

STRUCTURAL AND FUNCTIONAL ANALYSES OF CEACAM1

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In memory to my aunt Elena and to my father-in-law Manfred

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Abbreviations

APC	Gene product of adenomatous polyposis coli gene
b FGF	basic Fibroblast Growth Factor
BNG	Blue Native Gel electrophoresis
BSA	Bovine Serum Albumin
C1N3	broad specific mouse anti-CEACAM monoclonal antibody
C1P83	mouse anti-CEA monoclonal antibody
CEA	Carcinoembryonic antigen
CEACAM1	CEA-related protein 1
DAB	Diaminobenzidine
DMSO	Dimethyl Sulfoxid
DNA	Desoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EtSH	β -mercaptoethanol
FACS	Flow Cytometry
FCS	Fetal Calf Serum
HRP	Horse radish Peroxidase
IEF	Isoelectric Focusing
IFNs	Interferons
IgG/IgM	Immunoglobulin G/M
IGF	Insulin-like Growth Factor
ILs	Interleukins
LPSs	bacterial Lipopolysacharides
mAb	monoclonal Antibody
NaF	Sodium Fluoride
OD	Optical Density
PBS	Phosphate Buffered Saline
PBS-T/TBS-T	Buffer with Tween 20
PVDF	Polyvinyl difluoride
PI	Propidium Iodide
PTK	Protein Tyrosine Kinase
RNA	Ribonucleic Acid

RT	Room Temperature
SDS	Sodiumdodecyl Sulfate
TBS	Tris Buffered Saline
TGFs	Transforming Growth Factors
TNFs	Tumor Necrosis Factors
VEGF	Vascular Endothelial Growth Factor

All other abbreviations are explained directly in the text.

1 Introduction

The very high prevalence of colon cancer in the Western countries, where nearly 50% of population develops a colorectal tumor by the age of 70, defined this disease as an important public health issue (Parker et al., 1996).

1.1 Molecular basis of colorectal cancer

The generally accepted theory of a genetic model of colorectal cancer etiology termed “the multistep progression pathway” was for the first time described by Fearon and Vogelstein two decades ago (1990). The theory collected knowledge about specific gene alterations appearing frequently during colorectal tumorigenesis.

Alterations in gene expressions are required to convert a normal cell into a malignant one. It was suggested that cancer develops from normal colorectal mucosa by a series of transitions, beginning as adenomas, some of which become progressively more dysplastic as foci of carcinoma appear within the neoplasm. Mutations of proto-oncogenes confer properties including uncontrolled proliferation, loss of intracellular adhesion, expression of enzymes to digest basement of membrane, enhanced cell mobility, and finally a capacity to survive in remote locations. Accumulation of mutations rather than their order of appearance seems to direct cancer behavior (Kinzler and Vogelstein, 1996).

Single gene alterations described in the multistep progression pathway in human colorectal neoplasia are mentioned below (Fig.1).

Normal epithelium lining lumen of colon is characterized by rapid cell turnover in colonic crypts. The highly proliferating cells are located in lower third of the crypts, while cells already moved in upper parts of crypts are mature, differentiated without ability to divide. Cells on the top of crypts are under physiological conditions undergoing apoptosis.

In adenoma, proliferative cells move up to the top of crypts, continue to divide and escape from apoptosis.

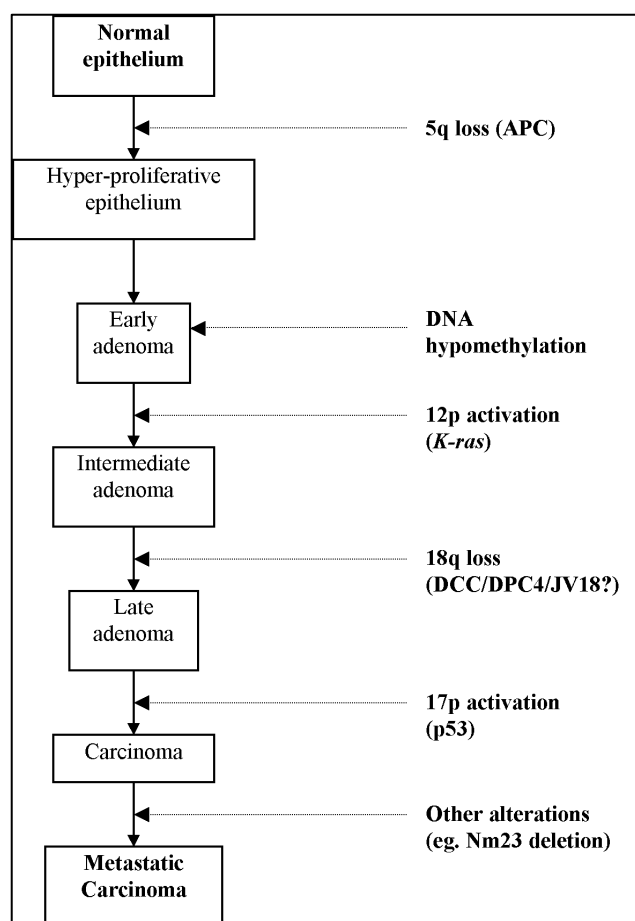


Figure 1: A genetic model for colorectal tumorigenesis. Modified from Kinzler and Vogelstein (1996).

1.1.1 Adenomatous polyposis coli (APC)

The initial mutations occurring in the early development of sporadic colon cancer and in hereditary Familial Adenomatous polyposis (FAP) is targeting the APC gene. Such mutations on chromosome 5q21 (Bodmer et al., 1987) cause C-terminal truncation of the protein product (Kinzler, and Vogelstein, 1996). *APC* is acting as a “gatekeeper” gene responsible for maintaining a balance between cell death and cell division (Kinzler and Vogelstein, 1998). Truncated APC protein is unable to carry out its growth controlling function. Additionally, APC is known to associate with β -catenin (Rubinfeld et al., 1993). This interaction mediates involvement of APC in cell adhesion processes, where cytoplasmic catenins binds to cadherins, a family of calcium-dependent homophilic cell adhesion molecules (Kemler et al., 1993). Finally, APC tumor suppressive effect lies in its ability to destabilize free β -catenin (von Kries et al., 2000). Consequently, APC antagonizes transcriptional activity of β -catenin required for tumorigenesis (Korinek et al., 1998).

1.1.2 Ras oncogene

Mutations within the *ras* family of oncogenes are found with equal frequency in both sporadic and hereditary colorectal tumors, suggesting that they may be an important factor in colorectal tumor progression (Farr *et al.*, 1988). The normal cellular homologues of *ras* oncogenes are G-proteins involved in the transduction of mitogenic signals across the cell membrane (Barbacid, M., 1987). Point mutations of the Kirsten-*ras* (K-*ras*) oncogene have been identified in up to 60% of colorectal carcinomas (Breivik *et al.*, 1994) as well as 42% of adenomas (Bos *et al.*, 1987), and are thought to occur primarily prior to malignant transformation (Vogelstein *et al.*, 1988). The high frequency of *ras* mutations in tumors at a relatively early stage in progression has led to the recent development of tests for detecting K-*ras* mutations in the stool of patients at risk for colorectal cancer (Sidransky *et al.*, 1992; Villa *et al.*, 1996; Nollau *et al.*, 1996).

1.1.2.1 The mitogenic Ras pathway

The *ras* proto-oncogene codes for a 21-kDa guanine-nucleotide binding protein (G protein), which adhere to the inner face of the cell membrane and its activity is dependent on GTP/GDP hydrolysis cycle. In its inactive state, Ras binds GDP. But after the activation of a receptor with which it associates, the protein exchanges GDP for GTP and becomes active, using its effector domain to initiate an intracellular signal cascade. In response to other signals, hydrolysis of GTP inactivates Ras and completes the Ras cycle.

Growth factors (GF)-stimulated mitogenic Ras pathway mediates effect on the cell proliferation in a part by the activation of kinases cascade: Raf, MEK, and ERK1/2 (Egan *et al.*, 1993) (Fig. 2).

Tyrosine kinase receptors (receptors for insulin, EGF, PDGF, and NGF) undergo ligand-induced autophosphorylation leading to the recruitment of the Grb2 (growth-factor-receptor-bound protein 2) adaptor protein and its associated Ras exchange factor “son of seven less” (SOS) (McCormick, 1993). Some receptors do not display phosphotyrosine, therefore another adaptor called Shc is needed to activate Ras. Subsequently, Ras binding to the serine/threonine kinase Raf-1 and their membrane translocation leads to phosphorylation of MEK kinase, which mediates further phosphorylation of ERK kinases (for review see Schlessinger and Bar-Sagi, 1994; White *et al.*, 1995). Upon activation, ERK kinases

phosphorylate the cytoplasmic targets and they are translocated into the nucleus, where the activity of different transcription factors is stimulated. One of these factors is Elk-1.

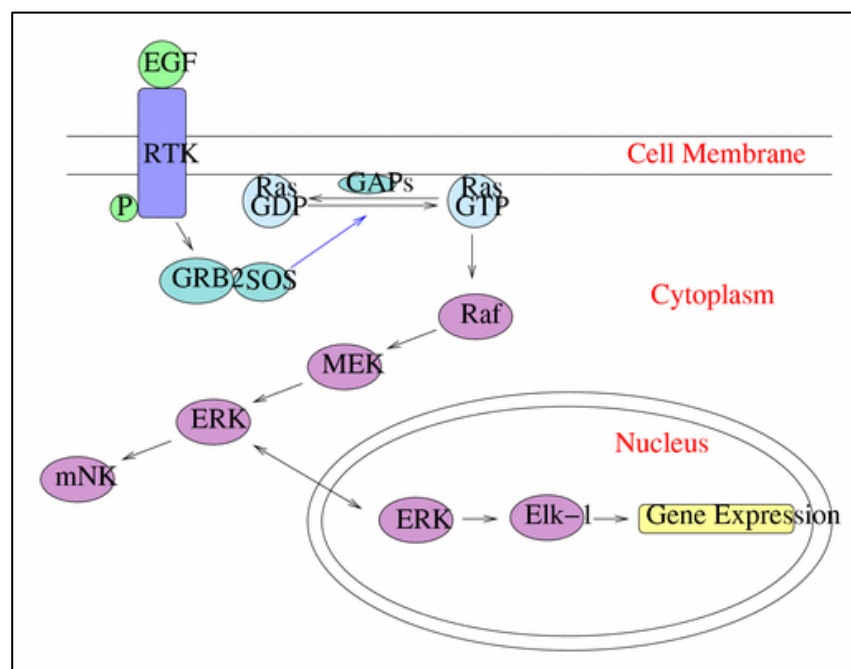


Figure 2: The Ras mitogenic pathway (from Vojtek et al., 1998)

Ras is a GTP/GDP regulated binary switch that resides at the inner surface of the plasma membrane and acts to relay extracellular ligand-stimulated signals to cytoplasmic cascades. A linear pathway where Ras functions downstream of receptor tyrosine kinases

(RTK) and upstream of a cascade of serine/threonine kinases (Raf-MEK-ERK) provides a complete link between cell surface and nucleus. Activated ERKs translocate into nucleus to phosphorylate and activate transcription factors, such as Elk-1 (Vojtek and Channing, 1998).

In about 25% of human cancers, mutated Ras proteins are able to mediate mitogenic signals to nuclei without external stimulation (Medema et al., 1993).

1.1.3 Tumor suppressor p53

The *p53* gene is a tumor suppressor and the most commonly mutated gene in human cancer (Kirsch and Kastan, 1998). The gene is localized on the short arm of chromosome 17 and the presence of *p53* mutations in patients suffering from tumor disease indicates poor prognosis and shorter survival (Kressner et al., 1999). Up to 75% of sporadic colorectal tumors exhibit *p53* inactivation (Vogelstein et al., 1988).

p53 is named also “guardian of the genome” because of its broad spectrum of functions including cell cycle control, DNA repair and apoptosis. Mutant p53 protein appears to be

over-expressed in tumors, where it has an extended half-life of 24 h in comparison to the wild-type protein, which has a half-life only 20 min (Rodrigues et al., 1990).

1.2 Introduction to CEACAM family

In the middle of the 70-ies Gold and Freedman (1965) have drawn the attention of the scientific community on the Carcinoembryonic Antigen (CEA), which was found to be expressed in colon cancer cells. The presence of CEA in extracts from colon carcinoma tissue suggested a specific tumor-associated function of CEA. They hypothesized that expression of CEA correlates with malignancy (Gold and Freedman, 1965). Today, the CEA family has been characterized and CEA is a widely accepted tumor marker for colorectal and other cancers.

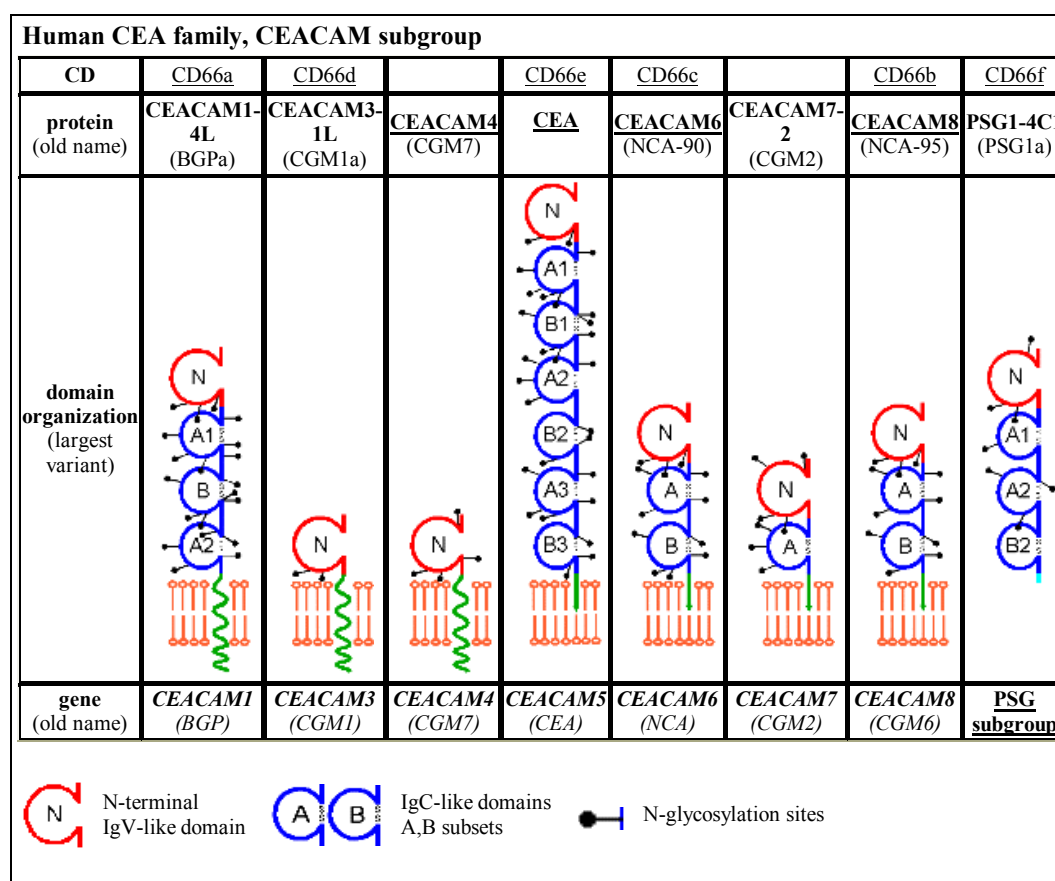


Figure 3: Schematic model of CEA family members nomenclature (from Beauchemin et al., 1999).

The human *CEA* gene family includes 29 different genes/pseudogenes (Olsen et al., 1994). According to the sequence homology, the group was divided into two major subgroups: (1) the CEACAMs and (2) the pregnancy-specific glycoprotein (PSG) subgroup. The CEACAM-proteins include CEA, the biliary glycoprotein (CEACAM1), non-specific cross-reacting antigen (CEACAM6) and *CEA* gene members (CEACAM3, 4, 7, 8) (Obrink, 1997; Beauchemin et al., 1999) (Fig 3).

CEACAM family proteins belong to immunoglobulin family of adhesion molecules, which differ in their posttranslational modifications and their splice variants of their genes (Beauchemin et al., 1999).

The CEA and PSG subgroups can be distinguish from each other at the C-terminus. The CEA subgroup members are attached to the cell membrane while PSGs are secreted. CEACAMs 1, 3 and 4 are anchored to the membrane with hydrophobic transmembrane region (TM) followed by short or long cytoplasmic domains, whereas CEACAMs 5, 6, 8 and 7 are anchored via a glycosyl phosphatidyl inositol (GPI) moiety into the cell membrane (Hammarström, 1999). GPI-anchored proteins usually have their final destination in the apical membrane and are excluded from basolateral membranes of polarized epithelial cells (Rodriguez-Boulau and Nelson, 1989). Proteins with a *bona fide* TM domain are targeted to basolateral surfaces by virtue of signals in their cytoplasmic domains. In the first case (GPI proteins), compartmentalization within the lipid bilayer is not result of protein-protein interaction (like it is true for TM proteins), but preferential miscibility of the glycolipid moiety of the GPI anchor with sphingolipids and cholesterol (Sceaton et al., 2000). As described above, CEACAMs belong to the immunoglobulin superfamily. They share two types of immunoglobulin domains: the N-terminal domain of 108 amino acids homologous to the Immunoglobulin (Ig) variable domain (IgV-like domain) and no or up to six domains homologous to the Ig constant domain of the C2 set (IgC2-like) (Williams et al., 1988). IgV-like N domain lacks intra-chain disulphide bridges. An important structural feature of the CEACAM proteins is their profound N-glycosylation on asparagine residues, where carbohydrate can represent up to 50% of the total mass (Kannicht et al., 1999).

1.3 Physiological functions of CEACAM family members

1.3.1 Role of CEACAM members in innate immunity

CEA is expressed by normal colon epithelial cells, where it is localized on the apical surface. In healthy individuals CEA is eluted to the colon lumen and is detectable at a very low concentration in the blood. CEA and CEACAM6, 7 play an important role in the innate immune defense protecting normal colon cells from microbial attack (Hammarström, 1999), CEACAM were previously shown to act as mediating receptors interacting with Opa (opacity associated protein) and thereby facilitating adhesion of pathogenic *Neisseria* species. This interaction is followed by opsonisation-independent phagocytosis of *Neisseria gonorrhoeae* by human granulocytes and each receptor individually can mediate gonococcal invasion of epithelial cells (Billker et al., 2002). Human pathogenic gonococcal and meningococcal strains associations with polarized epithelial cells *in vitro* induce CEACAM1 expression (Muenzner et al., 2000). *Neisseria gonorrhoeae*-triggered CEACAM1 up-regulation involves activation of the transcription nuclear factor NF- κ B. The ability of microorganisms to up-regulate its epithelial receptor CEACAM1 through NF- κ B suggest an important mechanism allowing efficient bacterial colonization during the initial steps of the infection process (Muenzner et al., 2002). Gonococcal infection activated a NF- κ B heterodimer consisting of p50 and p65, and inhibitors that regulate the nuclear translocation of activated NF- κ B complex inhibited CEACAM1 transcript expression (Muenzner et al., 2001).

1.3.2 Regulation of CEACAM family members expression by cytokines

1.3.2.1 Interferon gamma up-regulates CEA and CEACAM1 expression *in vitro*

Interferon gamma (IFN- γ) is a glycoprotein with a molecular weight of 17 kDa (Gray et al., 1982). Under physiological conditions, expression of IFN- γ is routinely observed after the activation of two major cell types, T lymphocytes and large granular lymphocytes (Young and Hardy, 1990). IFN- γ was reported to increase the expression of tumor-associated antigens (Giacomini et al., 1990; Kantor et al., 1989) resulting in increase in anti-tumor antibody

localization in tumors and tumor growth suppression (Gansbacher et al., 1990). Recently, it was shown that IFN- γ enhanced the sensitivity of tumor cell lysis for LAK cells by the induction of up-regulation of tumor cell adhesion molecules (Fady et al., 1992). In addition, IFN- γ is able to enhance the expression of TNF receptors on tumor cells (Tsujimoto et al., 1986). IFN- γ became interesting for treatment of human solid tumors (Welander et al., 1988; Malik et al., 1991; Ernstoff et al., 1992). However, the efficiency of treatment is strongly dose-dependent and effective concentration of IFN- γ is usually accompanied with toxicity manifestation (Quesada et al., 1988; Kurzrock et al., 1985).

Yan et al. (1992) were the first to observe effect of IFN- γ on the carcinoembryonic antigen expression of human colon cancer cells. Three different colon adenocarcinoma cell lines were sensitive for treatment with up to 1.000 U/ml of IFN- γ and cells were expressing CEA with three to five-fold enhancements during 5-6 days of continuous exposure (Yan et al., 1992). CEA, CEACAM1 and CEACAM6, members of CEACAM family were reported to be induced by IFN- γ treatment on the protein as well as on mRNA level. The difference in the kinetics of induction and effect of cycloheximide (protein synthesis inhibitor) on CEA and CEACAM1 mRNA levels suggested that two genes are regulated differently in colon HT29 cancer cells (Takahashi et al., 1993). Consequently, the same authors reported on the existence of an interferon-sensitive response element (ISRE) inside the CEACAM1 promoter and it was protected in *in vivo* footprints (Chen et al., 1996). Additionally, Fahlgren et al. (2003) had observed that IFN- γ is able to directly up-regulate CEACAM1 expression in colon epithelial cells and this is accompanied by a promotion of the cell differentiation resulting in indirect stimulation of CEA and CEACAM6 surface expression.

Anti-cancer therapy based on targeting of anti-CEA specific antibody requires appropriate cell surface expression of tumor marker, therefore cytokine induction of CEA expression by colon cancer cells is investigated. The moderately differentiated colorectal tumor cell line HT29-19A was shown to have enhanced CEA expression after treatment with combination of cytokines (IFN- γ , IFN- α and IL-6) in comparison to CaCo2, a more differentiated colon carcinoma cell line (Verhaar et al., 1999).

1.3.2.2 Effect of other cytokines on CEACAM family members

Induction of CEACAM1 by TNF- α is also shown to play a role in an invasive meningococcal disease and viral infection, if these infections have triggered the expression of CEACAM1 on

tissue where it is not normally present. TNF- α induced CEACAM1 expression by endothelial cells allows meningococcal adherence to vascular walls, and its exit from the bloodstream (Muenzner et al., 2000). The influence of CEACAM1 expression by different cytokines and transcription factor NF- κ B in the cause of *Neisseria* infection is not the only immunological based defense mechanism of CEACAM1 actions in the epithelial cells and granulocytes.

1.3.3 CEACAM family members and signal transduction

1.3.3.1 CEA involvement in signaling pathways mediated by protein kinase C

CEACAM1 is not the only member of the CEA family, which is involved in signal transduction. There is strong evidence that, CEA, despite of the lack of a cytoplasmic domain and possible phosphorylation sites interacts with protein kinase C (PKC). More than a decade ago, Chakrabarty and co-workers reported relationships between CEA expression and secretion and level of TGF- β 1 (Chakrabarty et al., 1988, 1989, 1990). In colon carcinoma cellular expression of CEA rapidly increased after treatment with TGF- β 1 and was abolished by Calphostin C (inhibitor of PKC) in a dose-dependent manner. Consequently, it was concluded that TGF- β 1 regulates the CEA responses through a signal transduction pathway associated with PKC (Chakrabarty, S., 1992). TGF- β 1 regulates proliferation and differentiation in various cell types including a suppressive effect on epithelial tumorigenesis (Sun at al., 1994). TGF- β 1 action is mediated via different cell surface receptors and extracellular matrix adhesion molecules, like CEA, fibronectin and laminin. Multiple TGF- β 1 signal transduction pathways exist (compare Fig. 4).

