CarcinoEmbryonic Antigen-related Cell Adhesion Molecule 8 (CEACAM8)

Purification, Characterization, Cellular and Clinical Studies

BY

LINSHU ZHAO

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Abstract

A 95-kDa protein was purified from normal human granulocytes. The protein reacted with a monoclonal antibody against CEACAM8. MALDI-Tof and MS/MS analyses revealed the protein to be a CGM6 gene product. Thus, the protein was proved to be identical to CEACAM8.

An ELISA for CEACAM8 was developed with detection range of 1-64μg/L. Data are presented on the levels of CEACAM8 in the blood of healthy individuals and patients undergoing surgery, as well as in patients with acute infection. The highly elevated levels of CEACAM8 in the blood of these patients were significantly correlated with the surface expression of CEACAM8 on neutrophils and the number of circulating neutrophils, which suggests that CEACAM8 could serve as a biological marker for granulocyte activity in vivo.

The cellular content of CEACAM8 in neutrophils was estimated to be 82.4 ± 8.9 ng/10⁶ cells. Subcellular localisation and mobilisation studies showed that the majority of CEACAM8 is present in the secondary granules of human neutrophils, with a small amount on the plasma membranes. Upon stimulation, CEACAM8 translocated to the plasma membranes from the secondary granules and was also released extracellularly (5.5 ± 0.7% of the total content of CEACAM8).

In eosinophils, the cellular content of CEACAM8 was estimated to be 73.8 ± 6.0 ng/10⁶ cells. In these cells, CEACAM8 is mainly stored in secretory vesicles. Upon activation, eosinophils released 5.1 ± 1.1% of the total content of CEACAM8.

Administration of granulocyte colony-stimulating factor (G-CSF) to healthy individuals resulted in an increased content of CEACAM8 in neutrophils on day 1, and decreased on day 4. However, the content of CEACAM8 in light membrane fractions was increased on day 4. The translocation of CEACAM8 observed in vivo after G-CSF administration is probably not directly related to this cytokine but to other cytokines such as TNF-a.

Keywords: human neutrophil, human eosinophil, granule proteins, CEA, CEACAM8, G-CSF, ELISA, subcellular fractionation

Linshu Zhao, Department of Medical Sciences, Clinical Chemistry, Uppsala University, Sweden

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To my family
List of Papers

This thesis is based on the papers listed below, which will be referred to in the following by their Roman numerals I-IV.


II  Linshu Zhao, Shengyuan Xu, Gustaf Fjaertoft, Karlis Pauksen, Lena Håkansson and Per Venge. An enzyme linked immunosorbent assay (ELISA) for human carcinoembryonic antigen-related cell adhesion molecular 8 (CEACAM8), a biological marker of granulocytes in vivo. (Accepted by Journal of Immunological Methods)


IV  Linshu Zhao, Shengyuan Xu, Lena Douhan Håkansson, Malgorzata Karawajczyk and Per Venge. Subcellular localization of carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8) in human eosinophils (Manuscript).

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Abbreviations

AP    Alkaline Phosphatase
AP 1   Alkaline Phosphodiesterase 1
BSA   Bovine Serum Albumin
CEA    CarcinoEmbryonic Antigen
CGM6   Carcinoembryonic Antigen gene family Member 6
CTAB  N-Cetyl-N,N,N-Trimethyl Ammonium Bromide
ECP   Eosinophil Cationic Protein
ELISA Enzyme-Linked ImmunoSorbent Assay
EPO   Eosinophil Peroxidase
EPX   Eosinophil Protein X
f-MLP Formyl-Methionyl-Leucyl-Phenylalanine
FPLC Fast Performance Liquid Chromatography
G-CSF Granulocyte Colony-Stimulating Factor
GM-CSF Granulocyte Macrophage Colony-Stimulating Factor
GPI   Glycosyl Phosphatidyl-Insitol
HBSS Hank’s Balanced Salt Solution
HNL   Human Neutrophil Lipocalin
HSA   Human Serum Albumin
IL-5  InterLeukin 5
LAP   Latent Alkaline Phosphatase
MALDI-ToF Matrix Assisted Laser Desorption Ionization-Time Of Flight
MFI   Mean Fluorescence Intensity
MMP-9 Matrix MetalloProteinase-9
MPO   Myeloperoxidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NCA</td>
<td>Non-specific Cross reacting Antigen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>PhenylMethylSulfonyl Fluoride</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio ImmunoAssay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SBTI</td>
<td>SoyBean Trypsin Inhibitor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
</tbody>
</table>
Introduction

Polymorphonuclear phagocytes, neutrophils and eosinophils, are crucial in the containment of infection. Neutrophils are armed with an array of microbicidal weapons, such as antibacterial proteins, proteases and hydrolases. They are the most abundant of the circulating white blood cells, and are the first cells to reach sites of pathogenic invasion. Therefore, they play a key role in the first-line defence against invading microorganisms and in inflammatory reactions in human disease. Eosinophils, much rarer than neutrophils, are equipped with weapons such as highly basic proteins able to kill helminths and to damage tissues. They are involved in parasite killing and antiviral defence, as well as in human diseases such as allergic inflammation. Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8), also known as NCA-95 (Non-specific Cross reacting Antigen-95), CD67, and CD66b, is a highly glycosylated membrane associated protein. It is expressed only in cells of the granulocyte-lineage and is capable of heterophilic adhesion to the closely related molecule CEACAM6 in vitro \(^{1,2}\). It has also been shown to be physically associated with CEACAM1, an adhesion and signalling molecule in many cells \(^3\). As a member of the carcinoembryonic antigen family CEACAM8 might play a role in the interaction between granulocytes or between granulocytes and epithelial cells expressing CEACAM6 and a role in signalling events in granulocytes. However, its precise function in vivo is not clear.

This thesis presents data on purification of native CEACAM8 from normal human granulocytes, establishment of the subcellular localisation of CEACAM8 in granulocytes, mobilisation of CEACAM8 from these granulocytes and development of an enzyme-linked immunosorbent assay for the measurement of CEACAM8 in body fluids and cell lysates, as well as the effect of granulocyte colony-stimulating factor (G-CSF) on the changes in the cellular content of CEACAM8 in neutrophils.
General Background

Neutrophil

The human neutrophilic granulocyte (neutrophil), the most abundant of circulating white blood cell plays an important role in the elimination of invading microorganisms by a process known as phagocytosis and in inflammatory reactions in human disease. During phagocytosis, neutrophils produce reactive oxygen species (ROS), including superoxide, hydrogen peroxide and hypochlorous acid, and release cytotoxic granule components into pathogen-containing phagocytic vacuoles, which results in the killing and digestion of invading microorganisms.

Neutrophil maturation and life span

Neutrophils are derived from a common progenitor of all hematopoietic lineage, the pluripotent stem cell and mature in the bone marrow, passing morphologic stages such as myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell and segmented cell. The earlier stages of neutrophil differentiation, myeloblast, promyelocyte and myelocyte are dividable, but the later stages of neutrophil differentiation are non-dividing. The mature neutrophil has a diameter of 12-15 μm, and has a typical multilobulated nucleus. After the neutrophils have left the bone marrow they migrate out into the circulation, where they remain for several hours. The final fate of the neutrophils is in tissues, such as spleen, liver and intestine, where they survive 1-2 days.

