concentration (pH 6.9)	NaH_2PO4 x 2 H_2O Na_2HPO_4 x 12 H_2O NaCl Aqua dest.	1.95 g 13.43 g 42.37 g Total volume 1 L
PBS 2 x salt concentration	PBS stock solution Aqua dest.	40 % 60 %
PBS 1 x salt concentration (pH 7.2 ± 0.2)	PBS stock solution Aqua dest.	20 % 80 %
PBS with 0.2 % BSA	PBS 1x BSA (Bovine serum albumin)	99,8 % 0,2 %
PBS-PFA	PBS 1x PFA (Paraformaldehyd)	99 % 1 %
RPMI buffer (RPMI+) for adhesion experiments	RPMI 1640 $CaCl_2$ + H_2O $MgCl_2$ Hepes	 25 mM 1 M 10 mM
3.2 M Phosphate buffer stock solution (pH 5.9)	Na_2HPO_4 NaH_2PO_4 Aqua dest.	113.91 g 149.77 g 500 mL
0.32 M Phosphate buffer (pH 5.4)	3.2 M Phosphate buffer Aqua dest.	25 mL Total volume 250 mL
0.25 M Phosphate buffer (pH 6.0)	3.2 M Phosphate buffer Aqua dest.	7.8 mL Total volume 100 mL
HDTM ammonium bromid buffer	Cetyltrimethyl ammonium bromid 0.25 M Phosphate buffer Aqua dest.	250 mg 10 mL 50 mL
80 mM TMB stock solution	Tetramethylbenzidin (T 2885, Sigma) N-N-dimethylformamide	0.231 g 12 mL
20 mM Tetramethylbenzidin	80 mM TMB stock solution N-N-dimethylformamide	3 mL 9 mL
2 M Hydrogen peroxide stock solution (3.4 %)	30 % H_2O_2 Aqua dest.	0.567 mL Total volume 5 mL
1.2 mM Hydrogen peroxide	2 M Hydrogen peroxide Aqua dest.	6 µL 10 mL

TMB reaction solution	PBS 1 x solution 0.32 M Phosphate buffer 1.2 mM Hydrogen peroxide 20 mM Tetramethylbenzidin	25 % 31.25 % 31.25 % 12.5 %
2 M Sodium acetate (pH 3.0)	Sodium acetate Aqua dest.	20.51 g 125 mL
200 mM Sodium acetate (stop solution)	2 M Sodium acetate Aqua dest.	10 % 90 %

Appendix 3: Antibodies

Table III: Anti-CD13 and anti-CD33 antibodies

Antibody	Lot. No	Specificity
CD13 APC (eBioscience, AH Diagnostics, Oslo, Norway)	E10286-1630	Mouse IgG1, κ
CD33 PE (eBioscience)	E021195	Mouse IgG1, κ

Table IV: Anti-CD44 and anti-syndecan-4 antibodies

Antibody	Lot. No	Specificity	Epitope	Control antibody	Lot. No	Specificity	Secondary antibody	Lot. No	Specificity
HCAM (DF1485) sc-7297 (Santa Cruz Biotechnologies, AH Diagnostics, Oslo, Norway)	H0608	Mouse monoclonal IgG1	Unknown	Sc-2025 (Santa Cruz Biotechnologies)	12208 (G0910)	Normal mouse IgG	Sc-2010	J1008	Goat anti-mouse IgG-FITC
HCAM (BU52) sc-65265 (Santa Cruz Biotechnologies)	12707	Mouse monoclonal IgG1	Unknown						
Syndecan-4 (5G9) sc-12766 (Santa Cruz Biotechnologies)	H1208	Mouse monoclonal IgG2a	Ectodomain on human syndecan-4	Sc-2025 (Santa Cruz Biotechnologies)	G0910	Normal mouse IgG	Sc-2010	J1008	Goat anti-mouse IgG-FITC
Syndecan-4 (H-140) sc-15350 (Santa Cruz Biotechnologies)	C1207	Rabbit polyclonal 1gG	Aminoacids 1-140 near N-terminus of human syndecan-4	Sc-2027 (Santa Cruz Biotechnologies)	F1608	Normal rabbit IgG	Sc-2012	G1708	Goat anti-rabbit IgG-FITC
Syndecan-4 (H-17) sc-33912 (Santa Cruz Biotechnologies)	L1007	Goat polyclonal IgG	Extracellular domain of human syndecan-4	Sc-2028 (Santa Cruz Biotechnologies)	D2808	Normal goat IgG	Sc-2777	K0207	Rabbit anti-goat IgG-FITC
Syndecan-4 (N-19) sc-9497 (Santa Cruz Biotechnologies)	B2310	Goat polyclonal IgG	N-terminus of human syndecan-4						