As PKC α inhibition aborts a late downstream event (induction of the CEA secretion) (Chakrabarty and Huang, 1995), it was hypothesized that PKC α is an upstream focal point controlling the induction of CEA by TGF- β 1.

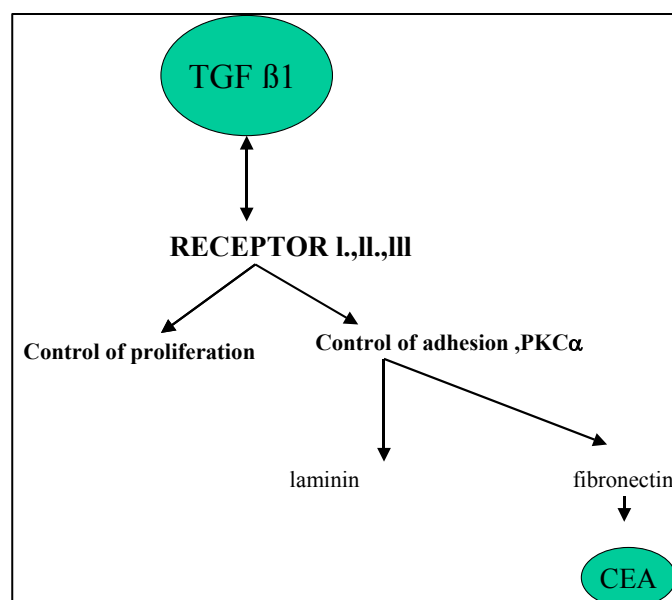


Figure 4: Schematic representation of hypothetical TGF- β 1 postreceptor signaling (Chakrabarty et al., 1998).

Another evidence for PKC being involved in the regulation of CEA expression is the enhancing effect of Staurosporine (ST, Aquino et al., 2000). ST is a microbial product and potent inhibitor of PKC (Tamaoki et al., 1986). This inhibitor is able to increase substantially the expression of CEA and reduce cell growth in a colon cancer cell lines which express marginal levels of CEA. The mechanism of ST action is not yet understood, but it is reasonable that ST effects on CEA and PKC and TGF- β 1-mediated up-regulation of adhesion molecules, such as CEA differ (Aquino et al., 2000).

1.3.3.2 Regulation of CEACAM1 phosphorylation state via associations inside its ITAM/ITIM motif

As described above, CEA family members differ in their structural features, at least, in respect to interactions within cell membrane components. One group is anchored via the phosphatidyl-inositol moiety (CEA, CEACAM6 and 8), others have a transmembrane region followed by a cytoplasmic domain (CEACAM1). CEACAM 1 participates in bile acid transport (Sippel et al., 1996), regulation of insulin receptor internalization (Formisano et al., 1995), and inhibition of tumor cell growth *in vivo* (Kunath et al., 1995). However, not all alternatively spliced isoforms of CEACAM1 are involved in the same signal transduction pathway. CEACAM1 with longer cytoplasmic domain contains a motif related to that of Immunoreceptor Tyrosine-based Activation (or Inhibition) motif (ITAM/ITIM).

ITAM/ITIM protein modules represent cytoplasmic subunits of the tyrosine kinase receptors, mediating interactions with other intracellular proteins in a phosphorylation dependent manner.

The cytoplasmic domain of the longer CEACAM1 splicing variant contains two tyrosine residues within ITAM. Both tyrosine's can be phosphorylated. Phosphorylation of ITAM/ITIM sequences that follow receptor-ligand interaction is likely to be induced by src-family PTK (Isakov, N., 1997). CEACAM1-L has been shown to be phosphorylated on tyrosine residues in its ITIM motif by src-kinases (Afar et al., 1992; Brümmer et al., 1995). Activation-dependent tyrosine phosphorylation of the receptor subunits creates temporary anchors for SH2-containing signaling proteins (Isakov, N., 1997). It has been proven that SHP-1, cytosolic protein tyrosine phosphatase containing two SH2 domains associates with CEACAM1, where phosphotyrosines 488 and 515 were required (Beauchemin et al., 1997). Changes in localization of src-kinases and their activity as well as changes in activity of SHP-1, SHP-2 phosphatases in response to CEACAM1 phosphorylation status suggested role of CEACAM1 in regulation of the tumor cell proliferation (Chen et al., 2001). CEACAM1-L can be phosphorylated *in vitro* on its tyrosine residues as well as on its serine and threonine residues (Afar et al., 1992; Culic et al., 1992).

1.3.3.3 Role of CEACAM1 in insulin action

In some cases the presence of a single phosphotyrosine is necessary for the ability to enter signal transduction pathways. Tyrosine 488 was convincingly shown to undergo phosphorylation in cells treated with insulin or protein tyrosine phosphatase inhibitor vanadate (Najjar et al., 1995).

The insulin receptor is a cell surface glycoprotein that consists of two α - and two β -subunits (Lee et al., 1994). The extracellular α -subunits are responsible for insulin binding, and the transmembrane β -subunits contain the tyrosine kinase domain. The activated insulin receptor can phosphorylate CEACAM1 (Najjar et al., 1995), adaptor protein Shc and others (White, M.F. and Kahn, C.R., 1994). Insulin and insulin-like growth factor-1 (IGF-1) receptors are structurally related and share many substrates, such as Shc (White et al., 1988; Gustafson et al., 1995). However, CEACAM1 undergoes ligand-stimulated phosphorylation by insulin receptor, but not by the IGF-1 receptor. This differential phosphorylation is regulated by a single amino acid (Tyrosine 1316) in the C-terminus of β -subunit of insulin receptor.

Phosphorylation of CEACAM1 by the insulin receptor appears to be essential and sufficient to mediate down-regulatory effect on the mitogenic action of insulin (Soni et al., 2000).

The adaptor protein Shc is a src homology 2 (SH2)-containing protein that undergoes phosphorylation by receptors of tyrosine kinase family (Kavanaugh et al., 1994). Activation of receptor caused redistribution of Shc from the perinuclear region to the cytosolic site of plasma membrane (Lotti et al., 1996). By binding to other signaling proteins, Shc exerts many effects on cells. Upon phosphorylation on its Tyrosine 317, Shc binds to the SH2 domain of Grb2 and couples it to receptor (Wolf et al., 1995). This leads to the association of Grb2 with the SOS causing translocation of p21^{ras} in proximity to the plasma membrane (Gale et al., 1993). Thus activation of the Ras/mitogen-activated protein kinase (MAP kinase) pathway influences the cell growth and proliferation. Poy et al. (2002) had reported that phosphorylated tyrosine 488 in CEACAM1 is required for its binding to the SH2 domain of Shc. CEACAM1 sequesters Shc, this major coupler of Grb2 to the insulin receptor and down regulates the Ras/MAP kinase mitogenic pathway (Poy et al., 2002).

1.3.4 CEACAM family members and cytoskeleton organization

CEACAM members are “capping” cells by building a glycoprotein network in normal colonic tissue, called the luminal glycocalyx (Frängsmyr et al., 1995). This special surface structure made up from homo-/heterophilic cross-linked CEACAMs can serve as a defense wall, which protects cells against entrance of different pathologic agents (Leusch et al., 1991; Chen et al., 1997; Hammarström, S., 1999) and facilitates recognition of the invasion agents (Ottonello et al., 1999). The glycocalyx represents more than only a mechanic barrier, but some protein components own properties of active signaling molecules. Cooperation between inside of the cell and CEACAMs are involved in interactions with cytoskeleton proteins by both, direct (Schumann et al., 2001) and/or indirect ways (Sadekova et al., 2000). Links between plasma-membrane-associated proteins and cytoskeletal elements determine cell shape, ensuring cell motility and facilitate cell-cell or cell-substratum adhesion. CEACAM1-L was suspected to interact with several cytoskeletal proteins and its usual localization at the cell-cell boundaries changed after treatment with cytochalasin D (in transfected Swiss 3T3 fibroblasts), present evidence for the existence of such a connection. Cytochalasin D is known as a filamentous actin disrupting agent. Small GTP-ases (Rho, Rac, Cdc42) cytoskeletal organisers (Braga et al., 1997; Kroschewski et al., 1999) are required for CEACAM1-L in the cell-cell contact sites. Rho GTP-ase plays a crucial role in control of cellular adhesion by directing adhesion

molecules to their final destination (Sadekova et al., 2000). The exact mechanism of regulation of the CEACAM1-L trafficking by Rho GTP-ases and their influence on actin-CEACAM1-L interaction has still to be established. Actin co-localization required treatment with pervanadate, suggesting tyrosine phosphorylation events to be necessary. It has been shown that truncated CEACAM1 isoform may be a site for actin polymerization, as it binds G-actin and continues to bind nascent F-actin during filament formation (Schuman et al., 2001).

1.4 CEACAM family members in malignancy

1.4.1 CEA-tumor-associated antigen

The stimulation of organisms to anti-tumor immune response required identification of tumor specific antigens serving as immunogenes. Most known antigens so far identified in human cancers are characterized as tumor-associated antigens (TAA), and are not tumor specific. Instead, they are over-expressed in malignant cells as opposed to cells of normal tissue. There is evidence for cytotoxic T lymphocyte (CTL) response to CEA derived 9-mer CAP1 peptide (Tsang et al., 1995; Nair et al., 1998; Marshall et al., 1999). By modification of 9-mer epitope CAP1 agonistic peptide, CTL switch on dramatically production of the inflammatory associated cytokines (GM-CSF, IFN- γ and TNF- α). These molecules accompanied by cytolytic activity of CTL producing them, are efficient against CEA-positive tumors in a HLA A2- restricted fashion (Salazar et al., 2000). Another possibility to generate tumor-specific antagonists is provided by genetic engineering methods. Hoffmann and co-workers (2001) published interesting results from investigations of the fusion proteins consisting anti-CEA single chain Fv fragment T 84.66 and murine B 7.1. This fusion protein was capable of enhancing the opsonisation of CEA-expressing tumor cells, thus providing the co-stimulation signals necessary to activate a T-cell response (Hoffmann et al., 2001).

1.4.2 Relations between expression of CEACAMs and malignancy

Upon malignant transformation, colon epithelial cells usually lose their polarity and express CEA on the entire cell surface. It is known that components from plasma membrane are continually exfoliated from the surface as plasma membrane-derived vesicles (Taylor and

Black, 1985), which through draining lymph and blood vessels can end up in the blood. CEA blood levels correlates with the tumor growth. The normal value of CEA in blood of healthy patients is usually lower than 2 ng/ml. Because of this high sensitivity CEA is used as diagnostic tumor marker for colorectal and other cancers, and CEA also serves as a prognostic factor for patients after the surgical treatment.

Zimmer and Thomas (2001) reported that mutation in CEA gene in colorectal cancer patients within the PELPK sequence which is responsible for hepatic clearance of CEA. This mutation may affect structural stability and binding affinity to the Chuffer cell receptor in the liver. It is quite probable that this fact may play a key role in manifestation of hepatic metastases (Zimmer and Thomas, 2001).

CEACAM1 protein as mentioned above has adhesive properties and is also involved in signal transduction. Niemeyer and co-workers (1993) have shown strong down-regulation of CEACAM1 mRNA in 80% of colorectal carcinomas. Both, truncated (S) and long (L) isoforms are needed for the proper cell surface architecture. The cell surface interactions between isoforms seem to be an important regulating factor for the initiation and the progression of malignant disease. Variation in the S/L ratio influences cell proliferation by regulation p27^{KIP 1} levels and DNA synthesis (Singer et al., 2000). CEACAM1-L is known to be responsible for inhibition of the tumor cell growth. A single point mutation in tyrosine 488 is sufficient to convert the inhibitory effect of the wild type protein to that of a dominant tumor developing phenotype. The cellular growth control by CEACAM1 (Uzi et al., 1999) in respect to the need of tyrosine phosphorylation site in the cytoplasmic domain of CEACAM1-L and its relationships with cytoskeleton compartments as well as the small GTP-ases responsible for modulation of cell morphology, differentiation and mobility has been proposed (Hall, A., 1998).

Down-regulation of CEACAM1 in prostate cancer has been shown to be associated with loss of the cell polarity. Consequently, the proliferative activity of prostate cancer cells was increased four-fold and non-polarized cells in Gleason grade 4 (Busch et al., 2002).

This et al. (2002) were observing the correlation between CEACAM1 expression in primary melanoma and the risk of metastasis. They summarized that the strongest CEACAM1 expression was at the invading front and predictive value of CEACAM1 expression was superior to that occurred of tumor thickness.

Other CEACAM molecules, which have altered expression patterns during tumorigenesis, are CEACAM6 and CEACAM7. Both CEACAMs are co-expressed in highly differentiated epithelial cells and become oppositely deregulated in hyper-proliferating mucosa, adenomas

and carcinomas. Whereas CEACAM7 is completely down-regulated, CEACAM6 is up-regulated, with a broader expression zone at the top of the hyper-proliferating polyps or adenomas, where the polarity of its expression is often lost (Schölzel et al., 2000). Clinical trials with patients after treatment with adjuvant fluorouracil-based chemotherapy with CEACAM-specific monoclonal antibodies, showed enhanced tissue expression of CEA, CEACAM1 and CEACAM6. Interestingly, CEACAM6 was suggested to be an independent prognostic factor for patient with colorectal cancer (Jantscheff et al., 2003).

1.4.3 CEACAM1 involvement in apoptosis

The long isoform of CEACAM1 (CEACAM1-L) is particularly abundant in the intestinal and colonic epithelium, where its expression seems to be specifically located at the apical plasma membrane of columnar cell surface (Frangmyr et al., 1995). CEACAM1-L is expressed preferentially in the upper part of colonic crypts, while the bottom of the crypts is devoid of it. Frangmyr et al. (1995) further confirmed glyocalyx localization of CEACAM1-L expression. This region of the colon is highly associated with apoptosis. When CEACAM1-L was stably transfected into the highly tumorigenic CEACAM1-deficient mouse colon carcinoma cell line CT51, tumor development was inhibited by 70-80% in *in vivo* tumorigenicity assays. The inhibition of tumor development is due to the expression of the long cytoplasmic tail-containing CEACAM1 and correlated with its tyrosine phosphorylation (Kunath et al., 1995; Izzi et al., 1999).

Upon apoptosis initiation of CEACAM1-L-transfected CT51 cells, caspase-mediated cleavage was observed. The conserved DQRD motif in the membrane-proximal cytoplasmic domain was identified to be a specific cleavage sequence recognized by caspase-3 *in vitro* (Houde et al., 2003).

Also the CEACAM1 isoform with a short cytoplasmic tail (CEACAM1-4S) acts in apoptotic processes. Mammary tumor (MCF7) cells transfected with CEACAM1-4S reverted to normal phenotype as evidenced by the formation of acini structures with central lumina. The lumen formation in MCF7 is mediated via apoptosis of central cells, as determined by caspase activation, Bax translocation to mitochondria, and cytochrome c release into the cytosol (Kirshner et al., 2003).

1.4.4 CEACAM1 and angiogenesis

CEACAM1 was shown to suppress tumor growth in several different cell types, including prostate cancer (Estera et al., 1999), colon (Kunath et al., 1995), bladder (Kleinerman et al., 1996), and breast carcinoma (Luo et al., 1997) independently of its adhesion activity. However, anti-tumor activity of the cytoplasmic tail of CEACAM1 was observed only under *in vivo*, suggesting an indirect involvement of CEACAM1 in angiogenesis. The expression of CEACAM1 in the prostate cancer cell line DU145 induced the production of specific inhibitors of angiogenesis, whose identity needs to be further investigated (Volpert et al., 2002).

1.5 The aims of the study

There are two important facts defining the background of the here presented study: (1) members of the CEACAM family glycoproteins are undergoing a strong dis-regulation upon malignant transformation of intestinal epithelia, and (2) loss of CEACAM1 was described to correlate with colorectal cancer and may contribute to early stages of tumorigenesis.

This work is orientated to reveal the possibility of specific regulation of CEACAM1 by different biologically active molecules such as cytokines, growth factors and antibiotics, etc. The effects on CEACAM1 subcellular localization, tyrosine phosphorylation and the ability to form high molecular weight complexes were to be investigated in response to treatment with regulatory active molecules.

Additionally, new protein-protein interaction partners for CEACAM1 and their possible role in signal transduction should be studied.

The aforementioned biochemical aspects of the CEACAM1 regulation were further evaluated with respect to the proliferative response of the model cell line HT29p derived from colorectal tumors.

2 MATERIALS

2.1 Cell culture

2.1.1 Cell line-HT29

Human colonic adenocarcinoma cell line–HT29 (ATCC; HTB-38) were used. The main cell model-HT29p cells derivate from HT29 cells and were prepared by repeated injection into the nude rats to obtain cell cascade series (HT29a-f) with growing tendency to invade and metastasize in animal model (Vogel et al., 1998).

2.1.2 Medium and supplements

RPMI 1640 medium was routinely supplemented with Glutamine 2 mM, Sodium pyruvate 1 mM, Penicillin 100 units/ml, and Streptomycin 100 µg/ml. All mentioned supplements were obtained from Life Technologies, Karlsruhe, Germany. Fetal bovine serum was from Hyclone Labs, Logan, UT, USA and was before use heat inactivated at 55°C for 30 min.

Agents: Trypsin-EDTA (Life Technologies, Karlsruhe, Germany) and Accutase (PAA Laboratories GmbH, Cölbe, Germany) were used for removing of adhesive cells from flask.

Trypan Blue solution (0.5%) necessary for cell counting in a Neubauer-Chamber was from Life Technologies, Karlsruhe, Germany.

Hybridoma medium was obtained from PAA Laboratories GmbH, Germany.

2.2 Apparatus

2.2.1 Incubators and centrifuges

- B6 incubator - Heraeus, Hanau, Germany
- Biofuge pico - Heraeus, Hanau, Germany
- Centrifuge 5402 - Eppendorf-Netheler-Hinz, Hamburg, Germany
- Rotina 48R - Hettich, Tuttlingen, Germany

2.2.2 Photometers and microscopes

- BioPhotometer - Eppendorf-Netheler-Hinz, Hamburg, Germany
- HT-2 - Anthos Labtech, Salzburg, Austria
- TECAN Elisa reader – TECAN, Crailsheim, Germany

- Axiovert - Zeiss, Jena, Germany
- BH-2 - Zeiss, Jena, Germany
- LSM 510 -Zeiss, Jena, Germany

2.2.3 Other apparatus

2D-electrophoresis apparatus was kindly borrowed from Biorad Laboratories, Germany.

Scintillation counter was purchased from Perkin Elmer Wallack GmbH, Germany.

2.3 Recombinant proteins and other tested substances

All cytokines (IFN- γ , Il-1 β , Il-4, Il-12, TNF- α) and growth factors (b FGF, EGF, TGF- α , TGF- β) were purchased from Strathmann Biotec AG (Hamburg, Germany). Geldanamycin, Tunicamycin, Staurosporin were obtained from Calbiochem (Merck, Darmstadt Germany) and LPS (from *Pseudomonas aeruginosae*, *Escherichia coli* B 6:26) as well as Acetaminophen, 5-azacytidin, Trichostatin, proteasome inhibitor Mg132 and different kinases and phosphatases inhibitors (Gö6983, ocadaic acid, sodium orthovanadate, Worthmannin) were from Sigma Chemical Co., Deisenhofen, Germany.

2.4 Primary and secondary antibodies

Primary antibodies:

- Mouse anti- β -actin -Sigma, Saint Louise, MI, USA
- Mouse anti-CD66a - Novocastra, Germany
- Mouse anti-CEACAM Ab-C1N3 (Schmiegel et al., 1985) and anti-CEA Ab-C1P83 (Hammarström et al., 1989)
- Mouse anti-CEACAM Ab-T84-1 (Neumaier et al., 1985) was kindly given from Professor M. Neumaier, Klinische Biochemie, Heidelberg, Germany.

- Mouse anti- EGFR - Calbiochem, Merck, , Darmstadt, Germany
- Mouse anti-Hsp90 α/β - Santa Cruz Biotechnology, CA, USA
- Mouse anti-Hsp90 - Calbiochem, Merck, Darmstadt, Germany
- Mouse anti-mucin RA96 (Matzku et al., 1988)
- Mouse anti-Phosphotyrosine - Cell Signaling, New England Biolabs, Frankfurt, Germany
- Rabbit anti- α -adaptin - Santa Cruz Biotechnology, CA, USA
- Rabbit anti-EGFR - Santa Cruz Biotechnology, CA, USA
- Rat anti-EGFR – Acris, ASA
- Rabbit anti-Hsp90 - Santa Cruz Biotechnology, CA, USA

Secondary antibodies:

Goat anti-mouse IgG1 direct conjugated with horseradish peroxidase was obtained from Dako (DakoCytomation, Denmark) and goat anti-mouse IgG1, goat anti-rabbit IgG+IgM, goat anti-rat IgG conjugated with Fluorescein was from Dianova (Dianova, Hamburg, Germany). Goat anti-mouse IgG+IgM and goat anti-rabbit direct conjugated with Alexa 555 was purchased from Mo Bi Tec GmbH, Göttingen, Germany as well as goat anti-mouse IgG+IgM direct conjugated with Alexa 353.

2.5 Kit systems

- Alexa 350 conjugation kit - Mo Bi Tec GmbH, Göttingen, Germany
- Alamar blue proliferation kit- Biosource International, California, USA
- BCA protein assay kit- Perbio Science GmbH, Bonn, Germany
- ECL, ECL plus Western blot detection- Amersham Bioscience, Freiburg, Germany
- EZ-Link™ Plus Activated Peroxidase Kit - Perbio Science GmbH, Bonn, Germany
- PCR-based mycoplasma detection kit - TaKaRa Bio Whittaker Europe, Taufkirchen, Germany
- Slow Fade kit - Mo Bi Tec GmbH, Göttingen, Germany
- Vectastain/Elite, Vectashild, DAB - Vector Labs, Burlingame, CA, USA

2.6 Chromatography materials

- AffiSep^R WGA adsorbent (GALAB Technologies GmbH, Geesthacht, Germany)
- Biorad ampholytes - Biorad Laboratories, Germany
- FreeZymeTM Conjugate Purification Kit - Perbio Science GmbH, Bonn, Germany
- IPG (immobilized pH gradient) strips - Biorad Laboratories, Germany
- NAP-5 desalting column - Amersham Bioscience, Freiburg, Germany
- PD-10 desalting column - Amersham Bioscience, Freiburg, Germany
- Protein G-agarose kit - KPL Laboratories, USA

2.7 Other reagents

- Coomassie blue G-250 - Merck, Darmstadt, Germany
- Coomassie blue R-250 - Serva Electrophoresis GmbH, Heidelberg, Germany
- Silver (AgNO₃) - Merck, Darmstadt, Germany
- PVDF membrane - Immobilon-P, Millipore, Ashburn, Germany
- Radioactive labeled [³H]-thymidine was purchased from Amersham Bioscience (Arlington Heights, IL., USA) and all other fine chemicals were obtained from the following companies and their grade of purity was at least *per analysis*.
- Merck, Darmstadt, Germany
- Sigma-Aldrich, Saint Louise, MI, USA
- Serva, Heidelberg, Germany

All standard laboratory plastic ware, tissue culture flask and dishes of tissue culture grade were obtained from Nunc (Roskilde, Denmark). Culture dishes of microbacteriological grade and plastic pipettes were bought from Greiner (Solingen, Germany).