The production of human neutrophilic granulocytes is controlled mainly by two myeloid-predominant growth factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), with the former being more specific for the neutrophilic granulocytes. G-CSF is a widely used therapeutic compound to combat the risk of infection resulting from both congenital and acquired neutropenias.
Granules and secretory vesicles

Human neutrophils obtain a number of proteolytic and bactericidal proteins, mobilisable cell-surface receptors and adhesion molecules, which are stored in mobilisable organelles, including primary, specific and gelatinase granules, as well as secretory vesicles. These organelles are formed in the bone marrow at subsequent stages of neutrophil maturation. The organelles may be classified on the basis of their size, morphology, density and content of granule matrix proteins. The primary (azurophil) granules, which take up the basic dye azure A due to their content of acid mucopolysaccharide, are formed exclusively during the promyelocytic stage. The primary granules are characterised by the content of myeloperoxidase (MPO) and may be further divided based on their content of defensins into large, defensin-rich granules and the smaller defensin-poor granules. The peroxidase-negative granules are divided into secondary (specific) granules and tertiary (gelatinase) granules. Specific granules are formed during myelocytic and metamyelocytic stages with a high content of lactoferrin, while tertiary (gelatinase) granules appear in band cell and segmented cell stages with a high content of gelatinase. Secretory vesicles are formed in the segmented cell stage, probably by an endocytic process. As shown in Table I, primary granules contain in their matrix hydrolytic and bactericidal proteins such as elastase, cathepsin G, bactericidal permeability-increasing protein, defensins and MPO, which kill and digest invading microorganisms. Secondary granules contain in their matrix collagenase, lysozyme, heparanase, gelatinase, vitamin B12-binding protein, α2-microglobulin-related protein, also known as neutrophil gelatinase-associated lipocalin (NGAL) and human neutrophil lipocalin (HNL), etc. Tertiary granules contain in their matrix gelatinase and lysozyme, etc. Secretory vesicles contain in their matrix plasma proteins such as albumin. Apart from these matrix proteins there are a number of proteins on membranes of granules and secretory vesicles. Primary granules contain in their membranes granulophysin (CD63) and CD68, etc. Peroxidase-negative granules (secondary and tertiary granules), contain in their membranes adhesion molecules, receptors for complement factors and chemotactic substances, and components of NADPH-oxidase such as CD11b/CD18 (Mac-1, CR3), and fMLP-receptor and soluble N-ethylmaleimide-sensitive fusion protein attachment proteins (SNAPs, SNAP-23 and SNAP-25), synaptotagmin II and neutrophil-specific cytochrome b558, etc. The membranes of secretory vesicles contain latent alkaline phosphatase and are particularly rich in CD11b/CD18, CR1, CD14, CD16 and fMLP-receptors, which are translocated to the plasma membranes after exocytosis.
### Table 1. Proteins in neutrophil granules and secretory vesicles

<table>
<thead>
<tr>
<th>Organelles</th>
<th>Matrix proteins</th>
<th>Membrane proteins</th>
</tr>
</thead>
</table>
| Azurophil Granules | Myeloperoxidase  
|                 | Defensin  
|                 | Lysozyme  
|                 | Cathepsin G  
|                 | Bacterial permeability-increasing protein  
|                 | Elastase, etc  | Granulophysin (CD63) |
| Specific Granules | Lactoferrin  
|                 | Collagenase  
|                 | Lysozyme  
|                 | Heparanase  
|                 | Gelatinase  
|                 | Vitamin B₁₂-binding protein  
|                 | Human neutrophil lipocalin, etc  | CD11b/CD18 |
| Tertiary Granules | Gelatinase  
|                 | Lysozyme, etc  | f-MLP receptor |
| Secretary Vesicles | Albumin, etc  | Synaptotagmin II  |
|                 |                                                                 | Cytochrome b₅₅₈, etc |
|                 |                                                                 | Latent alkaline phosphate |

In the process of resolving ongoing infection, the neutrophil interacts with its environment and fights with invading microorganisms by subsequently mobilising its organelles. Upon stimulation, the secretory vesicles are first mobilised followed by gelatinase granules, secondary and primary granules.\(^{43,44}\)
Mobilisation of the secretory vesicles is an important process for neutrophil interact with endothelium by up-regulation of receptors and adhesion molecules \[45\]. The mobilisation of secretory vesicles may be mediated by signalling through the selectins or by inflammatory mediators liberated from the endothelium. Exocytosis of the gelatinase and the secondary granules occurs subsequently. Exocytosis of granule content is a consequence of fusion of granule membrane with the plasma membrane and incorporation of granule membrane into the plasma membrane. In this way, membrane proteins located to the membrane of granules translocate to the surface membrane and furnish the cell with new receptors and other functional proteins. Exocytosis of gelatinase from tertiary granules may help degradation of type IV and V collagen in basement membrane. Exocytosis of zymogen collagenase from secondary granules like gelatinase from tertiary granules may facilitate diapedesis and migration of the cell into tissue by releasing matrix-degrading proteins \[46\]. Because, β2 integrin (CD11b/CD18) and the flavocytochrome b558, an essential component of the NADPH, are mainly stored in the membranes of gelatinase and specific granules, these granules are also related to phagocytosis and intracellular killing. Lastly, the primary granules fuse with phagosomes creates conditions for oxygen-dependent and –independent bactericidal activity.

Mobilisation of neutrophil secretory organelles is a calcium-dependent event. The individual secretory organelles require distinct Ca\(^{2+}\) concentration for mobilisation, with secretary vesicles requiring the lowest and primary granules the highest Ca\(^{2+}\) concentration \[44,47\]. This suggests the requirement of several calcium sensors responsible for mobilisation of the different secretory organelles. A protein, synaptotagmin II, was identified recently in specific granules in human neutrophils and translocated to the phagosome in a calcium-dependent fashion \[37\]. It was speculated to be a calcium sensor in these granules, since the synaptotagmins, calcium-binding proteins, are putative calcium-sensors of exocytosis in other cells \[48\].

Neutrophil function

Neutrophil microbicidal processes consist of the formation of a combination of reactive oxygen (and, possibly, nitrogen) species and various hydrolytic enzymes and antimicrobial polypeptides. During the inflammatory response, chemotactic factors such as C5a (complement factor 5a), PAF (platelet activating factor), LTB\(_4\) (leukotriene B\(_4\)), IL-8 (interleukin 8) and f-MLP (formyl-methionyl-leucyl-phenylalanine) released as a result of initial contact of infectious agents with phagocytes and other components of the immune system and generated by infectious agents themselves, attract neutrophils to sites of infection (chemotaxis) \[49\]. Usually, neutrophils roll along microvascular walls via low-affinity interactions of selectins with specific endothelial
carbohydrate ligands. Activation of neutrophil β2 integrins and subsequent high-affinity binding to intracellular adhesion molecules on the surface of activated endothelial cells initiates the neutrophil to penetrate the endothelial layer and migrate through connective tissue to sites of infection (diapedesis), where they finally congregate and adhere to extracellular matrix components such as laminin and fibronectin.

Neutrophil can internalise both opsonised and non-opsonised pathogens. Opsonisation of pathogens is an important mechanism to enhance the efficiency of phagocytosis. The main opsonins in the body are specific antibodies and complement fragments. The main Fc receptors of human resting neutrophils are FcγRIIa (CD32) and FcγRIIIb (CD16), while the high-affinity FcγRI (CD64) functions predominantly after neutrophils have been primed. Through specific receptors for IgG (Fcγ-receptors) and for the complement components, C3b (complement receptor type 1, CR 1) and C3bi (CR 3), neutrophil can attach the opsonised microorganism, which will be subsequently engulfed. The engulfed microorganism is destroyed by cytotoxic reactive oxygen species (ROS), reactive nitrogen species, and antibacterial components released from azurophilic and specific granules (intracellular killing). The killed microorganism is degraded by hydrolytic enzymes released into the phagocytic vacuole during the degranulation process (digestion). The highly accumulated neutrophils at sites of infection are a concomitant potential to cause severe tissue destruction should they undergo necrotic lysis and release cytotoxic granule contents and reactive oxygen species onto host tissues. Therefore, timely and vigilant execution of a controlled cell death (apoptosis) program, after phagocytosis is necessary for preventing damage to healthy tissues and necessary for resolution of infection. Phosphatidylserine (PS) appears to be a major ligand on the surface of apoptotic cells that triggers phagocytosis. In normal cells, PS is largely confined to the inner leaflet of the plasma membrane. During apoptosis, PS becomes exposed at the outer leaflet of the membrane. Once at the apoptotic cell surface, PS is recognised by different phagocyte receptors. The most prominent representative among them is the PS receptor. During apoptosis the neutrophil remains its granule contents and loses the ability to secrete them in response to secretagogues. In contrast to necrotic neutrophils, apoptotic neutrophils are ingested by inflammatory macrophages employing novel phagocytic recognition mechanism that fail to provoke a macrophage proinflammatory response, which is a healthy mechanism to clear host cells that have completed the task of containing and killing infectious microbes.
Regulation of neutrophil function

A number of soluble mediators including cytokines, bioactive lipids, hormones produced by immune cells, as well as secreted from endocrine, nervous and cardiovascular systems, influence the functions of neutrophils.