Appendix 4: Data with OD-values from adhesion experiments

Table V: OD values from adhesion experiments with anti-CD44 antibodies on differentiated HL60 cells

Antibodies	OD-values							
	Differentiated HL60 cells					Isolated neutrophils		
	Experiment 1	Experiment 2	Experiment 3.1	Experiment 3.2		Experiment 1	Experiment 2	Experiment 3
Blank	0,042	0,049	0,047	0,049		0,043	0,077	
	0,045	0,057	0,040	0,048		0,086	0,093	
	0,049	*	0,050	0,052		0,052	0,094	
Buffer	0,737	0,667	0,798	0,724		0,172	0,137	0,112
	0,757	0,688	0,737	0,731		0,163	0,145	0,112
	0,767	0,729	0,712	0,705		0,166	0,152	0,114
	0,670	0,718	0,746	0,592		0,167	0,140	0,121
	0,661	0,693	0,707	0,624		0,162	0,130	0,114
	0,655	0,699	0,731	0,582		0,163	0,132	0,118
Ctr sc-2025 10.0 µg/mL	0,600	0,601	0,636			*	0,110	0,089
	0,576	0,598	0,675			*	0,119	0,103
	0,601	0,630	0,686			*	0,124	0,094
	0,557	0,573	0,705			*	0,115	0,096
	0,562	0,597	0,736			*	0,116	0,103
	0,554	0,620	0,701			*	0,119	0,099
Ctr sc-2025 7.5 µg/mL	0,558	0,616		0,663		0,132	0,159	0,111
	0,591	0,614		0,658		0,155	0,166	0,126
	0,579	0,641		0,658		0,160	0,164	0,117
	0,638	0,591		0,614		0,171	0,145	0,118
	0,587	0,620		0,623		0,183	0,145	0,133
	0,603	0,628		0,624		0,180	0,153	0,127
CD44 sc-65265 10.0 µg/mL	0,298	0,414	0,499			0,068	0,093	0,084
	0,279	0,426	0,473			0,064	0,091	0,092
	0,292	0,432	0,493			0,065	0,087	0,088
	0,242	0,471	0,459			0,065	0,081	0,081
	0,254	0,445	0,438			0,064	0,077	0,085
	0,268	0,460	0,475			0,063	0,078	0,081
CD44 sc-65265 7.5 µg/mL	0,475	*		0,529		0,095	0,067	0,064
	0,495	0,520		0,492		0,093	0,073	0,062
	0,529	0,509		0,519		0,082	0,072	0,072
	0,464	0,522		0,558		0,065	0,075	0,069
	0,508	0,496		0,524		0,065	0,075	0,071
	0,548	*		0,567		0,072	0,074	0,070
CD44 sc-7297 10.0 µg/mL	0,607	*	0,669			0,168	0,176	0,119
	0,622	0,628	0,666			0,178	0,162	0,115
	0,677	0,546	0,702			0,173	0,162	0,115

	0,578	0,629	0,645			0,209	0,137	0,103
	0,656	*	0,669			0,193	0,144	0,110
	0,699	*	0,652			0,193	0,153	0,119
CD44 sc-7297 7.5 µg/mL	0,656	*		0,710		0,143	0,133	0,129
	0,721	0,623		0,690		0,137	0,143	0,134
	0,732	0,614		0,722		0,139	0,145	0,134
	0,601	0,698		0,719		0,164	0,157	0,158
	0,659	0,504		0,659		0,166	0,168	0,136
	0,713	*		0,685		0,160	0,168	0,140

* OD-values disregarded in this study