3 METHODS

3.1 Standard cell culture

The main cell model-HT29p, where index “p” means “parental”, the cells are originally characterized by low metastatic activity (Vogel et al., 1998). HT29p cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 units penicillin/ml, 100 µg streptomycin/ml at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were routinely checked for mycoplasma contamination by the PCR-based mycoplasma detection kit from TaKaRa (by Bio Whittaker Europe, Taufkirchen/Germany).

Fresh medium was added every second or fourth day, depending on the cell line. Old medium was discarded, cells were washed with PBS (room temperature) and new medium was added. Cells were split after they reached a confluence of approximately 80%. The old medium was discarded, cells were washed with PBS and Trypsin/EDTA-solution (0.25% Trypsin/ 0.01% EDTA) was added. Cells were incubated at 37°C for approximately 15 min, depending on the cell line. Trypsin-activity was stopped by adding an equal amount of medium and afterwards cells were centrifuged at 1,400 x rpm for 5 min. The supernatant was discarded; cells were resuspended in fresh medium and seeded into new cell culture flasks. The average splitting-ratio was 1:3.

In the experiments where EGF receptor was investigated, the use of trypsin for removal of the cells was avoided. EGF receptor as an integral protein inside cell plasma membrane has surface presented domains utilizable by Trypsin. Therefore, for this special proposes we used Accutase (PAA Laboratories GmbH, Cölbe, Germany). Accutase is a unique mixture of proteolytic and collagenolytic enzymes and can be used in same manner that Trypsin/EDTA solution to detach cells from adherent surface. Prior to some experiments cells were counted in a Neubauer-Chamber using Trypan Blue solution (0.5% in PBS) to discriminate living from dead cells.

For long-term monolayer culture, cells were grown in T175 cell culture flasks. For long-term storage cells were frozen in liquid nitrogen. The storage solution consisted of FCS/10% DMSO.

3.2 Hybridoma cell culture

Hybridoma cells (C1P83 1-1-1) producing monoclonal antibody raised against tumor-associated antigen- CEA (Hammarström et al., 1989) were used. Another hybridoma cells (C1N3 1-22) producing antibody reacting with pancreatic and various gastrointestinal tract tumors presenting on their cell surface CEACAM family molecules, were characterized in past as a broad specific antibody against several known members of CEACAM family (Schmiegel et al., 1985).

Both hybridoma cells were cultivated in the hybridoma medium (PAA Laboratories GmbH, Germany) with 10% heat inactivated FCS inside 6-well dishes for 1-2 weeks depending on their growth ability. Later, cells were adjusted to FCS-free conditions and were maintained at 37°C inside sterile glass bottles with 1 L volume. Every second day probes were taken for the analysis of antibody concentration and fresh medium added. When concentration reached maximum under used conditions, the cells were removed from the medium with centrifugation at 1,200 x rpm for 5 min and collected hybridoma supernatant was purified or was frozen at -80°C for maximal 5 months.

3.2.1 Purification of monoclonal antibodies from hybridoma supernatant

Collected hybridoma supernatants were using Protein G-agarose kit (KPL Laboratories, USA). The column material-protein G-agarose (0.01 ml agarose/ml hybridoma supernatant) was equilibrated with 10 CV binding buffer (20 mM NaPhosphate, 150 mM NaCl, pH 7.4).

Hybridoma supernatants were diluted at least 1:1 in binding buffer as well and let 1 h stay at room temperature for buffering .

The binding of monoclonal antibody to column was performed in batch process (equilibrated column material and supernatant were incubated together for 30-60 min at RT accompanied by gentle rotation). The column material with bound antibody was packed into the empty PD-10 plastic column (Amersham Bioscience, Freiburg, Germany) with the maximal volume of 10 ml. Hybridoma supernatant with unbound components flowed through the column and was collected for further analyses. The column with specifically bound antibody was washed with at least 10 ml of binding buffer and flow through was again collected for control. Appropriate number of 1.5 ml reaction tubes was prepared and each tube was filled with 100 µl of storage buffer (1 M Tris/HCl pH 9).Storage buffer is used for very important pH balance after elution

of antibody from the column with 4 CV elution buffer (100 mM Glycin/HCl pH 3). Several 1 ml aliquots of eluted antibodies were analyzed and the rest of antibodies was frozen at -20°C . The column material was reused several times and therefore it was usually regenerated with 2 CV of elution buffer followed by at least 10 CV equilibration buffer or for long term storage 80% Ethanol was used. Treated column material was placed at 4°C and was used again for the purification of exactly same antibody.

Fractions taken from each step of purification (hybridoma supernatant before purification, unbound fraction-respectively flow through, unspecific bound proteins in wash fraction and finally elute with concentrated antibody) were analyzed by classical biochemical methods like:

- Protein concentration estimation (BCA protein assay kit, Perbio Science GmbH, Bonn, Germany)
- Spectrometric measurement of OD_{280} (Extinction coefficient for IgG is 1.38 mg/ml if OD_{280} value is 1)
- SDS PAGE followed by Silver or Coomassie staining and Western blot developed with polyclonal anti-IgG antibody.

C1N3 antibody was analyzed for antigen specificity in comparison to C1P83 by Western blot (Fig. 5).

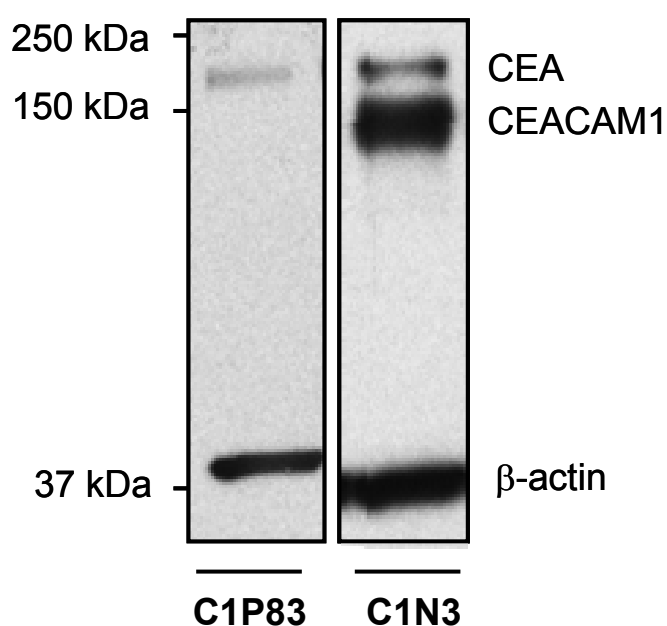


Figure 5: Western blot detection with the purified C1N3 and C1P83

Proteins were isolated under the denaturing conditions from HT29p cells and separated in SDS-PAGE followed by detection of CEA (about 180 kDa) with C1P83 mAb, while C1N3 mAb was able to recognize CEA (about 180 kDa) and CEACAM1 (about 150 kDa).

3.2.2 The labeling of C1N3 antibody with Horse reddish peroxidase

Prior to conjugation of purified C1N3 antibody with horse reddish peroxidase (HRP) was necessary to exchange storage buffer of antibody. For this propose NAP-5 desalting column (Amersham bioscience, Freiburg, Germany) containing Sephadex G-25 medium was used. After equilibration of the column with 10 ml of appropriate buffer (0.1 M Sodium bicarbonate buffer containing 0.15 M NaCl, pH 9) was antibody solution in maximal volume 0.5 ml separated according the special gel filtration based principle inside the column medium following by elution with 1.5 ml of Sodium bicarbonate buffer into prepared reaction tubes. Protein concentration was estimated by BCA protein assay kit and antibody was storage at -20°C.

C1N3 antibody was labeled with HRP using EZ-Link™ Plus Activated Peroxidase Kit (Perbio Science GmbH, Bonn, Germany). For the high efficiency of conjugation was important to optimize the ratio between amount of HRP and amount of IgG (in our cause C1N3) as well as duration of conjugation. Approximately 1 mg of C1N3 antibody in conjugation buffer (0.1 M Sodium bicarbonate buffer containing 0.15 M NaCl, pH 9) was added directly to 2 mg of lyophilized HRP and it was incubated for 2 h at RT. Reaction (conjugation) was stopped by the addition of a reductant and quench solution for 15 min. Final solution contained antibody-HRP conjugate and as well unbound HRP, which can later interfere and influence the action of conjugated antibody. Therefore, metal chelating chromatography (FreeZyme™ Conjugate Purification Kit, Perbio Science GmbH, Bonn, Germany) was employed to remove unbound HRP. After equilibration of the column containing immobilized iminodiacetic acid activated with Ni²⁺, C1N3-HRP conjugate was applied. The binding capacity of column resin ranges between 0.5 up to 50 mg of IgG. C1N3-HRP conjugates as well as HRP alone bind into chelating resin from where they were eluted with EDTA. EDTA presence caused that only C1N3-HRP conjugate containing resins formed blue colored ring and exactly counted volume of EDTA elution buffer had to be applied to flow out this fraction.

Purified fraction of C1N3-HRP conjugate was stored at 4°C.

3.2.3 The labeling of C1N3 antibody with Alexa Fluor 353

C1N3 antibody was replaced into appropriate buffer (0.1 M Sodium bicarbonate, 150 mM NaCl, pH 9) with using same procedure as described above for HRP conjugation. The conjugation of C1N3 with Alexa 350 was performed according company manuscript (Mo Bi

Tec GmbH, Göttingen, Germany). The amine-reactive dye in amount of 5 mg was dissolved in 0.5 ml of DMSO only short time prior to use because dye is not very stable in solution. The concentration of labeled target protein recommended by company ranges between 5-20 mg/ml, but we were not able fill this requirement. Therefore, we adjusted working conditions to our needs. During stirring of antibody solution with 1mg/ml of antibody, 50 µl of the reactive dye solution was slowly added. Obtained mixture was incubated 2 h at RT with continuous stirring followed by addition 0.1 ml of freshly prepared 1.5 M Hydroxylamine, pH 8.5 to stop conjugation reaction. Usually, only one-fourth to one-third of reaction dye binds to antibody. Unbound dye can be removed by use of gel filtration or dialysis, but in our cause it would mean lost of anyway very low amount of conjugate. Conjugate was stored at 4°C for up to 3 months.

3.3 Immunostaining

3.3.1 Immunohisto-/cytochemistry

For immunohisto-/cytochemistry either human colon tumor tissue or HT29p cell line was used. After surgical treatment of patients, one fraction of the resected tumor tissue was immediately frozen in liquid nitrogen. Another fraction was inspected by pathologists to determine tumor-type and grade. Prior analysis 5 mm blocks of tissue were cut of the primary tissue block and cut in 5 µm-thin slices using a cryotom. Three to five of these slices were mounted on a microscopic slide, air-dried for at least 3 h and fixed in acetone for 5 min. HT29p cells were harvested by mild trypsination and placed onto microscopic slides.

Cells on microscopic slides were fixed by incubation with acetone for 5 min fixed tissue and cells were stored at -20°C in plastic bags under vacuum conditions.

Prior analysis, the frozen samples were allowed to reach room temperature and were re-hydrated in PBS. Staining was then carried out by incubating for 1 h with a primary antibody-C1N3 directly conjugated with HRP or with Alexa 350. Concentration of C1N3 conjugates had to be optimized and we observed the most specific staining in concentrations ranging from 1 to 5 µg/ml. Both of C1N3 conjugates were diluted in PBS with 1% bovine serum albumin (BSA). C1N3-HRP staining pattern was detected with Diaminobenzidine (DAB) as a substrate for the HRP resulting in a brown precipitate in case of a reaction. Evaluation of results was carried out microscopically by following positive (brown) staining.

After incubation of tissue sections with C1N3-Alexa conjugate for 1 h at RT, slides were stringent washed with PBS and antifade solution (Slow Fade kit, Mo Bi Tec GmbH, Göttingen, Germany) was dropped onto stained tissue. Positive blue staining was investigated with an Axioskop2-fluorescence-microscope for epifluorescence from Carl Zeiss (Jena, Germany.)

3.3.2 Immunofluorescence

HT29p cells were treated with Accutase to detach them from an adherent surface and 2×10^5 cells were again seeded on sterile glass cover slip placed inside 6-well plate. Cells were maintained in complete RPMI medium with 10% FCS for at least 15 h at 37°C.

Following their deprivation of serum for 6 h in the presence of 0.5% BSA, the cells were treated with tested substances or RPMI alone for the assigned time points prior to their fixation and permeabilisation with 2 ml of ice-cold Methanol (approximately 70%) for 10 min at -20°C. Cells were then washed with ice-cold PBS containing 0.05% Sodium azide 1-2 times and blocked in blocking solution (0.2% BSA, 0.1% Glycin in TBS, pH 7.5) at 4°C for 18 h following by incubation with 0.1 µg/ml of C1N3 together with rabbit anti-EGFR antibody diluted 1:100 in blocking solution for 15 h at 4°C. After washing 2 x 10 min with TBS containing 0.1% Tween 20 and next 2 x 10 min with TBS alone, incubation with the goat anti-mouse IgG+IgM antibody directly conjugated with FITC (Dianova, Hamburg, Germany) and goat anti-rabbit IgG+IgM antibody directly conjugated with Alexa fluor 555 (Mo Bi Tec GmbH, Göttingen, Germany) was carried out for 1 h. Both of secondary antibodies were diluted 1:1000 in blocking buffer. Incubation with secondary antibodies was accompanied with nuclei staining (dilution also 1:1000). After washing step (same as described above) was on cells dropped antifade solution to prevent dissociation of fluorescence dyes and slides were cover with another one glass plate.

Visualization was carried out with a laser-scanning microscope LSM 510 for confocal microscopy from Carl Zeiss (Jena, Germany).

3.4 Flow cytometry (FACS)

3.4.1 Detection of CEACAM family members on the cell surface and in cytosol

HT29p cells were removed from plastic flasks with Accutase and counted with use of Trypan blue. All further steps were performed on ice and all of used solution was pre-cooled. The cell

number 5×10^5 was placed into each FACS tube and one part of cells was permeabilised with 80% cold Methanol for not longer like 2 min. Cells were washed 3 x 10 min with ice-cold PBS containing 0.5% Sodium aside. The incubation of permeabilised and un-permeabilised cells with 15 $\mu\text{g/ml}$ of C1N3 or 30 $\mu\text{g/ml}$ of C1P83 antibody diluted in ice-cold PBS containing 1% BSA for 1 h on ice was followed by washing step. Cells were washed 3 x with 1 ml of ice-cold PBS as mentioned above and centrifuged at 1,400 x rpm for 5 min. Supernatants were removed and goat anti-mouse IgG+IgM antibody directly conjugated with Fluorescein diluted 1:1000 in PBS containing 1% BSA was added for 1 h. Cells were resuspended in 1 ml of cold PBS prior to measurement. Flow cytometer (BD, Heidelberg, Germany) was used to estimate intensity of green fluorescence on the cell surface (in cause of un-permeabilised cells) and complete green staining in cytosol as well as on cell surface (in cause of permeabilised cells) corresponding to either by C1N3 or by C1P83 antibody recognizable specific antigen presence.

3.4.2 Cell cycle- measurement with Propidium Iodide

HT29p cells untreated or treated with tested substances were removed from flasks with Accutase and counted. 5×10^5 - 1×10^6 cells were resuspended in 1 ml of PBS with 5 mM EDTA solution (PBS-EDTA) followed by centrifugation at 1,400 x rpm for 5 min at RT. The cell pellets were incubated with 500 μl of 70% Ethanol for at least 30 min at RT to fixed and permeate the cells. RNA released from fixed cells was digested with 50 $\mu\text{g/ml}$ of RNase during 30 min incubation at RT. Washing step with PBS-EDTA was followed by incubation with Propidium Iodide (final concentration 50 $\mu\text{g/ml}$) for next 30 min in dark on ice. The measurements were performed with use of Flow cytometer (BD, Heidelberg, Germany).

3.5 Primary screening

Different organic and inorganic substances were tested on HT29p cell model system for their effect on growth, proliferation and apoptosis in the relation to expression alterations of CEA and/or CEACAM1 adhesion proteins. Substances were chosen according literature and some of them also because of the interest to test new components expressing regulatory activity (Table 1).

After mild trypsination 1.5×10^6 cells were maintained in complete RPMI medium containing 10% FCS and allowed to adhere inside 6-well plates for at least 15 h. After cell adhered, old

medium was replaced by the fresh one containing additionally tested substances in concentration dependent on substance nature and way of action. The cells were treated with different substances or medium alone for the assigned time points prior to further analyses. Only substances evoking strongest effect on CEA and/or CEACAM1 molecules (IFN- γ , TNF- α , EGF, and geldanamycin) were investigated into a detail.

3.6 Protein extraction, type of detergent and determination of concentration

Since this work is orientated on CEA and CEACAM1 as typical membrane associated proteins, the first and the most important step in discovery of biological properties of both of membrane glycoproteins is efficient extraction of them from cell membrane in intact status. Therefore, extraction had to be optimized in respect to buffer, salt and detergent kind and concentration. We have tested different buffer systems with or without detergents (as is shown in table 1) for extraction strength and compatibility with later described used methods. Cells were after mild trypsination counted and 1.5×10^6 cells pro well were placed into 6-well plates. Cells were allowed to adhere in complete RPMI medium supplemented with 10% FCS at least for 15 h following by their harvesting by trypsination again. After cells were washed 2 x with PBS, freeze-thaw method was used for separation of soluble proteins from membrane associated proteins. Briefly to this method: cells from each well were in separated tubes resuspended in approximately 200-350 μ l of Tris buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, protease inhibitors) and strongly mixed. Tubes with cells were three times frozen in liquid nitrogen following by thawing process. After last freeze/thaw cycle, disrupted cells were centrifuge at 14,000 x rpm for 15 min at 4 °C and supernatant containing all soluble proteins was removed to fresh tubes on ice. Pellet after last centrifugation contains all of cell membranes and organelles impossible to solubilize without detergent or high concentrated chaotropic salts. These pellets were resuspended in 250 μ l of same Tris buffer with addition of different tested detergents and salts. After strong vortex were placed samples on ice for 30 min followed by centrifugation under above described conditions. Supernatants were again removed into fresh tubes and pellets were solubilized in 20 μ l of Laemmli buffer and boiled at 95°C for 5 min. Soluble and membrane fraction proteins were concentrated with acetone as described later following by Western blot analysis.

3.6.1 Acetone precipitation of proteins

Supernatants, containing soluble and membrane proteins were checked for protein concentration with use of BCA system. 20 µg from each sample was precipitated with ice-cold 80% Acetone for 20 min at -20°C. Afterwards samples were centrifuged at 14,000 x rpm for 10 min at 4 °C and supernatants were discarded. Pellets were let dry on air allowing evaporating of the rest of acetone. Dry pellets were solubilized in 20 µl of Laemmli buffer and boiled at 95°C for 5 min. Samples were analyzed on immunoblot with C1N3 antibody.

Octyl β-D-glucopyranoside was selected for further use because of its strong extraction efficiency and non-denaturing characteristics.

Table 1: Determination of protein lysis buffer system

Different kinds of buffer systems were tested with respect to salt and detergents components for propose to isolate CEACAM1 and CEA from the cell plasma membrane of HT29p cells. The detergent with the best extraction efficiency, but keeping proteins more-less in native status was chosen. Cells were resuspended in 250-300 µl of appropriate buffer and three times freeze/thaw cycle was performed followed by 30 min incubation on ice. After 10 min centrifugation at 14,000 x rpm at 4°C, supernatants protein contents were measured by BCA kit. 20 µg of total proteins were acetone precipitated and separated in SDS-PAGE. Immunoblots checked for CEACAM family member presence in isolated faction in comparison to detergent-insoluble pellet prepared for electrophoresis by boiling at 95°C for 5 min in 20 µl of Laemmli buffer.

Extraction element	Character of compound	Dis-/Advantages	Extraction efficiency
PBS / TBS buffer	buffer	weak extraction of soluble proteins; native	very low
PBS / mechanical disruption	buffer	weak extraction of soluble proteins; native	very low
PBS / EDTA	buffer, chelator	weak extraction of soluble and surface presented proteins; native	low
Urea/EtSH	chaotropic salt, reducing agent	good extraction of integral membrane proteins, strong denaturation	high
Triton X-100	non-ionic detergent	good extraction of integral membrane proteins, incompatible with some methods	high
N- Lauroylsarcosine (Sarcosyl)	anionic detergent	average efficiency for membrane proteins under native conditions	middle
CHAPS/IPEGAL	Zwitterionic / non-ionic detergent	very good extraction also membrane proteins	very good
Laemmli- buffer / boiling	SDS anionic detergent and DTT reducing agent	very good extraction efficiency, absolutely incompatible with used methods	very good

Extraction element	Character of compound	Dis-/Advantages	Extraction efficiency
n-dodecylmaltoside	non-ionic detergent	good extraction efficiency, compatible with used methods	good
Octyl β -D-glucopyranoside	non-ionic detergent	very good extraction efficiency, used also for lipid rafts solubilization	very good

3.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The separation of single proteins under denaturing conditions inside continuous or discontinuous polyacrylamide gels was performed according Laemmli (1970). Sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) is a method used to fractionate proteins according to their size. Self-made gel with acrylamide concentration 8 to 12.5% (w/v) or ready to use (continuous gradient) gels with acrylamide concentration 4-20% (w/v) were used. Samples separated inside SDS-page were usually acetone precipitated and resuspended in up to 20 μ l of Laemmli buffer containing DTT. Afterward samples were boiled for 5 min at 95°C and loaded into the gel. Proteins were migrating through gel according molecular weight and run was facilitated by negative charged SDS presented in running buffer. Run took approximately 90 min at constant 135 V.

3.7.1 Coomassie staining of proteins

Gels with separated proteins were placed in to Coomassie blue solution (containing 40% (v/v) Methanol, 7% (v/v) Acetic acid and 0.18% (w/v) Coomassie brilliant blue R 250) and were developed for at least 30 min at RT. After gel was good homogeneous blue stained, destaining step was performed. Destaining solution (containing 40% (v/v) Methanol, 7% (v/v) Acetic acid and 3% (v/v) Glycerol) was permanently changed every 10 min or when to intensive blue solution stained. After replacing the buffer several times proteins as single bands were visible.

3.7.2 Silver staining of proteins

Silver staining is protein staining used usually where Coomassie staining is not sensitive enough. Gels were stained according protocol from Rabilloud (1988) and destained in same manner as described above for Coomassie staining.