The myelopoiesis is regulated by cytokines such as GM-CSF and G-CSF. GM-CSF stimulates the production of neutrophil, monocyte/macrophage, and eosinophil colonies. G-CSF is a relatively neutrophil-specific hematopoietin that stimulates the proliferation and differentiation of the haematopoietic progenitor cells of neutrophilic granulocytes. In addition, G-CSF like GM-CSF modulates the functions of mature neutrophils including survival, superoxide release, expression of adhesion molecules (CD11b/CD18, LAM-1), phagocytic and bactericidal activity. The pyrogenic cytokines, IL-1, TNF-α, and IL-6 effects various pathways that lead to the activation of NADPH oxidase. TNF-α primes HOCl production in vitro and in vivo and directly stimulates neutrophil degranulation. IL-8 is a potent chemoattractant and activating factor. Interferon-γ primes the neutrophil oxidative burst. Neutrophils also produce small amounts of some cytokines including interleukins (ILs) 1, 6, and 8, TNF-α, and G-CSF that may take part in the regulation of the inflammatory responses. Leukotriene B4 is a strong neutrophil chemoattractant and augments neutrophil phagocytosis. Platelet-activating factor (PAF) directly primes neutrophil superoxide production and neutrophil granule protein release. Growth hormone primes the oxidative burst of human neutrophils. Glucocorticoids weaken phagocytic and cytotoxic activities of neutrophils, as well as the capacity of neutrophils to produce ROS and secrete antimicrobial agents in response to stimulation with chemotactic peptides in vitro.

Eosinophils

Maturation

The eosinophil granulocyte (eosinophil) is derived from a pluripotent stem cell in the bone marrow. The differentiation of eosinophil in the bone marrow takes approximately 5 days. The eosinophil is released into the circulation, where it has a half-life of 6-12 hours, and then enters the marginated pool or migrate into the tissues and reside mainly in the submucosal tissue. The half life of the tissue eosinophil could be from several days up to weeks. Peripheral blood eosinophils are non-dividing fully differentiated cells with a diameter approximately 8 μm and a typically bilobulated nucleus. In the
circulation, about 1-4% of leukocytes are eosinophils. The production of eosinophils is controlled by hematopoietic cytokines such as IL-3, IL-5 and GM-CSF. Of these cytokines, IL-5 is the most specific for the eosinophilic lineage. IL-5 and GM-CSF are not only involved in the production of eosinophils, they also contribute to prolong the survival of eosinophils.

Eosinophil organelles

Eosinophil contains several organelles, including primary granules, secondary (specific) granules, small granules, lipid bodies, as well as a variety of vesicles. As shown in Table II, the primary granules contain the Charcot-Leyden crystal protein made up of a lysophospholipase in a crystallised form. The most prominent are the secondary granules, which consist of two compartments under electron microscopy: a crystal core and a surrounding homogeneous matrix. They contain highly basic proteins, which show an affinity to eosin that gives the eosinophil a pink colour and make it easily recognisable among other cells under the light microscope. Major basic protein (MBP) is localised in the core of the secondary granules, while eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil protein X (EPX) are localised at the matrix. The small granules contain acid phosphatase, arylsulphatase B, catalase, elastase, cytochrome b₅₅₈ and ECP. The lipid bodies contain cyclooxygenase, lipoxigenases, leukotriene C₄ synthase, elastase and EPO. The vesicles contain albumin and cytochrome b₅₅₈. Several classes of membrane proteins and receptors are present on the surface of eosinophil including immunoglobulin Fc receptors for Ig A, E and G, adhesion molecules such as CD11b/CD18, VLA-4 and L-selectin, chemokine receptor for RANTES, cytokine receptors for IL-3, IL-5 and GM-CSF and receptors for complement such as CR1 and CR3.
<table>
<thead>
<tr>
<th></th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Granules</strong></td>
<td>Lysoosphospholipase</td>
</tr>
<tr>
<td><strong>Secondary Granules</strong></td>
<td>Major basic protein (MBP)</td>
</tr>
<tr>
<td></td>
<td>Eosinophil cationic protein (ECP)</td>
</tr>
<tr>
<td></td>
<td>Eosinophil peroxidase (EPO)</td>
</tr>
<tr>
<td></td>
<td>Eosinophil protein X/Eosinophil derived neurotoxin (EPX/EDN)</td>
</tr>
<tr>
<td><strong>Small Granules</strong></td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td></td>
<td>Arylsulphatase B</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td>Elastase</td>
</tr>
<tr>
<td></td>
<td>Cytochrome b&lt;sub&gt;558&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>ECP, etc</td>
</tr>
<tr>
<td><strong>Vesicles</strong></td>
<td>Cytochrome b&lt;sub&gt;558&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>Albumin, etc</td>
</tr>
<tr>
<td><strong>Lipid bodies</strong></td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td></td>
<td>Lipoxigenase</td>
</tr>
<tr>
<td></td>
<td>Leukotriene C&lt;sub&gt;4&lt;/sub&gt; synthase</td>
</tr>
<tr>
<td></td>
<td>Elastase</td>
</tr>
<tr>
<td></td>
<td>EPO, etc</td>
</tr>
<tr>
<td><strong>Plasma membrane</strong></td>
<td>CD11b/Cd18</td>
</tr>
<tr>
<td></td>
<td>VLA-4</td>
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<tr>
<td></td>
<td>L-selectin</td>
</tr>
<tr>
<td></td>
<td>CR1</td>
</tr>
<tr>
<td></td>
<td>CR3, etc</td>
</tr>
</tbody>
</table>
Eosinophil function

During inflammatory reaction eosinophils are recruited in large number from peripheral blood to the inflammatory site. The mechanism behind the migration of eosinophils into the inflammatory site was suggested to be controlled by the interactions between receptors and adhesion molecules on eosinophils and their ligands on the encounter cells or matrix proteins under the influence of cytokines, chemokines or other locally produced soluble mediators.

Eosinophil cytotoxic mechanisms consist of a combination of oxygen-dependent process through oxidative metabolism which generates reactive oxygen metabolites, such as superoxide anions and hydrogen peroxide, and an oxygen-independent process through secretion of cytotoxic proteins such as MBP, ECP, EPX and EPO. Eosinophil is a poor phagocyte, which mediates its toxicity mainly by secretion of toxic mediators. Eosinophil is believed to play a role in host defence against parasitic worms and defence against respiratory pathogens. However, it is also recognised as major effector cell in the inflammatory processes underlying asthmatic and allergic diseases. The eosinophil not only stores and releases a wide variety of cytotoxic mediators, but also produces cytokines, chemokines and growth factors including IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-16, leukocyte inhibitory factor, GM-CSF, eotaxin, IL-8, RANTES, stem cell factor, tumour necrosis factor, interferon-γ, TGF-α and β, etc. It has been shown that the eosinophil also plays a role in presenting allergen to stimulate CD4+ T-helper (Th) cells. Epithelial damage and loss caused by MBP, EPO and ECP, the highly basic granule proteins in eosinophil is believed to be a major event in asthma pathogenesis. Thus eosinophil can make a major contribution to the inflammatory processes underlying asthmatic and allergic disease. Tissue eosinophils are undergoing apoptosis, the apoptotic eosinophils are mainly removed by macrophages. Other cells such as dendritic cells, fibroblasts, hepatocytes and epithelial cells are reported to be capable of phagocytosing apoptotic cells. Eosinophil apoptosis can be induced or accelerated by ligation of membrane receptors by specific MAb for Fas (CD95), CD69 and CD45. However, the apoptotic clearance pathway may be controlled different from that in neutrophils. For example, corticosteroids delay neutrophil apoptosis but greatly accelerate eosinophil apoptosis.
Carcinoembryonic antigen (CEA)

Carcinoembryonic antigen (CEA) is a marker widely used in clinical evaluations of human tumours of epithelial origin. The CEA gene family consists of 29 genes, of which more than 20 are transcriptionally active, with closely related genes clustered on the human chromosome 19q13.2. The CEA gene family belongs to the Ig gene superfamily. The CEA genes (as shown in figure 1) are divided into three main subgroups: the CEA subgroup, the pregnancy-specific glycoprotein (PSG) subgroup, and a subgroup of pseudogenes. The CEA subgroup consists of seven distinct members in humans: CEACAM1 (CD66a, BGP), CEACAM3 (CD66d, CGM1), CEACAM4 (CGM7), CEACAM5 (CD66e, CEA), CEACAM6 (CD66c, NCA, NCA-50/90), CEACAM7 (CGM2), CEACAM8 (CD66b, CD67, NCA-95, CGM6). Four of the CEA subgroup members, CEACAM1, CEACAM3, CEACAM6 and CEACAM8 are expressed in granulocytes. The members of the CEA family are believed to function as intercellular adhesion molecules. Furthermore, CEACAM1, CEACAM3, CEA, and CEACAM6 are known as receptors for some bacteria such as Opa protein expressing Neisseria and type 1 fimbriae expressing Escherichia coli in vitro.
Figure 1. The CEA gene family and CEA subgroup members
Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8)

Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM 8) 147, also known as NCA-95, CD67 and CD66b, has been identified in human neutrophils since many years 148. Based on the reactivity of a CGM6-expressing transfectant with antibodies against CEACAM8, the protein was suggested to be a product of the CGM6 (NCA-W272) gene 149-151. CEACAM8 has been assigned to the CEA family on the basis of the homology of the deduced amino acid sequence 152. CEACAM8, like many members of the CEA subgroups, is anchored to the membrane through a glycosylphosphatidylinositol anchor and specifically released from the membrane by treatment with phosphatidylinositol-phospholipase C 150. CEACAM8 consists of an immunoglobulin variable-like N-terminal domain of 108 aa (amino acid), two immunoglobulin constant-like domains (A and B domain) of 92 aa and 86 aa, and 29 aa hydrophobic domain, which is replaced by a glycosyl-phosphatidylinositol moiety in the mature protein 150. It is heavily glycosylated (11 N-glycosylation sites), and contains four cysteine residues which conform two sulfide bonds. The theoretical isoelectric point (pI) of the protein is 5.75.

CEACAM8 is expressed only in cells of the granulocyte-lineage and is characterised as a granulocyte-specific activation antigen 150,153,154. So far, CEACAM8 has been described in neutrophils and eosinophils 148,155. In resting neutrophils, it is mainly present in the secondary granules with lower amounts on the plasma membranes 156. Upon activation, its expression on the plasma membrane is rapidly up-regulated, probably by mobilisation from intracellular pools 151. CEACAM8 has been studied less in human eosinophils. It was shown that the expression of integrins and CEACAM8 on peripheral blood neutrophils and eosinophils was enhanced in patients with rheumatoid arthritis 157. CEACAM8 exhibits heterophilic adhesion to the closely related molecule CEACAM6 12, and it has been suggested that CEACAM8 might play a role in the interaction between granulocytes or between granulocytes and epithelial cells expressing CEACAM6 in vivo. CEACAM8 also has been shown to bind galectin-3, a potent stimulus of human neutrophils, implying that neutrophil CEACAM8 may be a functional galectin-3 receptor 158. Recently, CEACAM8 has been shown to be physically associated with CEACAM1 (CD66a, biliary glycoproteins, or C-CAM), an adhesion and signalling molecule in many cells including epithelial, endothelial and hematopoietic cells 3,147,159. Moreover, the MAb (80H3) against CEACAM8 has show to activate extracellular signal-regulated kinases1/2 (Erk1/2) in human granulocytes 3, implying a role of CEACAM8 in
signalling events in granulocytes. However, the precise function of CEA-CAM8 in vivo is not known.
Aims of the present investigation

The overall aim of this investigation was to gain further knowledge about human neutrophil and eosinophil functions. The specific aims of the present investigations were:

- to purify native CEACAM8 from human granulocytes.
- to develop an enzyme-linked immunosorbent assay (ELISA) for the measurement of CEACAM8 in fluids and cell lysates.
- to evaluate the possible usefulness of CEACAM8 as a biological marker for granulocyte activity in vivo.
- to establish the subcellular localization and mobilization of CEACAM8 in human neutrophils and eosinophils.
- to determine the cellular contents of CEACAM8 in neutrophils and eosinophils, and its release upon stimulation.
- to study how the cellular content and subcellular localization of CEACAM8 is affected in human neutrophils during G-CSF administration.
Materials and Methods

Preparation of granule proteins
Granulocytes were obtained from buffy coats of healthy blood donors. The granulocytes were disrupted by nitrogen cavitation. The cavitate was then collected and centrifuged to sediment the granules. After one cycle of freezing and thawing, the granules were extracted with 50 mM acetic acid, which was further extracted with 0.4 M sodium acetate, pH 4. The granule extract was then concentrated to approximately 5 ml using a YM-10 filter.

Chromatographic procedures
Gel filtration was performed on a Sephadex G-75 superfine column, equilibrated with 0.2 M NaAc, pH 4.5. Ion exchange chromatography was performed using the FPLC system on the strong cationic exchanger Mono-S using a pre-packed column equilibrated with 0.1 M NaAc, pH 4.0. The proteins, remaining unbound to the Mono-S column, were applied to the same column equilibrated with 0.02 M NaAc, pH 3.3. The bound proteins were eluted with a linear gradient from 0 to 0.25 M NaCl in NaAc, pH 3.3. Gel filtration chromatography was performed on a Superdex 75 HR column. The proteinase inhibitors, phenylmethylsulfonyl fluoride (PMSF) (100 mg/L), were added to all buffers from the cell disruption step to the first ion exchange chromatography.

Proteins in the chromatograms were measured by their absorbance at 280 nm. Ultrafiltration of pooled fractions was performed on YM-10 filter. Buffer change was performed on a PD-10 column.

Enzyme linked immunoassay for CEACAM8
A 96-well ELISA plate was coated with monoclonal mouse anti-human CEACAM8 antibody (80H3, Immunotech, Marseilles, France) diluted 1:50 in 0.1 M Na2CO3/NaHCO3, pH 9.6. Additional binding sites were blocked by incubation with 3% BSA in 0.1 M Na2CO3/NaHCO3, pH 9.6. Samples and standards diluted in TBS-0.4% Triton X-100 plus 0.1% CTAB containing 1% BSA were then added and incubated overnight at room temperature fol-
lowed by addition of rabbit anti-human CEA diluted 1:1500 and by goat anti-rabbit IgG conjugated with HRP diluted 1:15000. The enzyme reaction was visualised using tetramethylbenzidine (Sigma) for 30 min at room temperature and was stopped by adding 2 M H₂SO₄. Absorbance was read at 450 nm in an ELISA reader. The standards were prepared with purified native CEACAM8, the concentration of which was deduced from data of amino acid composition. Samples were measured in duplicate.