3.7.3 Western blot and antibody detection

After efficient separation of proteins inside SDS-PAGE were proteins transferred onto the Polyvinylidene difluoride membrane (PVDF, Immobilon P, Millipore Corp. Bedford, USA) during wet transfer inside electric field (Western blotting). Blotting buffer (25 mM Tris base, 192 mM Glycine, 10% Methanol) was used and the transfer was performed at 400 mA for 1 h. To prove presence of proteins of interest onto the PVDF membrane, immunostaining with appropriate antibody was performed. The membrane with proteins was incubated for 1 h at RT in blocking solution to prevent unspecific binding of antibody. Different kinds of blocking solutions can be used in dependency on used antibody. Usually used blocking agents are 2-5% (w/v) non-fat milk and 0.5-5% (w/v) BSA diluted in classical PBS buffer with an addition of 0.1% (v/v) Tween 20 (PBS-T). After the blocking step was solution with properly diluted primary antibody in blocking solution added to the membrane. The incubation time and temperature differed for different antibodies. Generally, if antibody is incubated with a membrane for more hours, incubation takes place at 4°C. The short time incubation with antibody runs at RT. This incubation was followed by washing of membrane from unbound antibody 3 x 10 min with blocking solution and next incubation with secondary anti-mouse IgG+IgM direct conjugated with HRP diluted in blocking solution was performed at RT for 50 to 60 min. After three times washing with PBS-T were membranes developed with chemiluminescence detection system (ECL or ECL plus, Amersham Bioscience, Freiburg, Germany). ECL is the substrate for HRP and its metabolism results in light-emission which is detected with an x-ray film (Kodak Biomax). All blots were normalized with an antibody against β -actin (42 kDa) to prove the loading of equal amounts of protein per lane.

3.8 Blue Native Gel electrophoresis (BNG)

BNG is a native electrophoresis method described by Schägger et al. (1994) and it was developed for the separation of native protein complexes from biological membranes, but that also separates both basic and acidic water-soluble proteins at a fixed pH of 7.5. In combination with a second dimension SDS-PAGE it provides an analytical method for the determination of molecular mass and oligomeric state of non-dissociated complexes (Schägger et al., 1994).

3.8.1 Protein sample preparation

1×10^7 HT29p cells non-treated or treated with studied substances were removed from flasks with Trypsin treatment and washed with PBS. Cell pellets separated by centrifugation were resuspended in BNG lyses buffer (50 mM Bis-Tris/HCl, pH 7.5, 750 mM ϵ -aminocaproic acid, 2 mM EDTA, protease inhibitors) and vortex. Freeze/thaw method was used to separate soluble proteins from membrane fraction. BNG sample buffer additionally containing 48 mM Octyl β -D-glucopyranoside had sufficiently isolated membrane associated CEACAM1 containing protein complexes from pellets. Protein concentration was determined with BCA kit. Prior to starting BNG, Coomassie blue G-250 (Serva Electrophoresis GmbH, Heidelberg, Germany) was added from stock solution in 500 mM ϵ -aminocaproic acid to the membrane proteins (150-200 μ g).

The high molecular standards were self mixed from HMW standards kit for gel filtration (Amersham Bioscience, Freiburg, Germany) and they were running parallel with the samples.

3.8.2 First (native) dimension

First dimension separates protein complexes under native conditions inside continuous polyacrylamide gradient gel with the acrylamide concentration 6 to 16% (w/v). Protein complexes were running according size and whole separation was performed at 4°C for 15 h at constant 150-200 V. Proteins were moving from upper chamber containing cathode buffer (50 mM Tricine, 15 mM Bistris/HCl, pH 7+ 0.002% Coomassie blue G-250) through gel (6-16% (v/v) acrylamide/*bis*-acrylamide solution, 50 mM Bis-Tris/HCl with pH 7, 500 mM ϵ -aminocaproic acid, 5% (v/v) Glycerol) to lower chamber containing anode buffer (50 mM Bis-Tris/HCl, pH 7). Coomassie blue in cathode buffer inducing a charge shift facilitated run of protein complexes in similarly manner as SDS does, but without denaturing effect.

Gel from first dimension was equilibrated 20-30 min in SDS-PAGE running buffer with addition of 1% SDS (v/v) and β -mercaptoethanol (0.05% (w/v)) followed by washing one time with only SDS-PAGE running buffer. The single lane from first dimension belonging to one sample was cut out and placed to second dimension.

3.8.3 Second dimension (classical SDS-PAGE)

Single cut strips from first dimension were further separated in classical SDS-PAGE under denaturing conditions. Single proteins derivate from one size complexes were separating

according molecular mass in one lane. Afterward gels were transferred onto PVDF membrane and developed with antibody as described above (point 3.7.3).

3.9 Immunoprecipitation assay

Human colon adenocarcinoma HT29p cells were maintained in RPMI medium either with 10% FCS or in medium supplemented with 0.5% BSA. Treated and non-treated cells were removed from flasks by mild Accutase treatment and placed into solubilization buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 20 mM Sodium orthovanadate, protease inhibitors) followed by freeze/thaw separation of soluble proteins. Membrane pellets were resuspended in same buffer with additionally added 48 mM Octyl β -D-glucopyranoside and let act on ice for 30 min. After centrifugation (10 min, 4°C, 13,000 x rpm), protein concentration in supernatants was determinate. The protein G-agarose (20 μ g) was equilibrated 3 x 5 min in binding buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, proteases inhibitors and 1% NP-40). Primary antibody (5-10 μ g) was allowed to bind onto the protein G-agarose during 1 h incubation in binding buffer at RT. Unbound antibody was washed away with washing buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 100 mM NaF, proteases inhibitors, 0.5% NP-40). Membrane proteins (100 μ g) were added to the agarose G-antibody complex and were incubated for next 1 h under same conditions as described above.

Immunopellets were stringent washed twice with washing buffer and next two times with same buffer without NP-40. After short centrifugation at 2,200 x rpm for 5 min, immunopellets were resuspended in 20 μ l of Laemmli buffer, boiled and separated in SDS PAGE followed by transfer onto PVDF membrane. Membranes were developed with antibody directly conjugated with HRP and chemiluminescence reagents ECL.

3.10 2 D-electrophoresis

Two-dimensional electrophoresis (2D-electrophoresis) was first introduced by P.H. O'Farrel (1975) and J. Klose (1975). The 2D-electrophoresis is analytic method including two steps: the first-dimension step (isoelectric focusing (IEF) separating proteins according to their isoelectric points (pI) and the second-dimension step (SDS-PAGE separating proteins

according to their size). Each spot on the resulting two-dimensional array corresponds to a single protein, and this single protein is characterized according to its pI and the molecular weight.

3.10.1 Sample preparation for IEF

HT 29p cells were removed from flask by gently trypsination and proteins were extracted and further immunoprecipitated as described above (part 3.9). Immunopellets after incubation with protein lysates (cytosolic and membrane fractions) and centrifugation at 2,200 x rpm for 2 min at RT were washed three times with buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaF, protease inhibitors, 1% NP-40). Immunopellets in Boston buffer (6 M (w/v) Urea, 2% (v/v) NP-40, 0.8% (v/v) Biorad ampholytes, 0.5% (v/v) β -mercaptoethanol) were incubated for 30 min at 50°C in thermo-mixer at 500 x rpm followed by addition of Rehydration buffer (8 M Urea, 2% (v/v) NP-40, 0.5% (v/v) Biorad ampholytes, 0.3% (w/v) DTT) and next 30 min incubation under same conditions as before. Subsequently, samples were centrifuged at 2,200 x rpm for 2 min at RT and supernatants were removed to fresh tubes. Pellets were resuspended in same amount of Rehydration buffer and incubated again for 30 min at 50 °C to flow out rests of proteins bound onto protein G matrix. Collected supernatants were further analyzed.

3.10.2 Isoelectric focusing

The first dimension separation of immunoprecipitated proteins was performed with use of IPG strips including immobilized pH gradient. Linear pH gradient 4-7 L strips (IPG strips, Biorad Laboratories, Germany) with the length of 7 cm were used. Samples with a final volume 150 μ l (exactly adjusted to the strips parameters) were placed into separated slots inside re-swelling tray and strips were rehydrated. IPG strips were overlaid with mineral oil minimizing evaporation and crystallization. Proteins were separated inside IPG strips according to protocol recommended by company.

3.10.3 Equilibration of IPG stripes

During equilibration step were IPG strips saturated with the SDS buffer system required for second dimension. Strips with separated proteins were removed from slots and gently washed from rest of mineral oil. Subsequently, equilibration was performed in a buffer (50 mM

Tris/HCl, pH 8.8, 6 M Urea, 30% (v/v) Glycerol, and 2% (w/v) DTT or 2.5% (w/v) Iodacetamid) for two times 15 min at RT.

3.10.4 The second dimension

After equilibration procedure were strips washed one time with distilled water and were placed into classical SDS-PAGE with acrylamide concentration 4-20%. Gel was afterward transferred onto the PVDF membrane and immunoblot was developed with antibody.

3.11 Lectine affinity chromatography (Wheat Germ Agglutinin, WGA column)

Affinity chromatography based on the reversible interaction between lectin WGA and glycoproteins containing sialic acid or N-acetylglucosamine residues in their structure. AffiSep[®] WGA adsorbent (GALAB Technologies GmbH, Geesthacht, Germany) was equilibrated with 5 CV of adsorption buffer (Phosphate buffer, pH 7.2) followed by incubation of the column material with a membrane lysate for 30 min at RT during permanent rotation. A flow through and washing fractions were stored for further analysis. Elution buffer (Phosphate buffer, pH 7.2 containing N-acetylglucosamine) in one column volume was used to elute specifically-bound glycoproteins. Column material was regenerated and stored at 4°C in 80% Methanol. The protein concentration in all of obtained fractions was estimated.

3.12 The viability and proliferation assay

To check alterations in the cell viability and the proliferation after treatment with selected substances, Alamar Blue fluorescence assay was used. The bioassay was designed to measure quantitatively the proliferation of various human cell lines. Alamar blue assay incorporates a fluorimetric/colorimetric growth indicator based OD detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that fluoresces in response to chemical reduction of growth medium resulting from cell growth. Continued growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator to change from oxidized (non-fluorescent) form to reduced (fluorescent) form (Lancaster et al., 1996) HT 29p cells were treated with mild trypsination and after centrifugation at 1,400 x rpm for 5

min, and cell number 1×10^4 (this cell number was estimated during optimization procedure where Alamar blue detection limit for cell numbers between 5×10^3 – 5×10^4 /well was tested) was seeded.

After HT29p adhered, properly diluted tested substances were added and allowed act for either 6 h (in TNF- α case) or 12 h. The incubation of cells with Alamar Blue solution had duration at least 6 h prior to measurement. Increase of fluorescence measured by TECAN ELISA reader (TECAN, Crailsheim, Germany) linearly correlated with the number of viable cells.

3.13 [^3H]-thymidine incorporation assay

Thymidine incorporation assay was employed to visualize effect of selected substances on DNA synthesis. DNA synthesis includes newly synthesized DNA during the cell division and also DNA repair. HT29p (1×10^4 /well) were seeded in 96-well plate and maintained for 20 h in complete 10% FCS containing RPMI medium following by the substitution of FCS via 0.5% BSA containing medium. Cells were adapted to these conditions for next 4 h and subsequently, tested substances were added in certain concentrations for either 3 h (in TNF- α case) or 15 h. Incubation of cells with $1 \mu\text{Ci}$ /well [^3H]-thymidine for 3 h was followed by permeabilisation of cells with 0.5% SDS in PBS for next 1h. The rate of DNA synthesis was measured after a cell harvesting with the scintillation counter (Perkin Elmer Wallace GmbH, Freiburg, Germany). Methods were modified in respect to the different stimulation duration for each studied molecule.

4 RESULTS

4.1 The cell model system and the characterization of C1N3 monoclonal antibody

4.1.1 Selection of the cell model

The human colon adenocarcinoma cell line HT29 has been used as a model cell system for studying a cell cascade with increasing potential in respect to their invasion and metastatic properties (Vogel et al., 1998). HT29 cells were injected into the *venae portae* of nude rats. After the tumor had developed, re-isolated tumor cells were used for next injection. This process led to the observation that metastatic potential is proportionally increasing with number of inoculation repeats. A cell cascade series called HT29p, a, b...f was obtained to study malignancy in colon cancer (Wang et al., 2003).

Index “p” for HT29p cells means “parental” and cells are characterized by a moderate metastatic activity derived from the inoculation of original HT29 cells. The entire HT29 cell culture series (p, a-f) was tested for the presence of CEACAM family members on the cell surface. Cells without permeabilisation were incubated with either C1N3 (broad specific anti-CEA/CEACAM1 members antibody) or C1P83 (specific anti-CEA antibody) followed by the incubation with a secondary anti-mouse IgG antibody conjugated with Fluorescein. Flow cytometry staining results suggested that the increasing malignancy of cells along HT29 variants is accompanied with a decreased surface expression of CEACAM1 (data not shown). CEA expression and release into the culture medium in non-polarized cancer cells was also shown in our model system (Fahlgren et al., 2003). CEA surface expression in HT29p was investigated with the use of C1P83 mAb (Fig. 6). C1N3 staining profiles showed that HT29p cells express CEACAM1 still in a significant rate (Fig. 6). The aim of these investigations in the mentioned cell model was to obtain a colon cancer cell line with a more or less still functionally active glycocalyx network. Therefore, HT29p variant with a detectable expression of CEACAM1 was chosen for further studies.

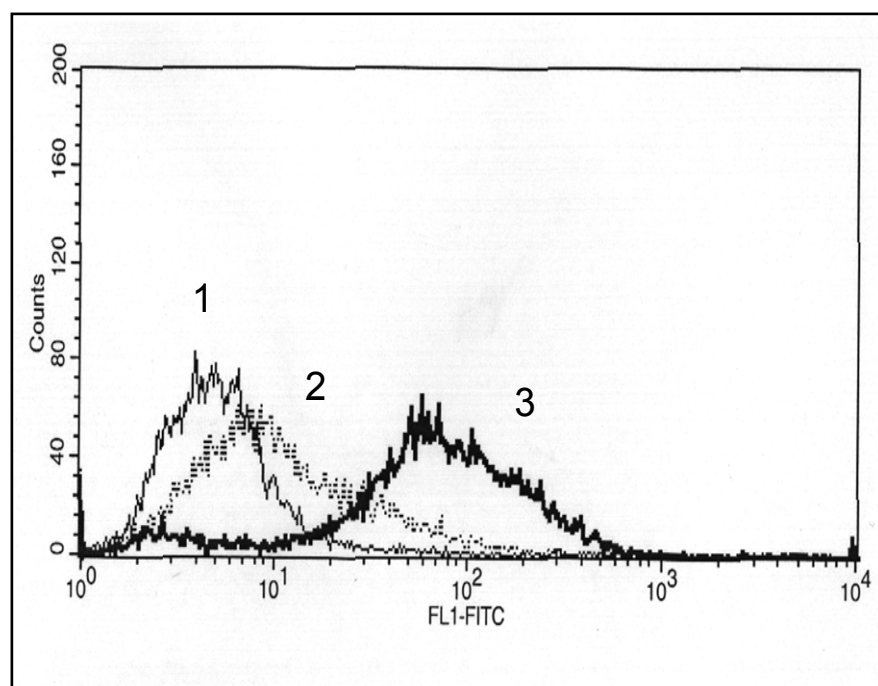


Figure 6: The expression of CEA and other CEACAM members on the cell surface of HT29p

The flow cytometry was used to investigate the surface expression of either CEA or all CEACAM members. HT29p cell surface-presented CEACAMs were stained with the purified C1P83 (2) or

C1N3 mAb (3). Autofluorescence of the cells was also measured to define background (1).

4.1.2 C1N3 and C1P83 monoclonal antibodies production, purification and characterization

The hybridoma cells (C1P83 1-1-1) were prepared already two decades ago as a mouse monoclonal IgG₁ (Hammarström et al., 1989). C1P83 mAb recognized CEA in a very specific manner. The hybridoma cells (C1N3 1-22) producing antibody reacting with the pancreatic and various gastrointestinal tract tumors CEACAM family molecules presented on their cell surface, were characterized in the past (Schmiegel et al., 1985).

Mentioned hybridoma cells were maintained in the medium without FCS supplementation. Subsequently, when the Ab production reached maximum, medium was harvested by centrifugation. The efficiency of antibody production as well as the purity of the fraction bound specifically to protein G-agarose was further controlled by silver staining. Monoclonal Ab was the only protein detected in the purified fractions.

After the purification C1N3 antibody was tested for its ability to recognize the appropriate antigen. Figure 7 shows staining of the colon tissue section with ALEXA 350-conjugated C1N3. The apical cell-surface staining of CEACAM members was visualized on tissue sections with C1N3. Epithelial cells investigated with fluorescence microscopy were lining the interior of the colon lumen and they were visualized by the light blue staining of nuclei

(negative control, Fig. 7, panel A). Darker blue fluorescence indicated a specific staining of the apical glycocalyx structure (Fig. 7, panel B). C1N3 binds in specific manner CEACAM members as part of glycocalyx network.

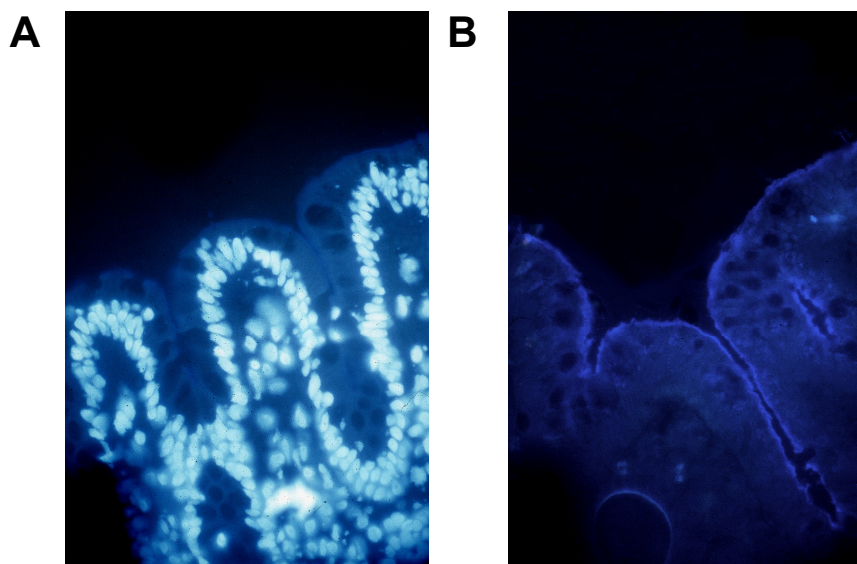


Figure 7: Immunofluorescence staining of colon tissue with C1N3 mAb

The colon tissue sections fixed on the glass slides were stained with C1N3 mAb directly conjugated with fluorescence dye (Alexa 350). The blue fluorescence staining indicated apical pattern of glycocalyx structure containing CEACAMs (panel B). The cells were stained for nuclei in negative control sample (panel A) without presence of C1N3-signals.

In order to compare C1N3 antigen specification with a previously characterized T84-1 antibody (Neumaier et al., 1985) 2D-electrophoresis was performed. CEACAM members were immunoprecipitated from the HT29 cell lysate with C1N3 and separated in an IEF according to their pI. Subsequently, in the second dimension proteins were separated according to their molecular weight (SDS-PAGE). Detection with T84-1 antibody shows spots belonging most likely only to CEACAM1 isoforms. CEACAM1 with its Mr of 150 kDa was identified according to pI and Mr (Fig. 8). Because of the pI of 5.28, the strongest spot was identified as CEACAM1. However, the pI was determined only for the peptide part of CEACAM1, where the influence of the carbohydrate part on the pI value was not reflected. The less intensive spots about 150 kDa could be represented by glycosylation variants of CEACAM1, because no splicing variant of CEACAM1 with found characteristics has been

previously described. On the other hand secondary modifications, i.e. phosphorylation of CEACAM1 (Culic et al., 1992), contribute to a change of the pI value. Most probable, the less intensive spots (Fig. 8) are represented by some kind of modification.

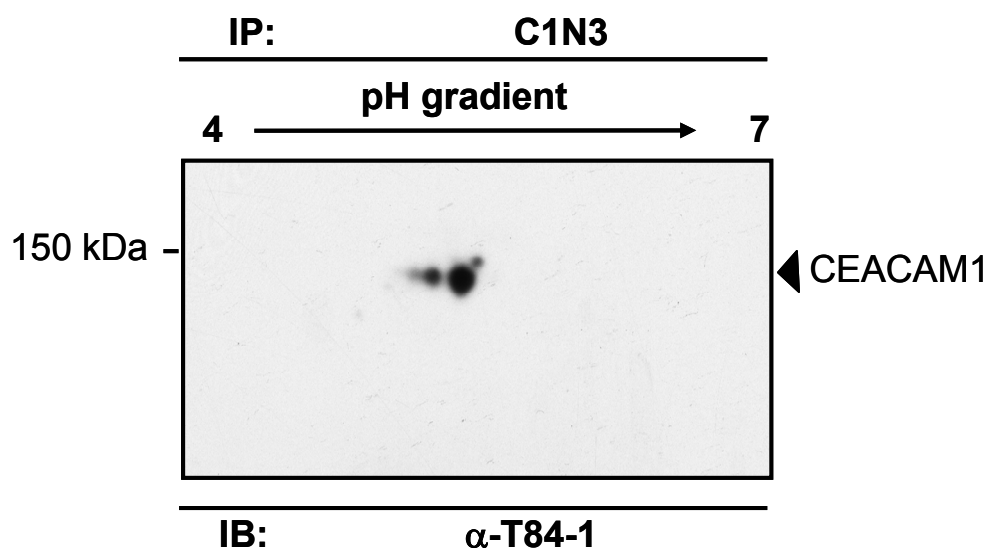


Figure 8: Antigen specificity of C1N3 is comparable with T84-1

The membrane fraction of HT29p cells was prepared with three freeze/thaw cycles followed by the detergent-dependent solubilization of the membrane pellet. Subsequently, during immunoprecipitation assay, C1N3-recognised CEACAM members were eluted with urea containing buffer. Proteins were separated inside the pH gradient (4-7) during IEF. The immunoblot after second (SDS-PAGE) dimension was developed with T84-1 antibody.

4.2 Primary screening of active substances regulating CEACAM family members

4.2.1 Detergent optimization

To optimise conditions under which respective proteins are extracted from human cancer cells, different extraction systems containing denaturing and non-denaturing agents as chaotropic salts, reducing agents and detergents were investigated (see Table 1). As a result of this selection an extraction system for further analysis was found.

It could be shown that Octyl- β -D-glucopyranoside at the concentration of 48 mM showed the best extraction efficiency to isolate CEA and especially CEACAM1 from the membrane fraction of the used cell system. Beside this fact, this non-ionic detergent has the ability to keep membrane proteins in native status (Gould et al., 1981). Additionally, Octyl- β -D-glucopyranoside is also compatible with co-immunoprecipitation assay and Blue native gel electrophoresis. Therefore the described extraction method was used in subsequent experiments.

4.2.2 Tested regulatory active substances

Different biologically active substances were tested for their ability to regulate expression of CEACAM family members with special respect to CEACAM1 expression (Table 2). Substances were chosen according to a special relationship to cancer signaling pathways or metabolism or they were suspected to influence CEACAM1 expression.