The detection limit of the assay was estimated by measuring 20 replicates of the blank. The mean absorbance plus 3 SD of these measurements was defined as the detection limit of the assay.

Isolation of neutrophils

Human neutrophils were isolated from heparinized whole blood by density gradient centrifugation at room temperature over 67% isotonic Percoll (Pharmacia, Uppsala, Sweden). After centrifugation, the mononuclear cells in the interface were removed. The erythrocytes in the bottom of the tube were lysed by hypotonic shock in ice-cold water for 1 min. The solution was normalised by adding an equal volume of 2 times higher concentration of phosphate-buffered saline (PBS). The neutrophils were washed twice with PBS. The neutrophils obtained by this procedure had a purity of > 95%.

Isolation of eosinophils

Human granulocytes were isolated as described above. Eosinophils were isolated via removal of neutrophils (CD16 positive) according to a negative immunomagnetic selection technique. Briefly, isolated granulocytes were incubated for 1 h at 4°C with anti-CD16 MAb-coated magnetic microbeads (at a proportion of 10⁷ granulocytes in 20 μL PBS with 2% newborn calf serum to 10 μL microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were subsequently allowed to pass through a steel matrix column in a magnetic field. Thereafter, the eosinophils that passed through were collected, washed, and suspended in HBSS medium. The purity and the viability of the eosinophils were > 96% and 99%, respectively. The neutrophils were bound to column were flushed out from bottom (after taking the column away from magnetic field).

Subcellular fractionation

Subcellular fractionation was performed either on a three-layer Percoll gradient as described by Borregaard ¹⁶⁰ or on a sucrose density gradient or on a floatation Percoll gradient as described by Dahlgren ¹⁶¹. In short, isolated neutrophils or eosinophils (with or without stimulation) were treated with 5
mM di-isopropylfluorophosphate for 5 min on ice. Cells were then pelleted by centrifugation at 400 g for 7 min and resuspended in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM ATPNa2, 3.5 mM MgCl2, 10 mM Pipes, pH7.2) containing 0.5 mM phenylmethylsulfonylfluoride (PMSF). Cells were disrupted by ultrasonication. Nuclei and intact cells were pelleted by centrifugation at 450 g for 15 min. The postnuclear supernatant was placed on top of three-layer Percoll gradient (1.05 kg/l/1.09 kg/l/1.12 kg/l) or an eight layer discontinuous sucrose gradients consisting of a 60% cushion overlaid with 55%, 50%, 46%, 42%, 38%, 32% and 20% (W/W) (all solution were prepared in 10 mM Hepes, pH 7.4), or on the middle of the gradient between Percoll of densities 1.04 and 1.12 kg/l after mixing the postnuclear supernatant with an equal volume of Percoll solution (1.12 kg/l). After centrifugation at 11000 rpm for 50 min for the Percoll gradient or at 25000 rpm in a SW 28.1 rotor (Beckman) for 16 h at 4°C for the sucrose density gradient, the gradient was collected in infractions of 0.42 mL from the top of the tube.

Release studies
The release studies were performed as described previously. Briefly, neutrophils (1 x 1010 cells/l) or eosinophils (5 x 109 cells/l) were resuspended in HBSS as indicated cell densities. For stimulation, separated aliquots (150 µl) of cells were exposed to a volume of 300 µl of washed, serum-treated Sephadex G-15 particles (83 g/l). Incubation was terminated after 20 min by adding 150 µl of ice cold buffer 10 mM of ethylenediaminetetraacetic acid in 0.15M of NaCl, followed by centrifugation at 600 g for 10 min at 4°C. A volume of 400 µl of supernatant was removed and mixed with 200 µl of 0.5% N-cetyl-N, N, N-trimethylammonium bromide (CTAB) in 0.15 M NaCl and stored for the measurement of granule proteins. The released granule protein concentrations in the supernatants were calculated as relative concentrations on the basis of the total granule content determined after extraction with 0.5% CTAB in 0.15 M NaCl.

Cellular content of CEACAM8
Neutrophils (300 µl) at a density of 1 x 1010 cells/l and eosinophils (150 µl) at a density of 5 x 109 cells/l were extracted with 1.5 ml (for neutrophils) or 0.75 ml (for eosinophils) of 0.5% CTAB in 0.15 M NaCl for 1 hour at room temperature and centrifuged at 600 g for 10 min at 4°C. Supernatants were removed and measured for CEACAM8.
Effects of G-CSF on the production of CEACAM8 in neutrophils

Glycosylated recombinant human granulocyte-colony stimulating factor (rHuG-CSF, lenograstim; Chugai-Rhone-Poulenc) 10 μg/kg s.c. was administered to four healthy volunteers once daily (8 AM ± 30 min) for six days (days 1-6). Heparinized peripheral blood was obtained immediately before the administration of G-CSF (day 0) and one day after the first injection of G-CSF (day 1), thereafter on day 4, and three weeks post administration of G-CSF. Neutrophils on days 0, 1, 4 and three weeks were obtained and disrupted by ultrasonication, and the post-nuclear supernatants were loaded on the eight layer discontinuous sucrose density gradients as described above.

Incubation of isolated neutrophils with cytokines

Granulocytes were isolated by dextran sedimentation as described by Håkansson and the isolated cells were diluted to a concentration of 3 x 10^9 neutrophils/l in Gey’s buffer. One hundred μl of cells were incubated with G-CSF (Chugai-Rhone-Poulenc, France) at final concentrations of 10, 100 or 1000 μg/l or with TNF-α (R&D systems, USA) at final concentrations of 1, 10, 100 μg/l. Incubations with PBS/HSA served as control. All incubations were performed for 45 minutes at room temperature.

Labelling of neutrophils with antibodies to CEACAM8

To each tube containing 100 μl cell suspension, 2 μl of undiluted fluorescein isothiocyanate (FITC) anti-CEACAM8 (Immunotech, Marseille, France) or FITC labelled isotype control antibodies (DAKO A/S) were added. The samples were then incubated on ice for 30 minutes. After this the cells were washed twice with ice-cold PBS/citrate.

Flow cytometry

The samples were analysed by the use of the EPICS-PROFILE II cytometer (Coulter Company Inc., Hialeah, FL, US). The neutrophils were identified on the basis of their forward and side scatter pattern. A gate was set around the neutrophil population and the FITC-fluorescence within the gate was measured. The cell surface expression of CEACAM8 was measured as specific MFI (mean fluorescence intensity) of the whole populations of neutrophils. The specific MFI of the neutrophil expression of the receptor was calculated by subtracting the background MFI obtained with the negative isotype control MAb from the value obtained with the CEACAM8 MAb. The intra-assay variation was 7%.
Reference populations and patients
Sera from two reference populations were analysed. One comprised 83 apparently healthy adults, 63 women and 20 men (mean age 43, range 30 - 69). The other population comprised umbilical cord blood at birth from vaginally delivered neonates (n = 28) born after a normal single pregnancy and normal delivery.

Sera from two patient groups were obtained. Group one consisted of nine patients undergoing hip replacement surgery (6 women, 3 men, mean age 58, range 47 - 68) and scoliosis surgery (2 boys, 1 girl, age range 12 -18). The second group consisted of 25 patients with acute infections and blood was drawn within 2 days after admission to the hospital. Sixteen of these patients (5 women, 11 men, mean age 66, range 24 - 91) had bacterial infections such as bronchial pneumonia (7 men, 3 women), sepsicaemia (1 woman, 2 men), erysipelas (1 woman, 1 man) and urinary vesicle infection (1 man). Nine patients (5 women, 4 men, mean age 78, range 23 - 89) had influenza A infections without any signs of bacterial superinfections.

Blood samples
Serum samples were prepared by allowing blood to clot at room temperature for 60-90 min, followed by centrifugation twice at 1350 g for 10 min. EDTA containing samples were prepared by means of centrifugation twice at 1350 x g for 10 min. The serum or plasma was stored at -70°C until analysis.

Marker protein assays
Alkaline phosphatase (marker for plasma membranes of neutrophils) and latent alkaline phosphatase (marker for secretory vesicles of neutrophils) activities were measured by hydrolysis of p-nitrophenyl phosphate (2 g/l) in the absence or the presence of Triton-X-100 (0.5%) in 50 mM triethanolamin-HCl and 10 mM MgCl2 pH 9.8. Gelatinase (marker for tertiary granules of neutrophils) was measured by ELISA. Human neutrophil lipocalin (HNL) (marker for secondary granules of neutrophils) and myeloperoxidase (MPO) (marker for primary granules of neutrophils) were measured by RIA. Alkaline phosphodiesterase I activity (marker for plasma membranes of eosinophils) was measured by hydrolysis of sodium thymidine 5'-monophosphate p-nitrophenyl ester (2 mM) in 20 mM Tris buffer, 0.5% Triton X-100, pH 9.0. Albumin (marker for secretory vesicles) and ECP (marker for secretory granules of eosinophils) were measured by RIA.
Statistical analysis

Student’s t-test, Tukey’s post hoc test, Wilcoxon’s test and Mann-Whitney’s test were used. All statistical calculations were performed on a personal computer by means of the statistical package Statistica for Windows (Statsoft, Tulsa, OK, USA) or Prism 4 (Graphpad, San Diego, CA, USA). $P < 0.05$ was considered significant.
Results and Discussion

PAPER I

Purification and characterisation of a 95kDa protein (CEACAM8)

A 95 kDa protein was purified, with a recovery of 13.5%, from acid extracts of granules from normal human granulocytes by means of a simple, three-column procedure consisting of Sephadex G-75, Mono-S cation exchange and Superdex HR-75 chromatography. The purified protein showed only one broad band at a molecular weight of 95 kDa on SDS-PAGE (figure 2). It reacted with polyclonal antibodies against CEA and a specific monoclonal antibody against CEACAM8 (80H3), but did not react with monoclonal antibodies against CD66acde and CE66c when analysed by immunoblotting. The molecular weight of the protein shifted to 40 kDa on SDS-PAGE after deglycosylation with N-glycosidase F, indicating that the protein is highly N-glycosylated. However, Endoglycosidase H treatment caused no change in its molecular weight, indicating the absence of a high-mannose structure. To characterise the protein further, MALDI-Tof and MS/MS analyses were performed. Tryptic peptide analysis by MALDI-Tof identified four peptides (50-63, 79-98, 199-207 and 293-308) with spectra of m/z matching the expected tryptic peptides from a CGM6 gene product. Furthermore, the nanoelectrospray mass spectrometry (MS/MS) analysis of two selected tryptic peptides of the protein revealed two amino acid sequences corresponding to residues 79-98 and 199-207 of the CGM6 gene product. These combined data identified the purified 95 kDa as a product of the CGM6 (W272) gene.
CEACAM8 has previously been identified using specific antibodies in both immunoblotting and immunoprecipitation procedures. Since no native CEACAM8 was available, structural and functional studies were not possible. Following the identification of the CGM6 (W272) gene from a leukocyte cDNA library as an NCA gene and the production of its recombinant protein, CEACAM8 was suggested to be a CGM6 (NCA-W272) gene product. Northern blot and immunological analyses have shown that CGM6 mRNA and its product are only expressed in cells of the granulocytic lineage. Therefore, CEACAM8 has served as a specific marker for the activities of granulocytes.

Some functional studies have been carried out using recombinant CEACAM8. Like other members of the CEA family, CEACAM8 has shown heterophilic binding to the closely related CEACAM6. However, the molecular weights of the recombinant proteins were much lower than that of the corresponding native protein, probably due to incomplete glycosylation.
Therefore, functional studies that may be related to the carbohydrate chains can not be addressed using the recombinant protein.

Recently, CEACAM8 was shown to bind a lectin, galectin-3\(^{158}\). This binding was likely to be mediated by carbohydrate chains, suggesting that the carbohydrate portion of CEACAM8 may play an important role in the regulation of cell activities. Therefore, further structural and functional studies of CEACAM8 undoubtedly require a pure, native protein. In our experiments, purified CEACAM8 showed no binding to galectin-3 as determined by immunoblotting, ELISA or gel filtration chromatography (data not shown). The reason for this discrepancy is not known. One possible explanation for this could be that the binding between CEACAM8 and galectin-3 needs a cofactor so far unknown. Another explanation could be that the protein changes its conformation during purification.

In this paper we present, for the first time, a method for the purification of a 95 kDa protein from granule extracts of normal human granulocytes and identify it as CEACAM8, a product of the CGM6 (NCA–W272) gene. The availability of native CEACAM8 will be essential for further studies of structure-function relationships.

PAPER II

Determination of extinction coefficient of CEACAM8

Using the data on the amino acid composition of purified CEACAM8 supplemented with the tryptophan and cysteine values deduced from the comparison of the amino acid compositions of CEACAM8 and the product of the CGM6 gene, the extinction coefficient (\(E^{1%}, 1\text{cm}\)) of CEACAM8 was determined to be 13.44 at 280 nm.
Development of an enzyme-linked immunoabsorbent assay (ELISA) for CEACAM8

An ELISA was developed for the measurement of CEACAM8 in fluids and cell lysates. The ELISA measures CEACAM8 within the range of 1-64 µg/l, with a detection limit of 0.7 µg/l.

The specificity of the assay was tested in two ways. First, a serum sample was spiked with purified CEACAM8 and serially diluted. A parallelism was obtained between the standard curve and the serially diluted serum. Second, proteins which were immunologically reactive with antibodies to CEA and CD66acde were tested in the assay. None of these proteins were detected by the CEACAM8 assay nor did they interfere with the assay, indicating the specificity of the assay.

A recovery of 91.7% - 97.5% was obtained when purified CEACAM8 was added to serum at three different concentrations (4-16 µg/l).

The precision of the assay was assessed by repeated measurements of sera of three different CEACAM8 concentrations. The intra- and inter-assay coefficients of variation were less than 3.3% (n = 10) and 7.0% (n = 10), respectively.

The stability of CEACAM8 in serum was determined by measuring CEACAM8 in serum samples subjected (or not) to six cycles of freezing and thawing. The result showed that there were no differences in the levels of CEACAM8 comparing sera not frozen and thawed (100%) with sera that had been frozen and thawed [101.55 ± 3.28% (mean ± SE, n = 6)].

Therefore, an accurate, specific, and reproducible ELISA for CEACAM8 was developed which will prove useful in studies about the role of CEACAM8.

Clinical evaluation of the CEACAM8 ELISA

Previously, Grunert et al measured serum levels of CEACAM8, as well as CEA and CEACAM6, in patients with solid tumours of various types and with chronic myeloid leukaemia (CML) and chronic lymphatic leukaemia, in order to evaluate their possible usefulness as tumour markers. The measurement of CEACAM8 in sera from patients with uterus or kidney carcinoma showed a higher sensitivity than the determination of CEA and CEACAM6, suggesting serum CEACAM8 as a candidate marker for uterine and kidney carcinomas. However, CEACAM8 has only been found in cells of the granulocytic lineage by means of immunohistochemistry, Northern blot
and FACScan analyses \(^{150,167}\). It is still not known why such a strong release of CEACAM8 is observed when uterus and kidney tumours are present.