Table 2: Summary list of all tested substances

Treatment of HT29p cells with listed compounds was performed for 6 and 18 h, respectively and used concentration of molecules are indicated. Subsequently, 20 μ g of each of total membrane extracts was separated in SDS-PAGE (4-20% acrylamide concentration) and analyzed by Western blot. Monoclonal antibody C1N3 was used to detect alterations in expression pattern of CEACAM family members. Symbol “*” indicates complete detachment of HT29p cells after the treatment with Tunicamycin.

Substance	Characteristics	Effect on CEACAM1	Concentration
Acetaminophen	Anti-inflammatory agent	No effect	50-100-250-500 μ M
5-azacytidin	Inhibitor of methylation gene silencing	Mild down-regulation	1-3 μ g/ml
EGF	Growth factor	Strong down-regulation	20 ng/ml
b FGF	Growth factor	Strong down-regulation	10 ng/ml
Geldanamycin	Hsp90 inhibitor	No effect	100 ng/ml; 1 μ g/ml
Gö 6983	PKC inhibitor	No effect	200 nM; 20 μ M
IL-1 β	Cytokine	No effect	5 μ g/ml
IL-4	Cytokine	Strong down-regulation	10 ng/ml
IL-12	Cytokine	No effect	50-100 μ g/ml
INF- γ	Cytokine	Strong up-regulation	500 U/ml

Substance	Characteristics	Effect on CEACAM1	Concentration
LPS	Lipopolysaccharide <i>Pseudomonas aeruginosae</i>	No effect	25-50-100-250 ng/ml
LPS	Lipopolysaccharide <i>Escherichia. Coli B6:26</i>	No effect	25-50-100-250 ng/ml
Mg132	Proteasome inhibitor	No effect	2 μ M
Ocadaic acid	Ser/Thr phosphatases inhibitor	No effect	10 nM
Orthovanadate Sodium	Tyr phosphatases inhibitor	No effect	100 μ M
PD 98059	MAP-kinases inhibitor	No effect	10 μ M
Staurosporin	PKC inhibitor	Mild up-regulation	25-50 nM
TGF- α	Growth factor	No effect	100-250 μ g/ml
TGF- β	Growth factor	No effect	50-100 μ g/ml
TNF- α	Cytokine	Up-regulation	5-10 ng/ml
Trichostatin	Histone-deacetylase inhibitor	No effect	10-50-100 ng/ml
Tunicamycin	Glycosylation inhibitor	*	100 ng/ml;1 μ g/ml
Wortmannin	PI3 kinase inhibitor	No effect	200 nM

IFN- γ is known to up-regulate CEACAM1 on mRNA as well as on a protein level (Chen et al., 1996; Fahlgren et al., 2003). Therefore it was used as a positive control, where the significant increase of CEACAM1 expression was obtained after treatment with INF- γ for 18 h. The increase of CEACAM1 after INF- γ stimulation was estimated by intensity scanning of the signals after chemiluminescence detection to be approximately six-fold higher in comparison to untreated cells (Fig. 9). Stimulation with TNF- α evoked a weaker, only 1.8-fold increase of CEACAM1 expression when cells were treated with TNF- α for 6 h. Geldanamycin–ansamycin antibiotic showed no regulatory effect on CEACAM1 expression in the concentration range tested (Fig. 9). Nevertheless it was further analyzed because of its characteristics as a potent tumor growth suppressor and a destructor of protein complexes involved in malignant signal transduction pathways.

Finally, for the growth factors EGF (20 ng/ml) and b FGF (10 ng/ml) a strong down-regulatory properties on HT29p cell line could be shown. The strongest CEACAM1 expression inhibitor-EGF was selected for more detailed studies. Immunoblot developed with C1N3 antibody (Fig. 9, upper panel) shows all candidates, which were able to significantly

modify expression of CEACAM family members. To control equal loading of proteins into each lane, β -actin detection was also performed (Fig. 9, lower panel). $\text{IFN-}\gamma$, $\text{TNF-}\alpha$ -molecules with up-regulatory activity, EGF as a potent suppressor of CEACAM1 protein expression and geldanamycin were subsequently studied.

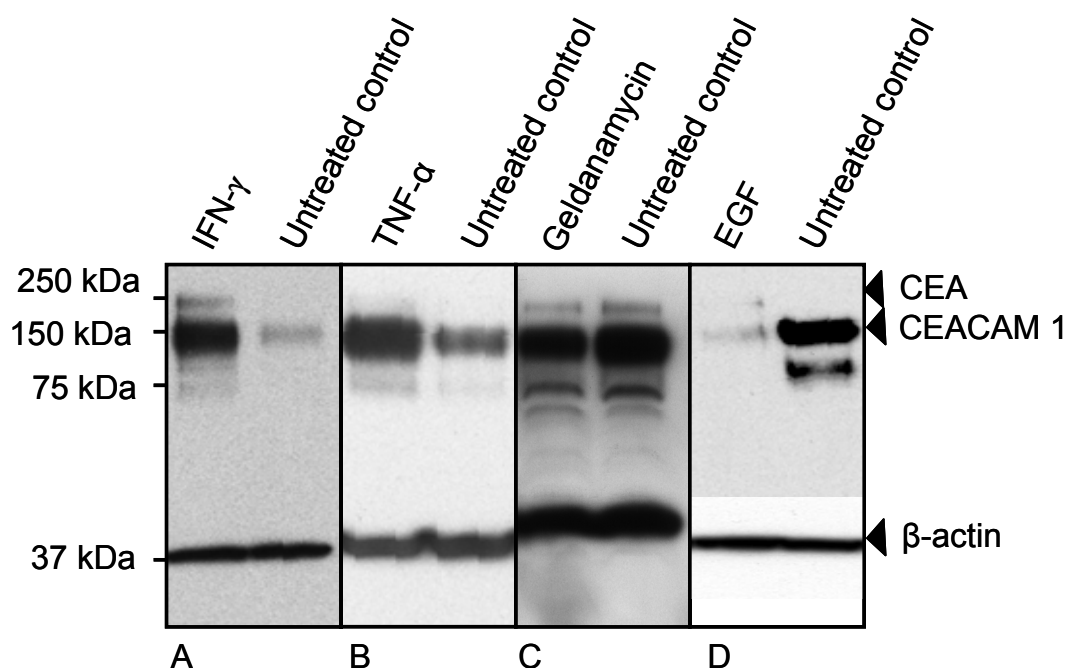


Figure 9: Effect of selected substances on CEA and/or CEACAM1 expression

HT29p cells were treated with $\text{IFN-}\gamma$ (panel A) for 18 h, $\text{TNF-}\alpha$ (panel B) for 6 h, geldanamycin (panel C) for 18 h, and with EGF (panel D) for 18 h. Membrane associated proteins from each experiment were separated in SDS-PAGE (4-20% linear gradient) and CEACAM family members were detected on Western blot with C1N3 mAb. Each panel was developed separately, because of individual film-expose duration needed to obtain a visible signal with ECL chemiluminescence substrate for HRP. Western blot showing CEACAMs in EGF-stimulated cells (panel D) was developed with above mentioned system for 40 min to visualize CEA and CEACAM1 bands. β -actin detection served to control equal loading of proteins on the gel.

4.3 The structural stability and functional alterations of CEACAM1 after the treatment with INF- γ , TNF- α , geldanamycin and EGF

The influence of the protein expression, namely CEACAM1 and CEA expression, can be accompanied by the structural and the functional alterations of a target protein. The synthesis of CEACAM1 as a transmembrane phosphorylatable glycoprotein includes a highly sophisticated complex stepwise co- and posttranslational modifications as well as a complicated trafficking to final destination probably along the vesicle transport.

4.3.1 The alterations in CEACAMs expression pattern

To find out if altered expression of CEACAM1 and CEA after the treatment with studied molecules is accompanied with localization changes, FACS analysis of HT29p cells was performed. Cells were, previously to antibody staining, permeabilised with Methanol and compared to unpermeabilised cells. In the case of the unpermeabilised cells, antibodies can only recognize the antigen present on the cells surface. Additionally, in the case of the permeabilised cells antibodies are capable of diffusing into the cytosol and recognizing antigen. Subsequently, cells were detected with secondary antibody directly conjugated with Fluorescein. The rate of Fluorescein staining of CEACAM1 and CEA was measured by Flow cytometry (Fig. 10).

Staining with C1P83 suggested no significant CEA presence in untreated HT29p cells (compare paragraph 4.1.1), probably because of CEA release into the medium as a result of the polarization lost of cancer cells.

IFN- γ -mediated up-regulation of CEACAM1 and CEA caused an increase of an intracellular (cytosolic) production of both molecules and it reached a maximum after 6 h incubation with IFN- γ (Fig. 10A). The cell surface expression of CEACAM1 and CEA had a progressive tendency with a maximal rate at the 48 h stimulation time point (Fig. 10B).

Cells, that are treated with TNF- α as a second up-regulatory active substance in the primary screening, were also analyzed by flow cytometry under the conditions as described above for IFN- γ . The analysis of the members of CEACAM family in permeabilised (Fig. 10C) as well as in unpermeabilised cells (Fig. 10D) showed no significant differences in comparison to an untreated cell. This can be explained by an insufficient sensitivity of the detection method to reveal small differences in protein expression. However, as was already mentioned both

cytokines differ in the intensity of their up-regulatory effects on CEACAMs expression (Fig. 9).

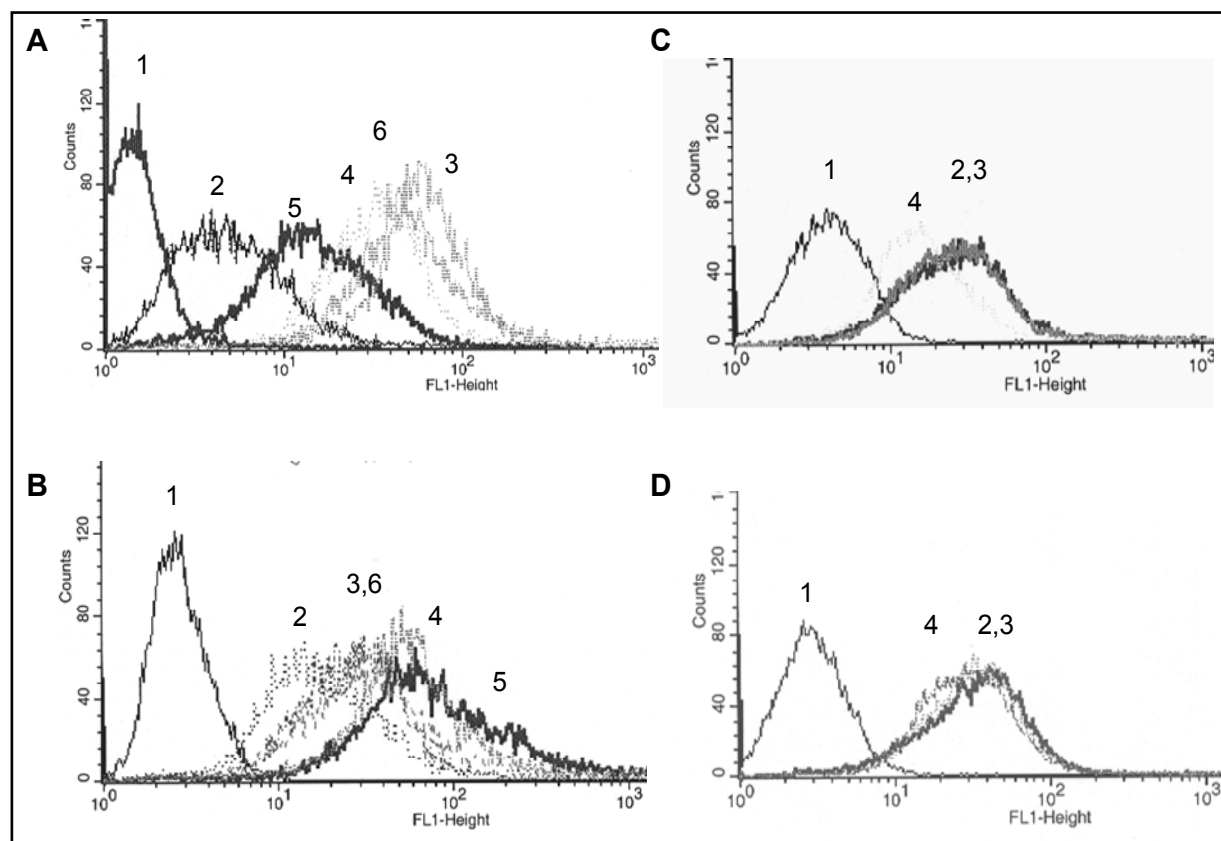


Figure 10: FACS analysis of total (A, C) and surface (B, D) expression of CEACAM family members.

HT29p cells were treated either with 500 U/ml of $\text{INF-}\gamma$ (panels A, B) for 0 h (2), 6 h (3), 24 h (4), 48 h (5), 72 h (6) or with 10 ng/ml of $\text{TNF-}\alpha$ (panels C, D) for 0 h (2), 6 h (3) and 24 h (4). Afterward, cells were permeabilised with 80% Methanol to allow C1N3 mAb diffusion into cells and the recognition of the cytosolic CEACAM members as well as the surface CEACAM members (A, C), while only surface CEACAM members were detected with mAb without previously permeabilisation of cells (B, D). Subsequently, cells were incubated with goat anti-mouse IgG+IgM antibody directly conjugated with Fluorescein and rate of fluorescence staining of cells was measured with flow cytometer.

Autofluorescence of treated cells was measured to estimate the background yield (1).

4.3.2 CEACAM1 Tyrosine phosphorylation status

CEACAM1 is a transmembrane glycoprotein. Its cytoplasmic domain contains a motif related to that of Immunoreceptor Tyrosine-based Activation (or Inhibition) motif (ITAM/ITIM) (Kammerer et al., 1998). CEACAM1 is suspected to be strongly involved in the signal transduction pathways through its ability to alter tyrosine phosphorylation status. We have investigated the CEACAM1 tyrosine phosphorylation response due to influences of the selected molecules (Fig. 11).

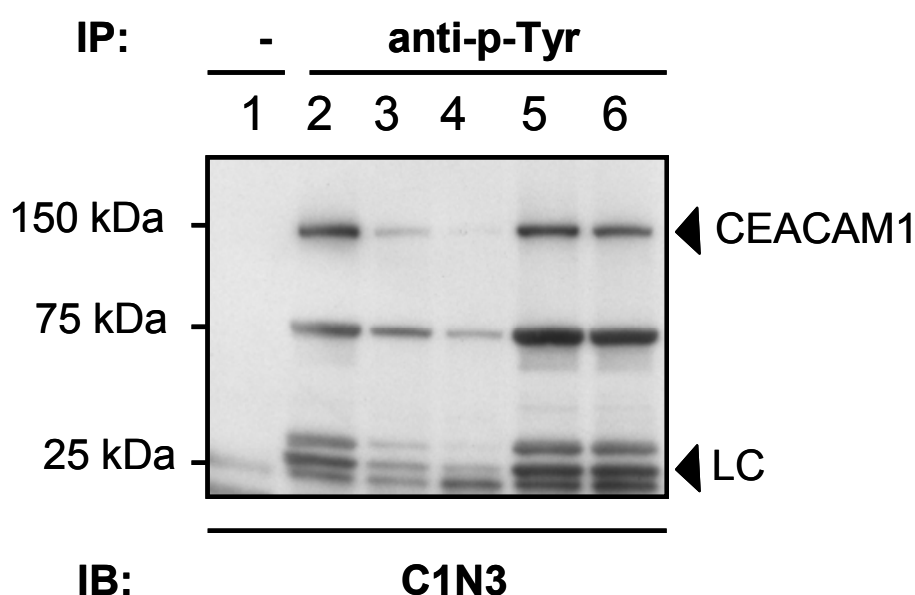


Figure 11: Phosphorylation assay: The determination of CEACAM1 phosphorylation

HT29p cells were treated with studied substances and the membrane proteins were isolated with a buffer containing phosphatases inhibitors. Proteins containing phosphotyrosine in their structure were immunoprecipitated with anti-phosphotyrosine specific mAb. Precipitated proteins were separated in SDS-PAGE. Western blot developed with C1N3 mAb directly conjugated with HRP visualized the alterations of CEACAM1 phosphorylation after treatment with EGF (lane 3), geldanamycin (lane 4), IFN- γ (lane 5), TNF- α (lane 6) in comparison to untreated cells (lane 2).

No signal was obtained in lane 1 (also developed with C1N3) showing that no unspecific binding to the protein G-agarose occurs. Bands detected around 25 kDa belong to Ig-light chain (LC).

HT29p cells were treated with selected molecules. Subsequently the cell membrane fractions were prepared under native conditions accompanied by an addition of strong tyrosine

phosphatases inhibitors to block any dephosphorylation activities inside the cells. Immunoprecipitation was performed using anti-phosphotyrosine antibody to specifically enrich all proteins containing phosphotyrosine. After separation by SDS-PAGE and Western-Transfer, detection of CEACAM1 was performed with C1N3 antibody. The intensity of signals representing phosphorylated CEACAM1 after IFN- γ as well as TNF- α stimulation of cells was comparable with that of untreated cells (Fig.11, lane 2). This finding suggests that IFN- γ up-regulation of CEACAM1 expression did affect phosphorylation dynamics inside ITAM/ITIM-like motif in a negative way (Fig.11, lane 5), as CEACAM1 increased protein expression due to IFN- γ stimulation was not accompanied with a proportional increase in the phosphorylation of CEACAM1. INF- γ did not trigger tyrosine phosphorylation of CEACAM1.

As it was already mentioned, TNF- α treatment moderately increase CEACAM1 expression at a low rate (compare figure 9 and 10 C, D). CEACAM1 phosphorylation is not significantly altered (Fig.11, lane 6) in comparison to untreated cells (Fig.11, lane 1). In contrast, geldanamycin (Fig.11, lane 4) and EGF (Fig.11, lane 3) treatments caused dramatic decrease of CEACAM1 detectable by C1N3 by Western blotting. In the case of EGF, the lower rate of phosphorylation after EGF treatment could be partially influenced also by decreased expression of CEACAM1 in HT29p cells, where the relative ratio between phosphorylated and dephosphorylated CEACAM1 is stored. After geldanamycin treatment CEACAM1 tyrosine-phosphorylation is decreased in comparison to the untreated cells. In contrast to any other agents tested, geldanamycin is the only one showing no influence on CEACAM1 steady state level (Fig. 9). Therefore down-regulation of CEACAM1 phosphorylation after treatment with geldanamycin is comparable with the control (without treatment). Thus, the tyrosine phosphorylation of CEACAM1 is directly influenced by geldanamycin suggesting the involvement of CEACAM1 in the signal transduction network.

4.3.3 Analysis of multi-protein complexes containing CEACAM1

CEACAM1 protein-protein interactions with the compounds of signal transduction network, receptor and non-receptor kinases (Afar et al., 1992; Najjar et al., 1995), phosphatases (Beauchemin et al., 1997), and some cytoskeleton proteins (Sadekova et al., 2000) were previously described. It is also known that members of CEACAM family of adhesion molecules are using homo-/heterophilic interactions between each other to create the glycocalyx structure on the apical surface of epithelial cells (Hammarström, S., 1999). All of

these findings are based on indirect (mostly co-immunoprecipitation) assays and therefore they can include also short time-transient associations due to the de-/phosphorylation dynamic character of CEACAM1. Until now, no method showing directly an evidence for the existence of long term interactions of CEACAM1 leading to protein complex formation was described.

Blue native gel electrophoresis (BNG) is a method which allows us to visualize protein complexes with their single compounds. In this study, BNG was established for examination of CEACAM1 containing complexes of HT29p cell membrane.

Glycoproteins enriched membrane fractions from treated and untreated HT29p cells were prepared with the use of WGA lectin affinity chromatography followed by BNG analysis (Fig. 12).

As shown in Figure 12 untreated cells contain several high molecular weight complexes containing CEACAM1 and the size of these complexes was estimated from 500-800 kDa (Fig.12, panel A). No CEA was detectable in visualized protein complexes with either C1N3 or T84-1, respectively. CEACAM1 was the only one molecule forming complexes form and also a protein monomer was detected. In comparison to untreated cells (protein complexes detectable between 6-8% polyacrylamide gradient gel), IFN- γ (Fig.12, panel B) and TNF- α (Fig.12, panel C) treatment had decreased number of different complexes, especially those with lower molecular weight. IFN- γ –stimulated increase of the intensity of signals presenting single complexes as well as CEACAM1 monomer suggested up-regulatory effect of this substance also on complex formation. After cytokines treatment we observed also other interesting alteration of complexes, i.e. the additional presence of C1N3 detectable protein with a size of approximately 120 kDa. This molecule very probably presents one of the different isoform of CEACAM1, because all tests to specify the identity of this molecule with antibodies against CEA, CEACAM6 and 7 failed. Another situation became visible after cells were treated with geldanamycin ((Fig.12, panel D). Geldanamycin caused under these experimental conditions complete disruption of CEACAM1 containing protein complexes while monomeric forms of CEACAM1 were not changed. EGF treated cells expressed so low levels of CEACAM1, that to analyze them with this method is not reasonable.

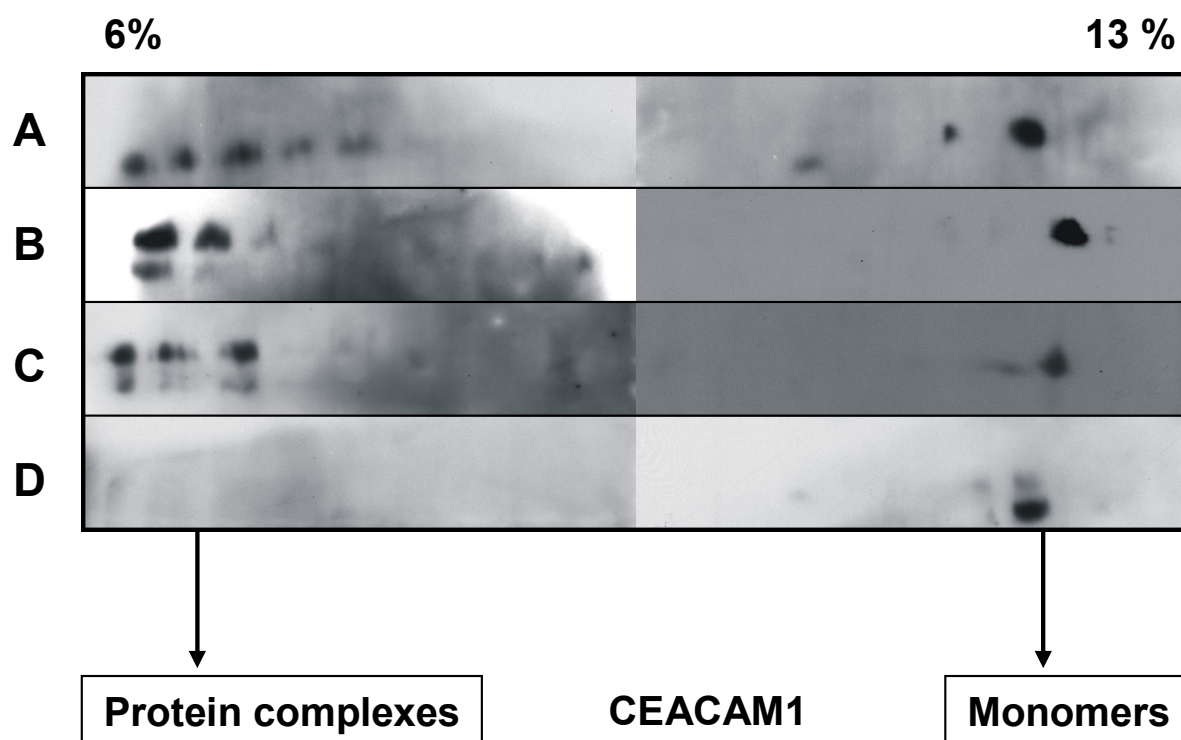


Figure 12: The investigation of CEACAM1 containing complex formation

The glycoproteins containing N-acetyl glucosamine in their structure were pre-purified from lysate of HT29p cells with use of WGA lectin affinity chromatography. Glycoproteins mobility inside the first native dimension was facilitated with addition of Coomassie blue G-250 to sample buffer as well as to cathode buffer. Subsequently, the second SDS-PAGE dimension was used to separate the single proteins according their Mr. Immunoblot from second dimension was developed with C1N3 to estimate number of complexes and to characterize CEACAM members forming complexes. Protein complexes were observed inside the gradient gel ranging between 6 and 8% acrylamide (v/v) concentration (left side of panels), while monomers of CEACAM1 were presented in 12-13% gradient gel (right site of panels). The influences of IFN- γ (panel B), TNF- α (panel C), and geldanamycin (panel D) on CEACAM1 containing complexes formation were compared with this in untreated cells (panel A).

4.4 Identification of a novel interaction partner of CEACAM1

How to explain the specific action of geldanamycin on CEACAM1 complex formation?

There is no evidence in published literature that CEACAM1 belongs to the geldanamycin-sensitive molecules. However, ansamycin antibiotics inclusive geldanamycin, are well characterized specific inhibitors of molecular chaperone Hsp90 and they are responsible for

ubiquitination of the large membrane proteins requiring Hsp90 guarding during the cellular trafficking and properly folding during internalization (Young et al., 2001).

Thus, already described results suggested a hypothesis about the involvement of Hsp90 in the CEACAM1 protein complexes formation. Therefore, the co-immunoprecipitation assay was performed to show possible association of CEACAM1 with Hsp90 chaperone.

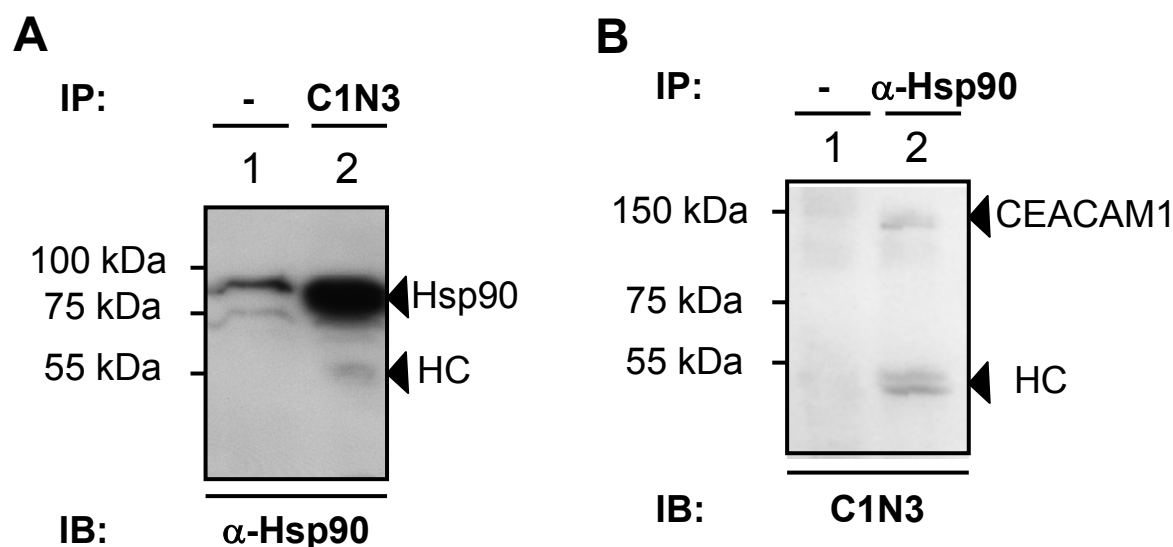


Figure 13: Co-immunoprecipitation of CEACAM1 with molecular chaperone Hsp90

The membrane proteins from HT29p cells were immunoprecipitated with C1N3 mAb coupled to protein G-agarose. The antibody-bound fraction was separated by SDS-PAGE and Western blot transfer was performed. The staining of membrane with mAb against Hsp90 showed positive Hsp90 signal in C1N3-bound fraction (panel A, lane 2). Hsp90 was partially bound unspecifically to protein G-agarose (panel A, lane 1).

In reverse scenario, α -Hsp90 mAb was used as a catching mAb. CEACAM members able to associate with Hsp90 were detected on Western blot developed with C1N3 mAb (panel B, lane 2).

Unspecific binding of CEACAM members onto protein G-agarose was also investigated (panel B, lane 1).

Hsp90 was detected in immunocomplex when C1N3 antibody was used as a catching antibody for CEACAM members (Fig. 13A, lane 2). Some of Hsp90-positive signals were detected also in agarose control representing unspecific binding (Fig. 13A, lane 1), but it was significantly weaker in comparison to C1N3-specifically bound immunocomplexes. However, used catching antibody C1N3 is define to be broad-specific and did not suffice for a

determination of binding partner for Hsp90. To decide if Hsp90 binds to CEACAM1 specifically, we repeated co-immunoprecipitation in reverse scenario. The anti-Hsp90 antibody was used for catching of the protein complexes hypothesized to contain CEACAM1 (Fig. 13B, lane 2). A single protein about 150 kDa was obtained and identified as CEACAM1. No CEA was shown to react with Hsp90. Geldanamycin effect on CEACAM1 complexes and down-regulation of CEACAM1 phosphorylation without any effect on protein expression and the fact that Hsp90 interacts with CEACAM1 suggested that CEACAM1 complexes include some kinases compound requiring Hsp90 for its biological action.

4.5 The effect of studied substances on cell biology

4.5.1 The effect on cell viability and proliferation

Already within the primary screening it was observed that some of tested substances evoke macroscopically visible cells alterations. To characterize such effects in more detail, Alamar Blue fluorescence assay was employed. Different numbers of HT29p cells were seeded to 96-well plates in RPMI medium containing 10% FCS (Fig. 14B) and second set of cells were maintained in RPMI medium supplemented only with 0.5% FCS (Fig. 14A). Subsequently, Alamar blue “ready to use” solutions was pipetted to each well and incubate for next 6-8 h. Fluorescence intensity was measured in proportional dependency on the cell number. This optimization experiment determinates the cell number by which fluorescence reached optimal value falling into linear range of optimization curve.

The up-regulatory active IFN- γ (500 U/ml), TNF- α (10 ng/ml), the down-regulatory active EGF (20 ng/ml) and geldanamycin (1 μ g/ml) were added into the cell culture to check out if cell viability can be also influenced under these conditions. Proliferation and cell viability alterations of HT29p cells treated for either 6 h (in case of TNF- α) or 18 h (in case of IFN- γ , EGF, and geldanamycin) were measured with Redox-sensitive substance, where increase in fluorescence intensity signaled linear growing up with number of live cells. Histograms (Fig. 15, left panel) are presenting graphic results of these measurements. IFN- γ stimulation of CEA and CEACAM1 evoked about 62% decrease in cell proliferation and viability rate what is probably accompanied also by cell death (apoptosis) of the originally seeded cells. Test does not distinguish between limited cell division and apoptosis. TNF- α influence on cell proliferation and viability had also a suppressive effect (about 31%). EGF, beside strong

down-regulatory effect on CEACAM1, caused also a clear increase of cell viability (about 60%). Geldanamycin with its lack of effects on CEACAM1 expression, but complex destructive properties caused the lowest decrease of cell proliferation rate (12. 6%).

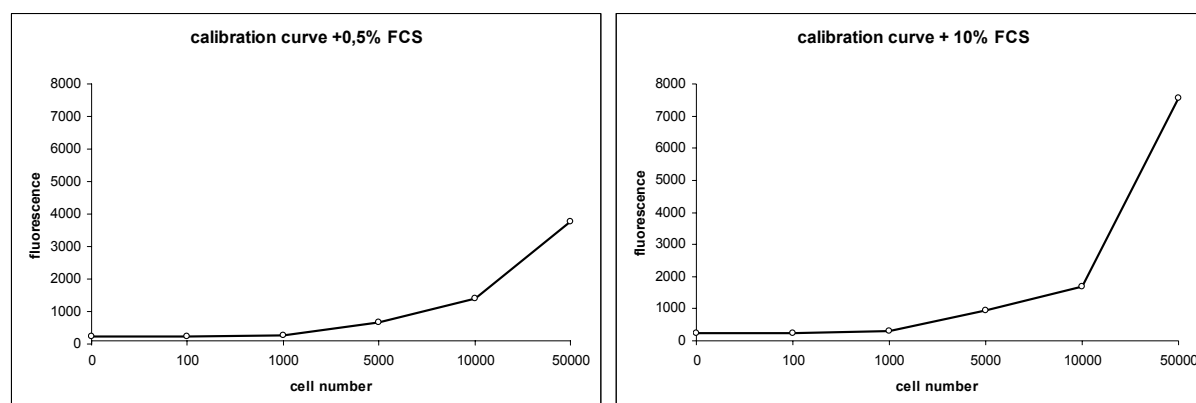


Figure 14: Optimization of the cell number and FCS condition for Alamar proliferation assay

The important step prior to the investigation of the cell proliferation changes caused by tested regulatory active molecules is to optimize a cell number and conditions under which experiments are performed. Two sets of cells were therefore seeded and maintained for 40 h either in RPMI medium supplemented with 0.5% FCS (left panel) or the same medium supplemented only with 10% FCS (right panel). Each cell set included cell number ranging between 0 and 5×10^5 cells per well.

The standard deviations to these experiments were under 10%.

4.5.2 DNA synthesis

As mentioned above the effect of tested CEACAM1 regulators on cell viability is already significant and results suggested biological effects of selected substances. However, cell number is the factor varying with environmental characteristic and does not answer question about exact determination of processes leading to decrease of cell viability.

To follow process of DNA synthesis, radioactive labeled thymidine was used. [^3H]-thymidine uptake is not increased only by synthesis of DNA *de novo*, but is observed also by the processes of DNA repair.

HT29p cells were seeded in 96-well plates and maintained for 20 h in complete RPMI medium containing 10% FCS following by substitution of FCS by 0.5% BSA in the medium. The mentioned FCS-BSA substitution was thought to prevent interfering influences of FCS components. Cells were adapted to these conditions and subsequently, tested substances were

added in certain concentrations. Incubation of cells with [^3H]-thymidine was followed by permeabilisation of the cells with SDS and harvesting. Rate of new DNA synthesis was measured with a scintillation counter.

To find out if effects of IFN- γ , TNF- α , EGF and geldanamycin on proliferation and viability are results of the alterations during the cell division, [^3H]-thymidine incorporation test was used (Figure 15, right panel).

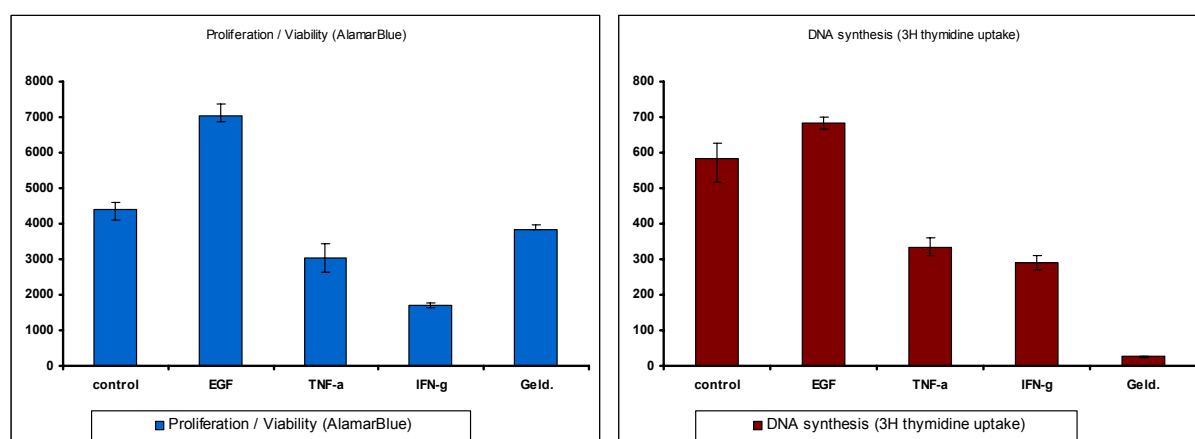


Figure 15: Effect of IFN- γ , TNF- α , geldanamycin and EGF on DNA synthesis and the cell proliferation

The cells treated either with IFN- γ , geldanamycin and EGF or with TNF- α in serum-free conditions followed by addition of [^3H]-thymidine and permeabilisation with SDS. DNA content was estimated in dependency on rate of [^3H]-thymidine incorporation. Results are illustrated with use of histogram graphs (right panel).

To check effect of studied substances on cell proliferation, an Alamar blue fluorescent proliferation kit was used (left panel). The intensity of measured fluorescence was proportionally related to alterations in cell number. Histograms (left panel) are presenting graphic results of these measurements.

The decrease in DNA synthesis crossly parallels to the reduced cell viability measured by Alamar proliferation assay. These observations support the fact, that IFN- γ stimulation of CEACAM1 and partially also CEA influenced DNA synthesis in significant rate. TNF- α (about 43%) decreased cell division in rate comparable to inhibition rate on proliferation. By EGF treatment of cells we have observed increase of [^3H]-thymidine incorporation about 17%, what probably represents complete proliferation increase. Both of used methods to look for EGF effect on cell viability and proliferation shows increase of cell number. More than

95% decrease of DNA synthesis after geldanamycin treatment is value describing the cell division arrest in G₀/G₁ phase.

4.5.3 Cell cycle alterations

Another possibility to monitor cell proliferation is to investigate cell cycle with respect to variations in single phases during cell division.

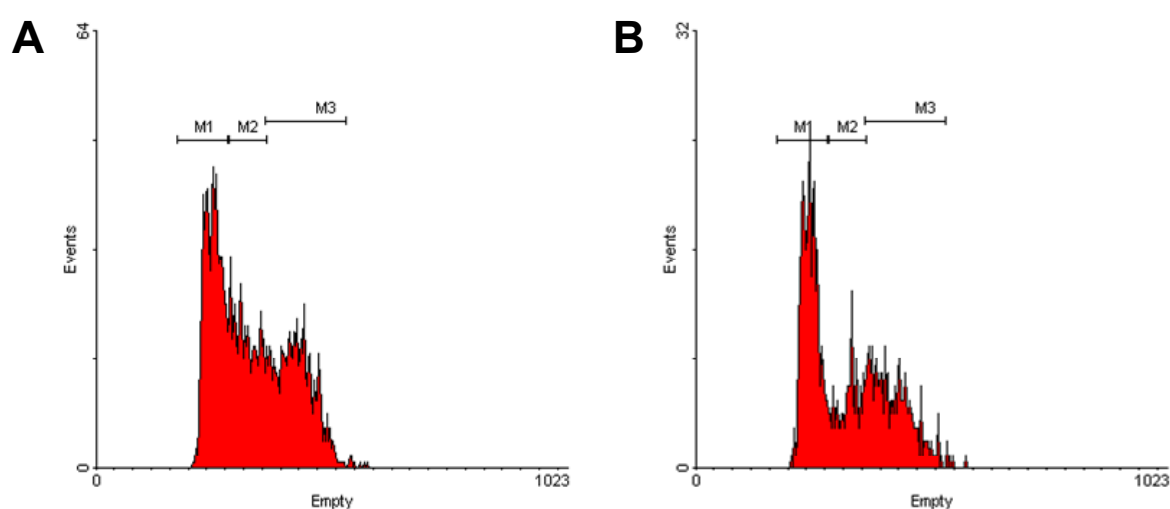


Figure 16: Geldanamycin-mediated G₀/G₁ arrest

HT29p cells were maintained in BSA-supplemented medium (panel A) or in medium additionally containing 1 $\mu\text{g/ml}$ of geldanamycin (panel B). The intensity of fluorescence staining with PI was proportionally correlated with total DNA content and fluorescence describing each cell cycle phase was statistically evaluated.

Cell number between 0.5×10^6 - 1×10^6 of HT29p cells untreated or treated with tested substances were permeabilised. Consequently, the appropriate incubation with RNA-se prevented false positive signal standing out from RNA. DNA was stained with use of Propidium Iodide (PI) with final concentration of 50 $\mu\text{g/ml}$ and results of this staining were measured with flow cytometry.

Table 3 shows statistic evaluated results of these experiments.

Both cytokines, IFN- γ and TNF- α did not evoke significant alterations in cell cycle course. More interesting results were obtained after geldanamycin treatment (Fig. 16).

The results of [3 H]-thymidine uptake experiments showed strong effect of geldanamycin on DNA synthesis following by the alteration in the growth and the cell proliferation. Propidium Iodide staining of DNA of HT29p cells treated with geldanamycin had also proved effect on cell cycle. Relative percent of cells entering G₀/G₁ phase was increased from 39.6 to 50.7 % accompanied by decrease in S phase suggesting G₁ arrest (Fig. 16). EGF treatment evoked a slight increase of the cells in S-phase proving stimulatory effect on HT29p cell proliferation shown by Alamar blue proliferation assay.

Table 3: Statistic evaluation of the cell cycle measurements in HT29p cells untreated and treated with tested substances

Cells were after treatment with IFN- γ , TNF- α , geldanamycin and EGF permeabilised and DNA was stained with a fluorescent dye PI. The intensity of staining was visualized by flow cytometry. Values are counted in relative percent and indicate participation rates of each cell cycle phase to complete DNA content.

	% events/ gate	% G ₀ /G ₁ phase	% S phase	% G ₂ /M phase
Untreated cells	100	39.6	29.5	35.4
IFN-γ	100	34.8	31.7	37.0
TNF-α	100	35.6	33.3	35.8
Geldanamycin	100	50.7	20.3	31.7
EGF	100	37.4	37.3	30.0

4.6 Antagonistic effect of CEACAM1 on EGF-stimulated cell proliferation

According previously described results a relation between CEACAM1 expression in HT29p cells and EGF stimuli. C1N3 mAb is able to bind CEA and CEACAM1 under the native conditions and to estimate effect of antibody on behavior of EGF-stimulated cells, a

competition experiment was performed. Thus, results suggested possible agonistic or antagonistic function of CEACAM1 on EGF-stimulated cell proliferation.

Cells were seeded in 96-well plate in complete RPMI medium supplemented with 10% FCS in different cell numbers and allowed to adhere for at least 24 h. Afterward cells were washed with PBS and fresh medium with 20 ng/ml of EGF, 50 μ g/ml of C1N3 or combination of both EGF/C1N3 were added for next 12 h. Subsequently, the fluorescence intensity was measured to estimate rate of proliferation.

Histogram was used to visualize schematically results obtained from this experiment (Fig. 17). Monoclonal anti-mucin RA96 mAb was used in combination with EGF as a control for C1N3 antibody.

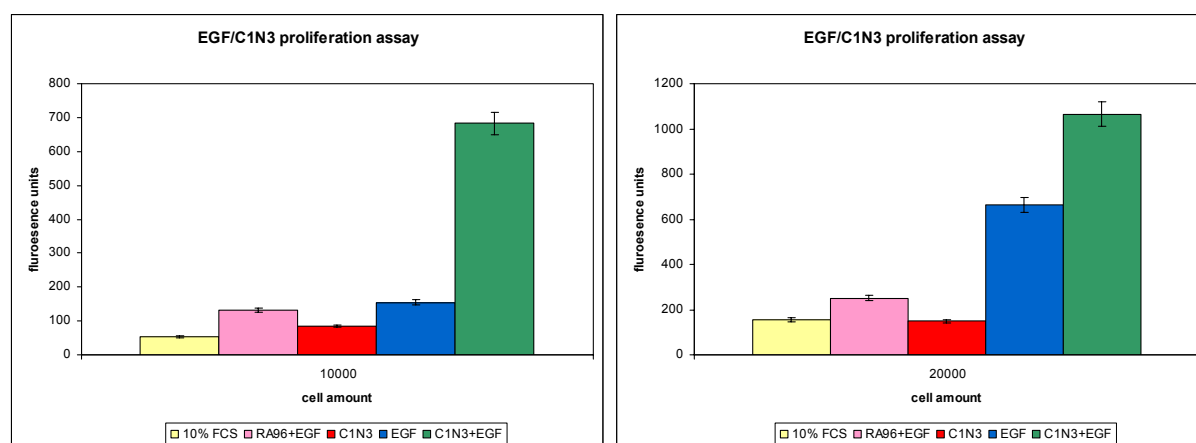


Figure 17: The effect of EGF, C1N3 and combination of EGF/C1N3 on the cell proliferation

Alamar blue fluorescence cell viability and proliferation assay was performed to estimate effect of EGF (blue barrow), C1N3 (red barrow) antibody and their combination (green barrow) on HT29p cells in comparison to untreated cells in complete medium with 10 % FCS supplementation (yellow barrow). EGF (20 ng/ml), C1N3 (50 μ g/ml) or combination of both of them were added to the cell numbers 1×10^4 (left panel) and 2×10^4 (right panel) per well for 12 h followed by the addition of Alamar blue substrate and measurement of fluorescence intensity proportionally reflecting alterations in cell number after treatment. Combination of EGF and R96 (anti-mucine mAb) was also investigated (pink).

The incubation of cells with medium containing C1N3 antibody evoked decrease in cell number in comparison to untreated cells suggesting that C1N3 inhibit tumor growth *in vitro* or stimulate apoptosis of HT29p cells under used condition. Toxic effect of antibody can not

be excluded and need further investigation. EGF alone stimulated cell proliferation as expected. Interestingly, cells exposed to EGF/C1N3 combination treatment revealed a 5 (Fig. 17, left panel) and 7-fold (Fig. 17, right panel) increase of cell number in comparison to untreated cells (control) and 1.6 (Fig. 17, left panel) -4-fold (Fig. 17, right panel) increase of cell number in comparison to cells treated only with EGF alone. These results suggested an antagonistic effect of CEACAM1 on EGF stimulated pathway and for further growth and proliferation of HT29p cells after EGF treatment.