To exploit the possible clinical relevance of CEACAM8, we established the ELISA for CEACAM8 and measured CEACAM8 levels in blood from patients undergoing surgery as well as the number of circulating neutrophils and their surface expression of CEACAM8. It is known that surgical trauma induces the production of cytokines such as IL-6 and TNF-\(\alpha\), which are believed to modulate a number of cell functions, particularly those of leukocytes \(^{168,169,170}\). To determine the effect of surgical trauma on neutrophil activities, blood was taken pre-operatively and at 6, 24, 48 and 72 h post-operatively from patients undergoing surgery and CEACAM8 was measured. As shown in figure 3, CEACAM8 increased at 24 h, reached a maximum at 48 h and tended to decline slightly at 72 h. The number of circulating neutrophils showed a very similar time course (the value at 6 hours post-operation is missing). The surface expression of CEACAM8 increased at 24 h and seemed, however, to increase further at 72 h. A possible explanation for this is that CEACAM8 might have been translocated from an intracellular pool to the plasma membranes of activated neutrophils, at 72 h. Overall there was a significant correlation between CEACAM8 in serum and the neutrophil surface expression (\(r = 0.4, p = 0.008, n = 44\)), suggesting the shedding of CEACAM8 from the cell surface.

Serum levels of CEACAM8 were measured in healthy subjects and patients with acute infections. Of 83 healthy subjects, only three showed levels of CEACAM8 above the lower limit of the assay. However, most patients with bacterial infections (15 out of 16) and influenza A (8 out of 9) showed levels of CEACAM8 above this limit. There were significant differences in the levels of CEACAM8 between healthy subjects and those with bacterial infections (\(p < 0.0001\)) or influenza A (\(p < 0.0001\)). It is characteristic that viral infections seldom bring about neutrophil involvement. Therefore, the highly elevated levels of CEACAM8 seen in patients with influenza A infections were somewhat unexpected. However, it has been reported that influenza A virus up-regulates the expression of integrins as well as CEACAM8 on neutrophils \(^{171}\). Therefore, it is probable that the release or shedding of CEACAM8 from neutrophils activated by influenza A virus may be taking place in the circulation and account for its elevated serum levels. On the other hand, the surface expression of CEACAM8 on neutrophils from infected patients and their serum levels of CEACAM8 were significantly correlated (\(r = 0.62, p < 0.0001, n = 33\)).

CEACAM8 was measured in serum and EDTA-plasma from the cord blood of neonates. In eight out of 28 children we found levels of CEACAM8 above the lower limit of the assay, a significantly higher proportion of positive
Fig. 3. Serum levels of CEACAM8, the expression of CEACAM8 on neutrophils and the number of circulating neutrophils in patients undergoing hip replacement surgery and scoliosis reconstruction surgery. Blood was taken pre-operatively and 6, 24, 48 and 72 post-operatively. Data are expressed as mean ± SD.
levels compared with our findings in healthy adults ($p = 0.0025$, Fisher’s exact test). Whether this is a reflection of a higher turnover of CEACAM8 in neonate neutrophils is unknown at present. The higher levels could also be a consequence of maternal labour. In fact, expression of several surface antigens including CEACAM8 were increased on neonate neutrophils and were found closely related to the length of labour. Serum levels of CEACAM8 were shown to be significantly correlated with plasma levels of CEACAM8 from twenty eight new born children ($r = 0.97$, $p < 0.001$), with approximately 30% higher levels in serum.

These results suggest that both serum and EDTA plasma may be used for the measurement of CEACAM8 as an indicator of granulocyte activation and turnover. The blood levels of CEACAM8 reflect well the number of circulating neutrophils and surface expression of CEACAM8 on neutrophils. Nevertheless, the possible contribution from activated eosinophils remains undefined. In preliminary studies on asthmatics with increased numbers of blood eosinophils, however, no elevations of CEACAM8 were observed, suggesting that eosinophils may not contribute to serum CEACAM8 levels.

PAPER III

Subcellular localisation and mobilisation of CEACAM8 in human neutrophils

In resting neutrophils the majority of CEACAM8 was present in the secondary granules, which were identified by the presence of human neutrophil lipocalin (HNL). A small amount of CEACAM8 was present in a light membrane fraction containing plasma membranes and secretory vesicles, identified by alkaline phosphatase and latent alkaline phosphatase, as shown in figure 4.

Stimulation of the neutrophils with PMA caused a dramatic increase in the content of CEACAM8 in the light membrane fraction, and a decrease in the secondary granules, suggesting a translocation of CEACAM8 to the plasma membranes from the intracellular pool of secondary granules. Because no quantitative assay for CEACAM8 had been previously available, the subcellular localisation of CEACAM8 in human neutrophils was not conclusive. Previous works suggested that CEACAM8 was located in secondary granules, gelatinase granules and a small amount in secretory vesicles and plasma membranes. In the present study, the distribution profile of CEAC-
CEACAM8 after subcellular fractionation showed no peak or increments of 
CEACAM8 in the fractions containing gelatinase granules. Furthermore, 
stimulation of neutrophils with fMLP, a stimulator of the easily mobilisable 
gelatinase-containing granules, induced only a small increment of CEAC-
AM8 in the light membrane fractions, while strong stimulation with PMA 
induced a much higher increment of CEACAM8 in the light membrane frac-
tion. This again indicated a translocation of CEACAM8 to the plasma mem-
branes from secondary granules. Our data suggest that the gelatinase gran-
ules are not the main intracellular pools of CEACAM8. One possible expla-
nation for the reported detection of CEACAM8 in gelatinase granules by 
immunoblotting is that there may be overlaps between different granule 
populations when these granules are fractionated on either sucrose or percoll 
gradients. This is typically the case for secondary and gelatinase granules. 
When pooling the fractions corresponding to the secondary and the gelati-
nase granules, the overlapping between both populations would result in 
CEACAM8 being detected in both secondary and gelatinase granules when 
analysing by immunoblotting.

Figure 4. Subcellular localisation of CEACAM8 in human neutrophils on a three-
layer Percoll gradient
Cellular content of CEACAM8 in neutrophils and its release

The content of CEACAM8 in neutrophils was estimated to be 82.4 ± 8.9 ng/10^6 cells (mean ± SE, n = 10). To study the release of CEACAM8, neutrophils were incubated with serum-opsonised particles G-15 at 37°C for 20 min. The amount of CEACAM8 released was 5.5 ± 0.7% (mean ± SE, n = 4) of the total content of CEACAM8. For comparison, we measured HNL in the same secreted material. The amount of HNL released was 28.6 ± 4.3% (mean ± SE, n = 4), much higher than that of CEACAM8.

The effect of G-CSF administration on the content and subcellular localisation of CEACAM8 in neutrophils

To investigate the effects of G-CSF on the cellular content of CEACAM8, neutrophils were obtained from healthy volunteers to whom G-CSF had been administrated once daily for six days. Neutrophils isolated from heparinized peripheral blood on day 0, 1, 4 and three weeks post administration of G-CSF were disrupted and the post nuclear supernatants were loaded on eight layer discontinuous sucrose density gradients. After fractionation, CEACAM8 was determined in each fraction. The content of CEACAM8 increased on day 1 and decreased on day 4 significantly compared with day 0. Besides, an increased content of CEACAM8 was obtained in the light membrane fraction on day 4.