4.7 CEACAM1 involvement in EGF-triggered response

EGF stimulate in colon cells phosphorylation of epidermal growth factor receptor (EGFR) followed by further phosphorylation steps mediated by this receptor tyrosine kinase and activation of the Ras/MAP kinase mitogenic signaling pathways that regulate cell growth, proliferation and apoptosis (Blenis et al., 1993). All our findings in respect to EGF were observed under conditions where cells were maintained in RPMI medium supplemented with 10% FCS. Foetal calf serum may contain plasmatic fatty acids which are also able to activate EGFR. To distinguish specifically between EGF action on CEACAM1 molecular characteristics (like phosphorylation state, protein complex formation and possible association with EGFR) fatty acids-containing FCS was removed from culture medium and it was substituted by 0.5% BSA. Concentration of EGF used for cell stimulation was decreased from reasons described above to 100 nM. HT29p cells were not able to grow under FCS-reducing conditions for long term. Therefore cells were seeded and let adhere for 24 h period of time followed by 6 h adaptation phase in medium containing 0.5% BSA and only thereafter they were stimulated with 100 nM EGF.

4.7.1 EGF-dependent phosphorylation of CEACAM1 in fatty acids-free conditions

We hypothesized that if CEACAM1 is involved in the Ras pathway as suggested via its interaction with Shc adaptor protein, phosphorylation steps leading to pathway activation will lead be reflected on CEACAM1 tyrosine phosphorylation.

Co-immunoprecipitation assay was employed to prove this idea. HT29p cells were maintained in medium containing 0.5% BSA followed by EGF stimulation in different time points ranging between 0 and 60 min. The membrane associated proteins containing phosphotyrosine were bound to anti-phosphotyrosine antibody. Subsequently, the immunocomplexes were analyzed by Western blotting with C1N3 antibody directly conjugated with HRP to detect quantitatively CEACAM1 molecules phosphorylated on their tyrosine residues as a result of EGF stimulation (Figure 18A).

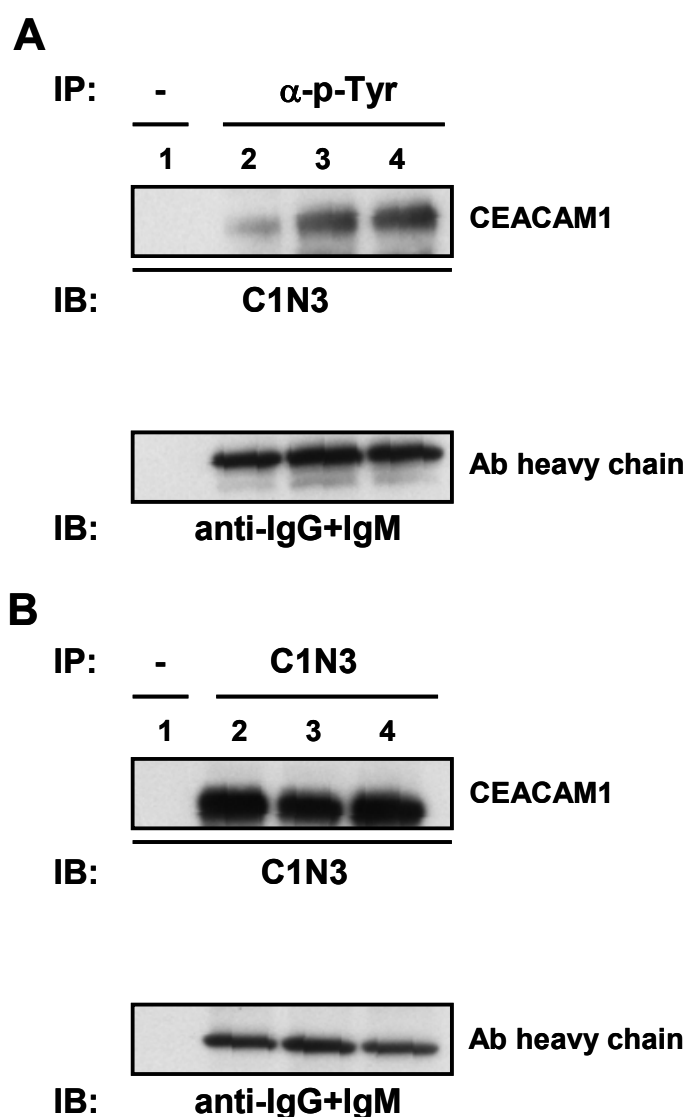


Figure 18: EGF-stimulated CEACAM1 phosphorylation under the FFA-free conditions

HT 29p cells were growing for at least 18 h in complete RPMI medium supplemented with 10% FCS followed by 6 h maintenance in 0.5% BSA containing RPMI medium (upper panels A and B, lane 2). Subsequently, cells were treated with 100 nM EGF for 5 (upper panels A and B, lane 3) and 10 min (upper panels A and B, lane 4). Membrane fraction from HT29p cells was immunoprecipitated with either anti-phosphotyrosine mAb (panel A) or with C1N3 mAb (panel B). Western blot detection was performed with C1N3 mAb (upper panels A, B)

To check unspecific binding of proteins to agarose (upper panels A and B, lane 1) same amount of proteins was incubated without addition of Ab. To control equal

amounts of Abs used for immunoprecipitation, lower panels in A, B represent the 55 kDa heavy chain of IgG, detected with goat anti-mouse IgG Ab.

As expected, CEACAM1 tyrosine phosphorylation is a transient process and phosphorylation differences became significantly recognized at time points 0, 5, 10 min. Any further prolongation of EGF stimulation caused the significant alteration of the phosphorylation state of CEACAM1.

EGF stimulation evoked CEACAM1 phosphorylation in time dependency while amounts of CEACAM1 provided for each time point were concurrent (Fig. 18B).

EGF stimulation of cells increased phosphorylation rate after 5 min (Fig. 18A, upper panel, lane 3) and 10 min (Fig. 18A, upper panel, lane 4) in comparison to untreated cells (Fig. 18A, upper panel, lane 2). No signal was detected with C1N3 antibody in control for unspecific binding to protein G-agarose (Fig. 18A, upper panel, lane 1).

To check out equal binding of anti-phosphotyrosine antibody (Fig. 18A, lower panel) or C1N3 (Fig. 18B, lower panel) antibody to protein G-agarose, blots were re-probed only with anti-mouse IgG antibody conjugated with HRP.

4.7.2 Protein complex formation after EGF stimulation

The ability of CEACAM1 to build up high molecular weight protein complexes was investigated with the use of already described BNG. EGF under FA-free conditions stimulated CEACAM1 phosphorylation and we were interesting if this alteration of primary structure will be reflected in CEACAM1 ability to associate in protein complexes. For this propose membrane proteins were separated in the native first dimension electrophoresis. Proteins were transferred onto the membrane and developed with C1N3 antibody (Fig. 19).

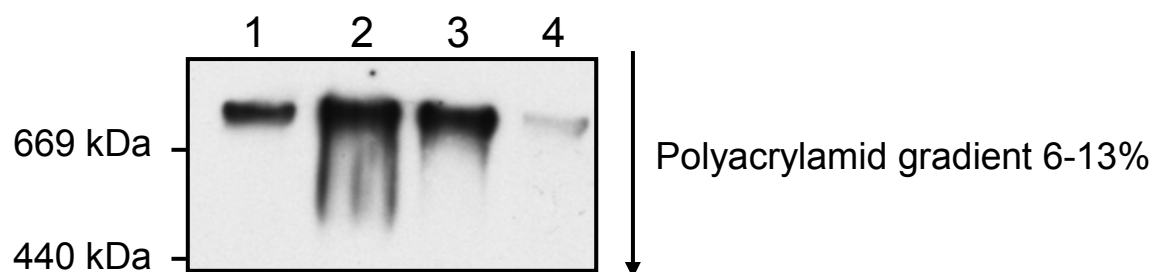


Figure 19: The identification of CEACAM1 containing complex formation after EGF treatment

HT 29p cells were adjusted to FFA-free conditions during 6 h incubation in 0.5% BSA containing RPMI medium (lane 1) and after treatment with EGF for 5 min (lane 2), 10 min (lane 3) and 60 min (lane 4), cells were harvested into BNG lyses buffer. Membrane fractions were analyzed in first (native)

dimension and Western blot analysis with C1N3 mAb was performed to detect CEACAM1 containing complexes. High molecular weight standards were used to estimate size of studied complexes.

The results showed that increased CEACAM1 phosphorylation is accompanied by the increase association activity of CEACAM1 with another proteins leading to an occurrence of complexes. Complex formation reached maximum after 5 min (Fig.19, lane 2) and association intensity is probably going down after 10 min (Fig.19, lane 3) stimulation with EGF in comparison to untreated cells (Fig.19, lane 1), while 60 min stimulation caused significant decrease of complex formation (Fig.19 lane 4).

4.7.3 EGF–stimulated interaction of CEACAM1 with EGFR

All of so far described findings suggest direct relationship of CEACAM1 to EGF-stimulated signaling. EGF-triggered pathway can be activated via autophosphorylation of different receptor tyrosine kinases and in mentioned case it is EGF receptor. Specific and short term response of CEACAM1 in phosphorylation dependent manner followed by increase intensity of CEACAM1 containing protein complex formation are leading to hypothesis about substrate relationship of CEACAM1 to EGFR.

To prove this hypothesis, co-immunoprecipitation assay with use of anti-EGFR antibody (Fig. 20A) and parallel control experiment using C1N3 antibody (Fig. 20B) as catching antibodies was performed.

Immunoprecipitated CEACAM1 isolated from the membrane fraction of HT29p cells after stimulation with EGF were analyzed by Western blot. Detection of CEACAM1 was performed with C1N3 antibody. Results manifested not only a physical association between CEACAM1 and EGFR, but also dependency of this interaction on duration of EGF stimulation. The increased association rate was observed after 5 min of stimulation with EGF (Fig. 20A, lane 3) in comparison to untreated cells (Fig. 20A, lane 2). Prolongation of EGF incubation with cells for 10 min did not significantly increase rate of association (Fig. 20A, lane 3) and the intensity of CEACAM1 band is identical with that of untreated cells. Possible unspecific binding of CEACAM1 to protein G-agarose leading to false positive interpretation of results was checked out (Fig. 20A, lane 1) as well. Parallel experiments were performed in same manner as described above with C1N3 antibody to prove equal amount of CEACAM1 protein in membrane fraction used for immunoprecipitation (Fig. 20B, lane 1-4).

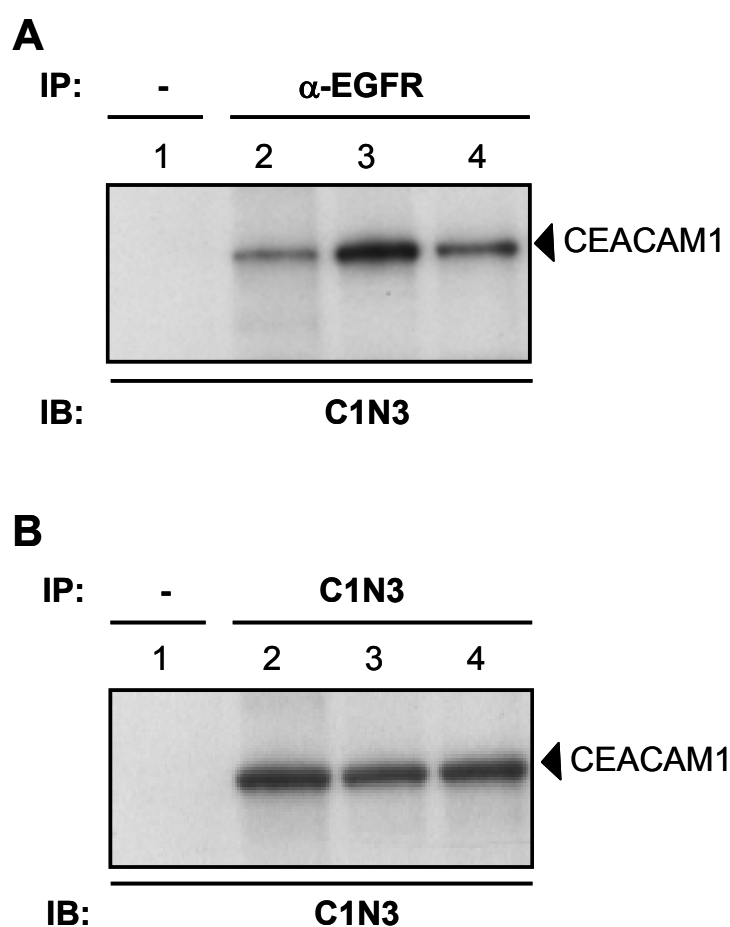


Figure 20: Physical association between CEACAM1 and EGFR co-immunoprecipitation experiments

HT29p cells were stimulated with EGF for 0 (panel A and B, lane 2), 5 (panel A and B, lane 3), and 10 min (panel A and B, lane 4) under serum free conditions followed by the isolation of membrane proteins. The membrane proteins were immunoprecipitated either with anti-EGFR mAb (panel A) or with C1N3 mAb (panel B) bound non-covalently to protein G-agarose. The antibody-specifically bound proteins were separated in SDS-PAGE. Immunoblot was developed with C1N3 mAb directly conjugated with HRP. Unspecific binding onto the protein G-agarose is shown in lane 1 (panel A and B).

It is interesting to recognize that despite of same rate of CEACAM1 phosphorylation after EGF treatment of cells for 5 and 10 min, complex formation reached maximum at 5 min time point and than was decreased. Interaction of CEACAM1 with EGFR had also maximal intensity after 5 min and than was going down. These results suggested that CEACAM1 is probably target of transient phosphorylation by EGFR.

4.7.4 Dynamics of CEACAM1 and EGFR trafficking

To define cellular localization of EGFR-CEACAM1 complexes, confocal microscopy was used. HT29p cells untreated or treated with EGF in fatty acids free conditions were stained with antibody labeled with fluorescence dyes.

In untreated cells, positive staining indicating co-localization between EGFR and CEACAM1 was observed only sporadically in the plasma membrane. Interestingly, there is some evidence for co-localization of both, CEACAM1 and EGFR in Endoplasmic reticulum-Golgi apparatus. These compartments are responsible for glycosylation and membrane protein sorting.

However, short-term EGF-stimulation (time range 1-5 min) evoked slow transport of EGFR (green) to the cell membrane, while CEACAM1 (red) localization was not significantly changed (Fig. 21B). Upon the prolongation of EGF stimulation (10 min) was EGFR cumulated on the cell surface accompanied with strong CEACAM1 translocation into the membrane (Fig. 21C).

Additionally, co-localization between CEACAM1 (blue), EGFR (green) and α -adaptin (red) (specific indicator for clathrin-coated vesicle formation and transport) was performed. No co-localization (indicated with white color) between mentioned molecules appeared in plasma membrane or intracellular. However, positive staining was observed on the cell boundary separating daughter cells before cytokinesis (data not shown).

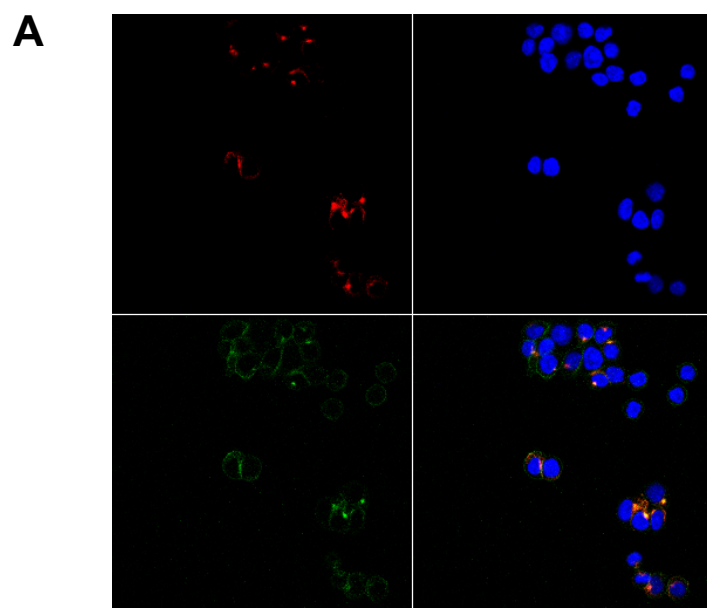


Figure 21A: For description see next page.

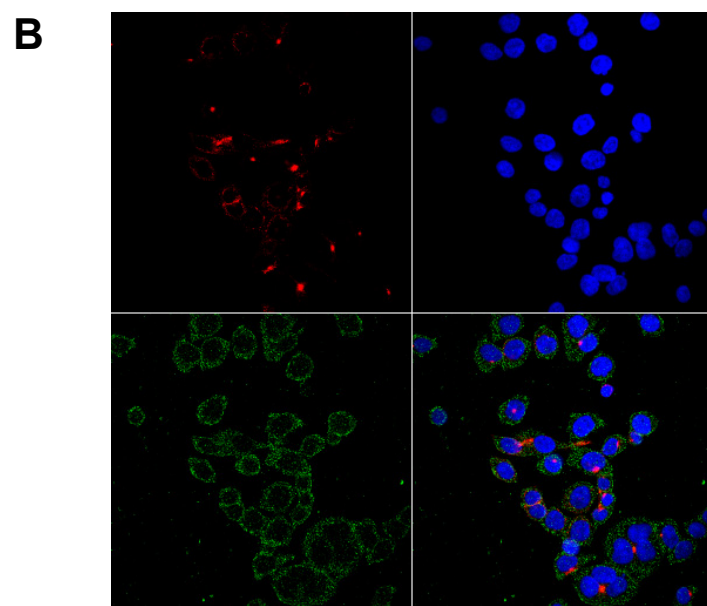


Figure 21B: For description see next page.

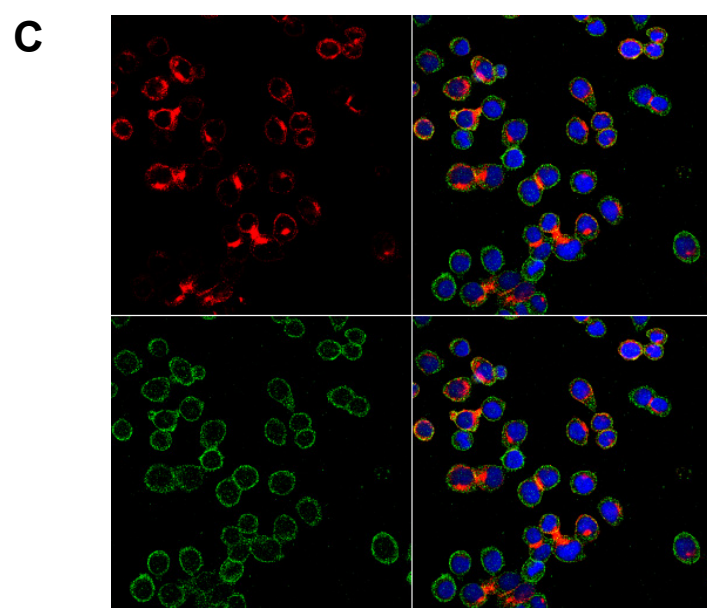


Figure 21C: For description see next page.

Figure 21: Immunofluorescence staining of HT29p cells after treatment with EGF

The cells grown on sterile glass cover slip were stimulated with 100 nM EGF for 0 (A), 5 min (B) and 10 min (C) prior to their fixation, blocking and the incubation with C1N3 mAb together with rabbit anti-EGFR specific antibody. Subsequently, incubation with the goat anti-rabbit IgG+IgM antibody directly conjugated with FITC (green) and goat anti-mouse IgG+IgM antibody directly conjugated with Alexa fluor 555 (red) was performed accompanied with nuclei staining. Visualization was carried out with a confocal microscope LSM 510.

Red fluorescence staining belonging to CEACAM1 (panel A, B, C; upper left figure) indicated antigen recognition by C1N3 mAb. EGFR presence was visualized by green fluorescence (panel A, B, C; lower left figure), while nuclei staining was determinate by blue color (panel A, B, C; upper right). Computer techniques allowed to lay each of these three fluorescence staining on each other and facilitated observation of localization of each of studied molecules (panel A, B, C; lower right figure).

5 DISCUSSION

5.1 Primary screening

Normal colon epithelium as well as cancer cells derived from colon epithelium, express up to four CEACAM members. These include CEA, CEACAM1, CEACAM6 and CEACAM7 (Frangsmyr et al, 1999). It was reported that CEACAM1 and CEACAM7, in contrast to CEA and CEACAM6, were expressed selectively in columnar cells at the upper third of the crypts and the luminal surface. However, all of the mentioned molecules were microscopically localized to the apical surface glycocalyx. This carbohydrate rich zone on the cell surface is generally believed to play an important role in innate immunity serving as a defense structure against microbial attack of the intestinal lumen mucosa (Hammarström and Baranov, 2001). Upon malignant transformation, members of CEACAM family undergo an altered expression. It has been previously reported that CEA is expressed prevalently in colorectal tumors compared to normal tissue (Kuroki et al., 1988). Because of loss of polarity, cancer cells express CEA over the surface entire followed by its release from cell to the extracellular space (Hammarström, S., 1999). Some researchers have found CEA mostly intracellular with limited membrane association in HT29 colon cancer cell lane (Fahlgren et al., 2003). Schölzel et al. (2000) reported down-regulation of CEACAM7 and up-regulation of CEACAM6 expression along hyperplastic polyps and early adenomas. The level of mRNA for CEACAM1 was described to be deregulated in a negative way, where down-regulation in more than 80% of colorectal carcinomas occurred (Neumaier et al., 1993). Its down-regulation in colorectal and other tumors led to CEACAM1 identification as a tumor suppressor (Neumaier et al., 1993, Kleinerman et al., 1995). CEACAM1 splicing variants differing in the length of the cytoplasmic tail exist (Huang et al., 1999). Through their interactions not only tumor development, but also cell polarization and luminal formation of mammary epithelial cells is influenced (Huang et al., 1999). Soluble forms of CEACAM1 were described to stimulate angiogenesis of endothelial cells (Ergun et al., 2000).

For potential therapeutic proposes, CEACAM members, especially CEACAM1 as a tumor suppressor, gained interest. As suggested, intact glycocalyx is indirectly involved in tumor etiology and its damage accompanied cancer development.

Here, this work is directed to identify specific molecules (cytokines, growth factors, antibiotics, etc.) able to regulate CEACAM1 and/or CEA. Thus, up-regulation of these glycoproteins would renew partially glycocalyx. Moreover, the investigation of down-stream

pathways influenced by CEACAM1 may identify further novel therapeutic intervention options.

Interferon gamma (IFN- γ) is a glycoprotein with a molecular weight of 17 kDa (Gray et al., 1982). Under physiological conditions, expression of IFN- γ is routinely observed after the activation of two major cell types, T lymphocytes and large granular lymphocytes (Young and Hardy, 1990). IFN- γ was reported to increase the expression of tumor-associated antigens (Kantor et al., 1989). IFN- γ became interesting for treatment of human solid tumors (Welander et al., 1988; Ernstoff et al., 1992). Yan et al. (1992) were the first to observe that IFN- γ treatment of colon cancer cells effected the expression of carcinoembryonic antigen, with the protein level being enhanced up to five-fold during 5-6 days of continuous exposure (Yan et al., 1992).

Tumor necrosis factor-alpha (TNF- α) is a pleiotropic inflammatory cytokine. It was first isolated by Carswell et al. in 1975 in an attempt to identify tumor necrotizing factors responsible for killing of the sarcoma Meth A. This cytokine produced mostly by macrophages possesses tumor growth inhibitory properties (Carswell et al., 1975).

IFN- γ was already mentioned to increase CEA and CEACAM1 protein as well as their mRNA levels in cancer cells (Fahlgren et al., 2003), therefore it was used as a positive control for our experiments. An increase of CEA and CEACAM1 protein expression up to 6-fold was observed. Interestingly, TNF- α was the only one molecule identified to up-regulate CEACAM1 specifically without effecting of CEA. The up-regulation was only moderated (about 1.8-fold by Western blot analyses) and no significant increase in surface expression of CEACAM1 was detectable by FACS analyses.

Geldanamycin belongs to ansamycin benzoquinone antibiotics, which is already well characterized as a potent tumor suppressor. Geldanamycin action is based on competitive association with chaperone Hsp90 inside ATP/ADP switch (Neckers et al., 1999). Because of geldanamycin cytotoxicity, its derivatives are studied and successfully entering clinical trials in oncology (Hostein et al., 2001).

Geldanamycin was here shown for the first time to be a specific inhibitor of CEACAM 1 protein-protein interactions without effecting CEA and CEACAM1 expression level.

Additionally, data obtained from flow cytometry experiments suggested that, in contrast to IFN- γ stimulated increase of CEA/CEACAM1 trafficking to and through the cell membrane, neither TNF- α nor geldanamycin treatments could significantly alter mentioned processes.

Another group of molecules selected for this study are growth factors.

EGF as well as basic FGF are ligands for receptor tyrosine kinases, the activation of which results in a number of intracellular responses regulating cell growth and proliferation (Haugh, J.M., 2002; Etscheid et al., 2004) and they are also reported to be involved in malignant transformation (Wells et al., 2002; Zhang et al., 2003).

Both, EGF and also b FGF were able to down-regulate CEACAM1 on protein level. It remained to be shown how these down-regulatory effects are related to CEACAM1 protein turnover and shedding.

Interestingly, another ligand of the EGF receptor, transforming growth factor- α (TGF- α) caused no alterations of CEACAM1 and/or CEA expressions in HT29p cell system.

The expression of b FGF enhancing vascular endothelial growth factor (VEGF) release in endothelial cell induces the formation of capillary-like structures by vascular endothelial cells grown on three-dimensional fibrin gels (Hollborn et al., 2004).

Moreover, since CEACAM1 was characterized as a major effector of VEGF in the early microvessel formation (Ergun et al., 2000; Wagener and Ergun, 2000) may serve as a target for the inhibition of tumor angiogenesis. Contradictory, b FGF either stimulates a release of VEGF followed with activation of CEACAM1 angiogenic potential, or it suppresses CEACAM1 expression, thus inhibit angiogenesis. CEACAM1 could be an important regulation point in process of angiogenesis.

Additionally, since HT29p cells shows limited expression of surface presented CEACAMs, it may be caused epigenetically. Therefore, the effect of trichostatin, a potent inhibitor of histone deacetylase, and 5-azacytidine as a demethylating agent turning on gene expression, on CEA and/or CEACAM1 protein expression were studied. No expression alterations on protein level were observed suggesting that these mechanisms of gene silencing do not contribute to CEA and CEACAM1 low level expression on HT29p cell surface.

5.2 CEACAM1-a potential key regulator of cell proliferation and viability

CEACAM1 was to be found rapidly down-regulated upon malignant transformation of colon epithelia (Neumaier et al., 1993) and it was already described as a potent tumor suppressor

(Kunath et al., 1995). The possible correlation between cancer cell proliferation and CEACAM1 expression was therefore investigated.

The here presented results of the cell proliferation studies support the relationship between loss of CEACAM1 and carcinogenesis. Moreover, TNF- α as well as IFN- γ specifically and efficiently up-regulating CEACAM1 protein expression caused a decrease in the cell proliferation and probably enhanced also apoptosis. The involvement of CEACAM1 in cytokine-triggered apoptosis needs to be further investigated.

In contrast, growth factor EGF responsible for strong down-regulation of CEACAM1 leads to enhanced cell proliferation.

Thus, the idea to use extracellular stimuli to intervene with malignant alterations *in vitro* and in the future also *in vivo* gains interest.

5.3 Biochemistry of geldanamycin effect on CEACAM1

Geldanamycin, an ansamycin antibiotic, that specifically inhibits heat shock protein-90 (Hsp90) and its endoplasmic reticulum homologue GRP94, have been identified as an agent with antitumor activity (Neckers et al., 1999; Brunton et al., 1998). Chaperone Hsp90 is distinguished from other chaperones in that most of known substrates are signal transduction proteins, the classical examples being steroid hormone receptor and signaling kinases (Picard et al. 1990, Xu and Lindquist 1993). Because signaling proteins with multiple regulatory stages often undergo the conformational alterations, the structural flexibility needed for these steps may render them inherently less stable and thus more likely to be recognized by Hsp90. Under stress conditions such molecules as heat shock Hsp90 contributes more generally to the refolding of denatured proteins (Nathan et al. 1997). Thus, interactions of substrate proteins with Hsp90 arise from structural properties at the molecular level rather than biological function (Young et al. 2001).

The interaction of geldanamycin with Hsp90 results in competition for ATP binding to Hsp90 and inhibition of chaperone function (Neckers et al., 1999). The hallmark of anti-tumor activity of geldanamycin has been attributed to destabilization and degradation of key client proteins, including receptor and non-receptor kinases (Erb-B2, epidermal growth factor receptor, Src family kinases), serine/threonine kinases (c-Raf, Cdk-4), cell cycle and apoptosis regulators (Brunton et al., 1998) in the ubiquitin-dependent proteasome pathway (Whitessel et al. 1994, Schulte et al. 1995).

Here it was found that geldanamycin is able to keep CEACAM1 molecule in a dephosphorylated state. Such a CEACAM1 dephosphorylation in geldanamycin-treated cells is accompanied with loss of its ability to form the high molecular protein complexes. Therefore we hypothesized: (1) CEACAM1 complexes include some component which is directly inhibited by geldanamycin, (2) Secondly, geldanamycin depletion of Ras and Raf kinases could affect up-stream proteins in case that CEACAM1 is a member of this pathway.

Ad1) According to knowledge about geldanamycin binding requirements, CEACAM1 is not a direct substrate for geldanamycin. Therefore we were looking for correlations between chaperone Hsp90 and CEACAM1. Thus, the interaction of Hsp90 with a novel client-protein CEACAM1 was specifically identified as a target of geldanamycin attack.

Ad2) interestingly, beside geldanamycin-induced biochemical influence on CEACAM1 protein, macroscopic alterations of the cell behavior were observed. CEACAM1 appearing in monomeric and dephosphorylated state correlated with decreased cell viability and DNA synthesis was dramatically suppressed as well. If we hypothesized about CEACAM1 as an up-stream member of Ras mitogenic pathway, as described later, any effect of geldanamycin on the mentioned signaling pathway would lead to its arrest followed by tumor growth suppression and cytostasis.

Hostein et al. (2001) had used a derivate of geldanamycin (17-Allylamino-17-demethoxygeldanamycin) with lower hepatotoxic properties to prove their hypothesis about tumor suppressive ability of 17AAG via specific targeting of signaling molecules from mitogenic Ras pathway. The fact that in approximately 30% of all colon cancers mutation of Ki-ras occurs (Andreyev et al., 1998) accompanied with constitutive activation of ERK-1/2 (Hoshino et al., 1999; Sebolt-Leopold et al., 1999), suggested the possibility to induce effectively growth inhibition of colon cancer cells. Treatment with 17AAG caused depletion of c-Raf and inhibition of ERK-1/2 phosphorylation. Additionally, N-ras, Ki-ras and c-Akt were degraded leading to cytostasis and apoptosis of colon adenocarcinoma cell line (Hostein et al. 2001).

Here, geldanamycin treatment of HT29p led to an accumulation of cells entering G₀/G₁ phase of cell cycle, supporting data from strongly reduced [³H]-thymidine incorporation.

It was concluded, that effect of geldanamycin reflects its multi-targeting character inside Ras pathway, whereas protein expression of the up-stream modulator of this pathway-CEACAM1 is not changed and following consequences are likely due to the loss of its ability to interact with other proteins.

5.4 The processing and intracellular trafficking of CEACAM1

5.4.1 CEACAM1 processing

It was already described that growth factors, namely EGF and b FGF, were able to down-regulate CEACAM1 protein expression. However, whether this regulation appears primarily on transcriptional level or reduced glycoprotein expression on the cell surface is caused by its increased processing and/or shedding has to be further investigated. Down-regulation of CEACAM1 after long-term treatment with growth factors was detected by Western blot analyses. Moreover, since a specific epitope recognized by C1N3 detection mAb was not yet characterized, thus it may be possible that in case of an extracellular epitope being recognized, digestion of CEACAM1 would decrease signal around 150 kDa and no shorter fragment would be detectable with this antibody.

Subsequently, the results from immunofluorescence confocal experiments showed CEACAM1 translocation into the cell membrane in short-term response to EGF.

However, the surface presentation of CEACAM1 may be accompanied with its proteolytic degradation. There is no evidence proving associations of CEACAM1 with extracellular proteases yet.

5.4.2 The intracellular trafficking of CEACAM1

Little is known about the impact of CEACAM1 on vesicle transport of other membrane proteins, their internalization and ubiquitination.

However, CEACAM1 has been described as an important part of a protein complex required for receptor-mediated insulin internalization in hepatoma cells followed by its degradation (Formisano et al., 1995). CEACAM1 undergoes tyrosine phosphorylation by the activated insulin receptor (Najjar et al., 1995), thus it is able to interact with an adaptor protein Shc (Poy et al., 2002) and regulate Shc-triggered action either in the signal transduction network or receptors endocytosis (Okabayashi et al., 1996). CEACAM1 involvement in insulin endocytosis and degradation was shown to be very specific. Moreover, there is strong evidence that CEACAM1 failed to alter endocytosis of the insulin-like growth factor-1 (IGF-1) receptor in response to IGF-1 (Najjar et al., 1998) and platelet derived growth factor (PDGF, Formisano et al., 1995).

Interestingly, there are some important similarities between insulin receptor and EGF receptor internalization.

First, Shc as is a common substrate for both receptors and since we were able to show that CEACAM1 undergoes tyrosine phosphorylation by EGFR upon EGF stimulation, we hypothesized CEACAM1 to be (as described in insulin receptor case) involved in vesicle transport and internalization of EGFR. Performed immunofluorescence staining (Fig. 21) of colon epithelial cell line expressing CEACAM1 and also EGFR led to several conclusions:

(1) Both of investigated molecules were less co-localized in plasma membrane, but inside the Endoplasmic reticulum-Golgi apparatus (ER/GA) region strong positive signal was observed. Moreover, only few signals indicating EGFR presence on the cell surface were detected in untreated cells.

(2) The short-term treatment with EGF triggered slow translocation of EGFR from cytosol to the cytoplasmic site of the cell membrane without any alterations in CEACAM1 localization.

(3) CEACAM1 as well as EGFR was both transported into the membrane during longer EGF stimulation. Additionally, most of co-localization signals still appeared in ER/GA region, where usually all large membrane proteins are glycosylated.

Additionally, co-localization studies suggested that either CEACAM1 interaction with EGF receptor is indirect or that EGF-triggered protein complex formation takes place during posttranslational modification in ER/GA.

Since EGFR function is maintained in these cells, thus ligand-evoked receptor translocation is correlating with CEACAM1 presentation on the cell surface at the same time.

However, extremely little co-localization between receptor and CEACAM1 was detectable in the cell membrane during EGF treatment. EGFR requires molecular chaperon Hsp90 to keep itself in proper folding structure during cellular trafficking to the final destination as well as for the internalization (Barzilay et al., 2003). Moreover, here we have identified Hsp90 as a novel interaction partner for CEACAM1. Thus, it does not seem unlikely that EGFR and CEACAM1 could form large complexes during post-translational modification and sorting for further vesicle transport.

The second important fact common for EGFR and insulin receptor internalization is that Shc binds to α - and β -adaptins. Adaptins are implicated in functions essential for the dynamic cycle of clathrin-coated pits and vesicles (Okabayashi et al., 1996).

Surprising results were obtained after cells were specifically stained with α -adaptin additionally to EGFR and CEACAM1. Only in sporadic cases co-localization between all of

three mentioned molecules was detectable, suggesting that in this cell system is neither EGFR nor CEACAM1 transported through clathrin-coated vesicles requiring α -adaptin presence (data not shown). In contrast to sorting of insulin receptor (Choice et al., 1998), where only α -adaptin was involved, both α - as well as β -adaptins were reported to play a specific role in vesicle transport of EGF receptors (Sorkin et al., 1995) The role of CEACAM1 in vesicle transport of insulin receptor thus seems to be different from that of EGFR. Co-localization of CEACAM1 with β -adaptin would be needed to prove this hypothesis.

Additionally, significant co-localization between CEACAM1, EGFR and α -adaptin was observed in contact cell boundaries of daughter cells shortly before cytokinesis. This is another interesting point for further research, because very little is known about the possible involvement of CEACAM1 in cell division.

5.5 Role of CEACAM1 in EGFR signaling

5.5.1 CEACAM1 has antagonistic effect on EGF signaling

As was described earlier, during “hunting” on molecules selectively regulating CEA and/or CEACAM1 protein expression, that EGF was identified as a strong inhibitor. Since b FGF is also able to suppress CEACAM1 expression, this down-regulatory effect seems to be common for more ligands of receptor tyrosine kinases. According our hypothesis CEACAM1 suppression would be characterized as an out-come from feedback regulation via downstream molecules included in signaling pathway common for receptor tyrosine kinases stimulated with EGF or b FGF. If this hypothesis is right, regulation would very probably take place on transcriptional level. More investigation of alterations on the gene expression as well as alterations on mRNA level has to be done to answer this question. If this kind of regulation is appearing in our cell model, it could be reasoned by antagonistic character of CEACAM1 in EGFR and/or b FGFR pathways.

In cells treated with EGF, inhibition of CEACAM1 expression was accompanied by increased cell proliferation supporting published data about tumor suppressive activity of CEACAM1. Co-incubation of EGF with monoclonal C1N3 Ab additionally increased the proliferation rate of HT29p cells, suggesting a possible antagonistic activity of CEACAM1 for EGF response. However, antibodies raised against different members of CEACAM family are acting either as an extra-cellular clustering factor to activate CEACAM1 containing protein complexes

following by different effects on cell physiology (Stocks et al., 1996; Nair et al., 2001; Budt et al., 2002) or antibodies with neutralizing effect on their target are known (Singer et al., 2002). C1N3 monoclonal antibody used in this work was not yet studied for its inhibitory or clustering activity.

5.5.2 CEACAM1—a potent linkage between obesity and cancer

A signaling regulatory activity of CEACAM1 on level of tyrosine kinase receptors was already described (Poy et al., 2002).

Upon insulin receptor activation CEACAM1 undergoes phosphorylation followed by its binding to Shc. Thus removal of this coupler of Grb2 with the insulin receptor was able to prevent a “switch on” of down-stream Ras/MAP kinase mitogenic pathway (Soni et al., 2000). CEACAM1 is in this regard involved in sugar as well as in lipid metabolism. Additionally, members of Ras mitogenic pathway are often altered in different kinds of cancer (Adrejev et al., 1998), thus providing a possible link between metabolic diseases and cancer.

To identify this linkage it was hypothesized that epidermal growth factor receptor, with its dual activation character by EGF and/or non-saturated fatty acids could be a good candidate. The activation of EGFR by fatty acids provides a direct connection to lipid metabolism (Mollerup et al., 1996) as well as fact that a lot of EGFR actions require phospholipase C, which hydrolyzes lipid phosphatidylinositol-(4,5)-bisphosphate (Haugh, J., 2002). Additionally, it is interesting that Shc is down-stream substrate for EGF receptor (Haugh et al., 1999) in the same manner like for insulin receptor.

Here, we have identified CEACAM1 as a specific substrate for activated EGF receptor, where CEACAM1 undergoes tyrosine phosphorylation in response to short-term exposure to EGF.

This phosphorylation seemed to be transient as was concluded from complex formation study and immunofluorescence co-localization assays. Additionally, observations on specific phosphorylation sites and experiments with CEACAM1-transfected cells and *in vivo* data in transgenic animals lead to the conclusion that CEACAM1 down-regulates EGF-triggered cell proliferation and growth (Abou-Rjaily et al., 2004, submitted).

In summary, CEACAM1 as a common substrate for insulin and EGF receptors represent a molecule which may bridge metabolism with neoplastic transformation.

6 Summary

The aims of the study were orientated to reveal more detail about members of carcinoembryonic antigen family. This group of glycoproteins is termed according the first described CEA molecule, which is used as a specific tumor marker for colorectal cancer. Here, interest was concentrated to CEACAM1 member because of its potent tumor suppressive properties.

The colon cancer cell line HT29p was used as an *in vitro* system to reveal in a more detail involvement of CEACAM1 in signal transduction.

Several biologically active molecules were characterized in their ability to regulate protein expression level of CEACAM1. The multifunctional cytokine IFN- γ triggering increase of protein expression of CEACAM1 was already identified and therefore it was used here as a positive control for expression regulation. However, the effect of this cytokine on biochemical properties of CEACAM1 protein was so far not studied. Another cytokine TNF- α , ansamycin antibiotic-geldanamycin and growth factor-EGF were here defined to initiate protein expression alterations of the membrane associated glycoprotein CEACAM1. The protein expression alterations due to the extracellular stimuli were conducted with differential effect of stimuli on tyrosine- phosphorylation of CEACAM1 followed by changes in a formation of the high molecular weight protein complexes of CEACAM1. Geldanamycin known to be a specific inhibitor of Hsp90 molecular chaperone had a strong destructive effect on mentioned complexes, additionally causing CEACAM1 dephosphorylation. Moreover, Hsp90 was here identified to be a novel interaction partner of CEACAM1.

The cellular growth was also dramatically changed upon geldanamycin-induced decrease in CEACAM1 phosphorylation and loss of CEACAM1 complex formation properties. Despite of mild effect on the cell viability, [3 H]-thymidine uptake, cell cycle analysis recovered a strong arrest of the G₀/G₁ phase of cell cycle.

Since ligands for the receptor tyrosine kinases (EGF, b FGF) were able to down-regulate CEACAM1 rapidly, a role of CEACAM1 in their signaling was hypothesized.

EGF-triggered signaling was further studied because of similarities with insulin pathway, in which the role of CECAM1 is well described (Poy et al., 2002). The results of the experiments searching for relationship between EGF signaling and CEACAM1 suggested that CEACAM1 regulate cellular trafficking of EGFR in response to EGF. Additionally, CEACAM1 was shown to be differentially phosphorylated after cells were treated with EGF.

Co-immunoprecipitation experiments visualized a physical association between CEACAM1 and EGFR.

Since CEACAM1 has been characterized as an important tumor suppressor for colorectal cancer, the reported biochemical and cell biological characterization of CEACAM1 in this HT29p *in vitro* model system provides novel insights for further preclinical *in vivo* therapeutic strategies.

7 Zusammenfassung

Die hier präsentierte Arbeit erbringt neue Erkenntnisse über die Mitglieder der Carcinoembryonic Antigen Protein-Familie. Diese Gruppe von Glycoproteinen ist nach dem zuerst beschriebenen CEA Molekül benannt, das als spezifischer Tumor-Marker für colorectalen Krebs verwendet wurde.

Die Darmkrebs Zelllinie HT29p wurde in einem *in vitro* System studiert, um tiefere Einblicke der Beteiligung von CEACAM1 während der Signal-Transduktion zu gewähren.

Es sind einige biologisch aktive Moleküle aufgrund ihrer Fähigkeit zur Regulation des Expressionsniveaus von CEACAM1 untersucht worden. Das multifunktionelle Zytokin IFN- γ , das eine Erhöhung der Protein-Expression von CEACAM1 verursacht, ist bereits identifiziert worden und wurde daher in dieser Arbeit als Positiv-Kontrolle für eine Expressions-Regulation verwendet, obwohl der Wirkungsmechanismus dieses Zytokins auf die biochemischen Eigenschaften von CEACAM1 noch nicht geklärt ist. Ein weiteres Zytokin, TNF- α , das Ansamycin Antibiotika Geldanamycin und der Wachstumsfaktor EGF sind in dieser Arbeit als Initiatoren der Beeinflussung der Protein-Expressionsrate des Membran-assoziierten Glycoproteins CEACAM1 definiert worden. Die Beeinflussung des Expressionsniveaus aufgrund der extrazellulären Anregung wurde mit unterschiedlichen Auswirkungen der Impulse auf die Tyrosin-Phosphorylierung von CEACAM1 untersucht. Weiterhin wurde eine hierdurch induzierte Bildung von hochmolekularen Komplexen, die CEACAM1 enthalten, beobachtet. Geldanamycin, das als spezifischer Inhibitor des molekularen Chaperones Hsp90 bekannt ist, zeigte einen ausgeprägt zerstörerischen Effekt auf die o.g. Komplexe. Ebenso bewirkte Geldanamycin eine Dephosphorylierung von CEACAM1. Zudem konnte Hsp90 in dieser Arbeit als neuer Interaktionspartner von CEACAM1 identifiziert werden.

Es wurde gezeigt, dass aufgrund der Geldanamycin-induzierten Verminderung der Phosphorylierung von CEACAM1 und der daraus folgenden Veränderungen der Fähigkeiten von CEACAM1 zur Bildung von Komplexen das zelluläre Wachstum dramatisch beeinflusst werden konnte: Obwohl Geldanamycin hierbei nur einen geringen Effekt auf die Zell-Lebensfähigkeit aufwies, zeigte der [^3H]-Thymidin Aufnahmeversuch einen ausgeprägten Arrest der G₀/G₁-Phase des Zellzyklus auf.

Da Liganden für Rezeptor-Tyrosin-Kinasen, z.B. EGF, b FGF, in der Lage waren, das Expressionsniveau von CEACAM1 sehr schnell zu senken, wurde für CEACAM1 eine Beteiligung an der Signal-Transduktion angenommen.

Besonders die von EGF ausgelöste Signal-Transduktion wurde weitergehend untersucht; insbesondere im Hinblick auf die Ähnlichkeiten mit dem Insulin-Signalweg, in dem die Rolle von CEACAM1 gut untersucht ist (Poy et al. 2002). Die erzielten Ergebnisse in der Suche nach dem Zusammenhang zwischen der EGF Signal-Transduktion und CEACAM1 legten nahe, dass CEACAM1 die intrazelluläre Verteilung und den subzellulere Transport von EGFR nach EGF-Stimulierung reguliert. Zudem konnte gezeigt werden, dass CEACAM1 nach Inkubation mit EGF differentiell phosphoryliert wurde. Co-Immunoprecipitationsexperimente belegten eindeutig die molekulare Assoziation zwischen CEACAM1 und EGFR.

Da CEACAM1 als wichtiger Tumor Suppressor für colorectalen Krebs charakterisiert ist, präsentiert die in dieser Arbeit im *in vitro* Zellmodell HT29p gezeigte biochemische und zellbiologische Charakterisierung von CEACAM1 neue Erkenntnisse für weitergehende präklinische und therapeutische Strategien.

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Erklärung

Hiermit versichere ich ausdrücklich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Ich erkläre weiterhin, dass ich diese Dissertation nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Kiel, 23.05.2004

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