G-CSF stimulates the proliferation and differentiation of haematopoietic progenitor cells to neutrophilic granulocytes 174,175. Highly elevated levels of endogenous G-CSF have been reported in acute bacterial infections 176, where G-CSF would be involved in the mobilisation of neutrophils from the bone marrow. In this study we observed that the administration of G-CSF resulted in dramatic changes in the content of CEACAM8 in human neutrophils in vivo and that this depended on the time elapsed after G-CSF administration. The highly increased content of CEACAM8 on day 1 after G-CSF is uncertain, but could have several causes. One could be that the amount of mRNA for CEACAM8 may be induced by G-CSF, as was shown for alkaline phosphatase both at mRNA and protein level 177. Another could be the change in blood cell populations after G-CSF administration, since there is a mobilisation of less mature cells from the bone marrow after such treatment 178,179. The decreased content of CEACAM8 on day 4 after administration of G-CSF is due to the reduced marrow transit time 180 and also to the secretion of these proteins from activated cells in the circulation, since the release from secondary granules was enhanced at day 4 during G-CSF administration.
The in vitro effects of G-CSF and TNF-α on CEACAM8 expression on neutrophils

To explain the fact that the content of CEACAM8 was decreased on day 4 compared with day 1 after administration of G-CSF, whereas the content of CEACAM8 was increased in the light membrane fraction at day 4, we investigated the in vitro effects of G-CSF and TNF-α on CEACAM8 expression on neutrophils. We incubated isolated granulocytes from normal healthy donors with increasing doses of G-CSF and TNF-α, for 45 min, at room temperature. The expression of CEACAM8 on neutrophil surfaces was then analysed by flow cytometry. G-CSF increased the expression of CEACAM8 by about 5% (p = 0.03, n = 6), whereas TNF-α increased it by almost 50% (p < 0.001, n = 4). The increased content of CEACAM8 in the light membrane fraction on day 4 is therefore not likely to be a direct consequence of the action of G-CSF, since G-CSF only marginally induced translocation of CEACAM8 to the plasma membrane in vitro. In earlier work, we showed that G-CSF administration caused a dramatic increase in cytokine levels inducing TNF-α \(^{181}\). It was therefore of interest that TNF-α potently increased CEACAM8 expression on neutrophil surfaces, in vitro. Our conclusion is therefore that cytokines such as TNF-α are responsible for this effect of G-CSF after in vitro administration.

PAPER IV

Subcellular localisation of CEACAM8 in human eosinophils

In order to establish the subcellular localisation of CEACAM8 in human eosinophils, disrupted eosinophils were fractionated on an eight layersucrose density gradient. The content of CEACAM8 in each fraction, as well as that of marker proteins, was measured by either RIA or enzymatic assays. The majority of CEACAM8 (≥95%) was found localised in plasma membrane fractions (sucrose density 1.095 – 1.135) as identified by alkaline phosphodiesterase 1 activity and dot blot assay of CD9 (not shown). A minor proportion of CEACAM8 was localised in secretory granule fractions (sucrose densities 1.267 – 1.280) as identified by the content of ECP.
Since the majority of CEACAM8 was localised to the light region of the sucrose gradient and since the resolution of the gradient in this region does not allow a distinction between plasma membrane-associated proteins and proteins localised in light density organelles such as vesicles, we used a floatation Percoll gradient to resolve intracellular organelles from plasma membranes, in the light density region. As shown in figure 5, the floatation Percoll gradient separated the CEACAM8 into three peaks. About 21% of CEACAM8 was present in the plasma membranes, and more than 70% of CEACAM8 was present in a broad vesicle peak identified by the marker protein albumin, while a small amount of CEACAM8 (6%) was present in secretory granules, as identified by ECP. These results indicate that CEACAM8 in eosinophils is mainly stored in intracellular vesicles of low density.

Figure 5. Subcellular localisation of CEACAM8 in human eosinophils, on a floatation Percoll gradient
Cellular content and release of CEACAM8

The content of CEACAM8 in eosinophils was determined to be 73.83 ± 6.03 ng/10⁶ cells (Mean ± SE, n = 7), similarly to neutrophils. Thus, CEACAM8 is a minor protein in human eosinophils. In order to study the release of CEACAM8 from eosinophils, cells were stimulated with serum-treated Sephadex G-15 particles. After removing the cells and Sephadex particles by centrifugation, the supernatants were used to estimate the release of CEACAM8 from activated eosinophils. The amount of CEACAM8 released from eosinophils was 5.07 ± 1.06% (Mean ± SE, n = 7) of the total cellular content, a similar amount to that released from neutrophils. For comparison, we measured ECP in the same secreted material. The amount of ECP released was 8.90 ± 1.27% (Mean ± SE, n = 6), which is significantly higher than that of CEACAM8.

CEACAM8 in human eosinophils is mainly stored in vesicles. The cellular content of CEACAM8 in eosinophils is similar to that in neutrophils, and upon stimulation eosinophils release a similar amount of CEACAM8 as neutrophils. The function of CEACAM8 in eosinophils in vivo remain to be determined. As a member of the family of carcinoembryonic antigen (CEA), it may play a role in interactions between eosinophils or between eosinophils and epithelial cells, and play a role as a modulator by binding ligands in vivo.
Concluding remarks

This thesis presents data on CEACAM8 in human granulocytes. CEACAM8 is a highly glycosylated membrane-associated protein with a molecular weight of 95 kDa. It is a member of the CEA family and a product of the CGM6 (NCA-W272) gene. CEACAM8 is expressed only in cells of the granulocyte-lineage and it is a minor protein in granulocytes. CEACAM8 is mainly stored in the secondary granules of human neutrophils and in intracellular vesicles of human eosinophils. Upon cell activation, CEACAM8 can be translocated to the plasma membrane and released extracellularly to some extent from the storage pools within granulocytes. As a member of CEA family, CEACAM8 might play a role in the interaction between granulocytes or between granulocytes and epithelial cells expressing CEACAM6, or may be involved in signalling events in granulocytes. However, further extensive investigations are needed to elucidate the precise function of CEACAM8 in vivo. Firstly, it is important to find the natural ligands of CEACAM8. The availability of native CEACAM8 through the procedures presented here will make possible further structural and functional studies of CEACAM8, particularly with those related with the possible role of its carbohydrate chains. CEACAM8 has been shown to bind the lectin galectin-3. The fact that no binding of galectin-3 to purified CEACAM8 was found in this work either by immunoblotting, ELISA or gel filtration chromatography, suggests that the binding between CEACAM8 and galectin-3 may need a cofactor still to be identified. Alternatively, a conformational change of CEACAM8 brought about during the purification procedure may account for the lack of binding to galectin-3.

The clinical data obtained show that serum CEACAM8 is a good marker for inflammation, though its measurement can not distinguish between bacterial and viral diseases. However, the determination of serum levels of CEACAM8 could be useful for the diagnosis of diseases involving both neutrophils and eosinophils such as inflammatory bowel disease. In general, CEACAM8 could serve as a biological marker for granulocyte activation in vivo.
Summary

- CEACAM8 was purified from normal human granulocytes.
- It was confirmed at the protein level that the CEACAM8 is a product of the CGM6(NCA–W272) gene.
- An accurate, specific and reproducible ELISA for CEACAM8 was established.
- Clinical data suggest that CEACAM8 could serve as a biological marker for granulocyte activity in vivo.
- The cellular contents of CEACAM8 was estimated to be 82.4 ng/10^6 neutrophils and 73.8 ng/10^6 eosinophils, and the amounts of CEACAM8 released upon cell stimulation were 5.5% and 5.1% of total cellular contents for neutrophils and eosinophils, respectively.
- CEACAM8 is mainly present in the secondary granules, with lower amounts in the plasma membrane, in resting neutrophils. Upon activation, it was translocated from the secondary granules to the plasma membranes. In resting eosinophils, CEACAM8 is mainly present in low density vesicles, with lower amounts in the plasma membrane and secretory granules.
- The translocation of CEACAM8 observed in vivo in neutrophils after administration of G-CSF is probably secondary to its effects, and probably caused by other cytokines such as TNF-α.
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References


114. in V, Grootendorst DC, Bel EH et al. CD11b and L-selectin expression on eosinophils and neutrophils in blood and induced sputum of patients with asthma compared with normal subjects. Clin Exp Allergy. 1998;28:606-615.


151. Kuroki M, Matsuo Y, Kinugasa T, Matsuoka Y. Augmented expression and release of nonspecific cross-reacting antigens (NCAs), members of the


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## ERRATA

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