**Rapid Communication**

**Increased basolateral sorting of carcinoembryonic antigen in a polarized colon carcinoma cell line after cholesterol depletion—Implications for treatment of inflammatory bowel disease**

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**Abstract**

**AIM:** To investigate a possible increase of basolateral expression of carcinoembryonic antigen (CEA) by interfering with the apical transport machinery, we studied the effect of cholesterol depletion on CEA sorting and secretion.

**METHODS:** Cholesterol depletion was performed in polarized Caco-2 cells using lovastatin and methyl-β-cyclodextrin.

**RESULTS:** We show that CEA is predominantly expressed and secreted at the apical surface. Reduction of the cholesterol level of the cell by 40%-50% with lovastatin and methyl-β-cyclodextrin led to a significant change of the apical-to-basolateral transport ratio towards the basolateral membrane.

**CONCLUSION:** As basolateral expression of CEA has been suggested to have anti-inflammatory properties, cholesterol depletion of enterocytes might be a potential approach to influence the course of inflammatory bowel disease.

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**Key words:** Inflammatory bowel disease; Cholesterol; Polarized secretion; Carcinoembryonic antigen; Caco-2

**Introduction**

Carcinoembryonic antigen (CEA, also named CEACAM5, gp180 or CD66e) is a 180 kDa glycoprotein that belongs to a subfamily of the immunoglobulin superfamily. In the intestine, CEA is mainly localized at the apical membrane of the goblet cells and colonic enterocytes[4]. Although CEA has been used extensively as a tumor marker for colon carcinoma, its physiological function remains unclear. The induction of CEA by cytokines[5,6], its in vitro cell-cell adhesion properties[6] and its inhibitory effect on natural killer cells[7] have suggested a significant role on immune function. It has been shown that apically expressed CEA can bind to several microorganisms like E.coli, Salmonella, Neisseria or Haemophilus, and possibly function as a sensor to trigger or prevent bacterial infection[6-10]. On the other hand, basolateral expression of CEA has been shown to act as a CD8 ligand that is involved in the activation of suppressor T cells[11]. Interestingly, patients with inflammatory bowel disease (IBD) show a significantly reduced expression of CEA regardless of the activity of the disease[12,13]. Therefore, it has been suggested, that reduced expression of CEA may be an inherited defect in IBD[14]. Immunohistochemical staining of CEA in intestinal tissues has shown that in normal mucosa, CEA is located predominately at the apical, but possibly also at the basolateral membrane of the enterocytes. These findings are different in IBD patients. There is loss of staining in the basolateral membrane in ulcerative colitis and reduction of both ap-
cal and basolateral staining in patients with Crohn’s disease. CEA is attached to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. GPI anchored proteins have been shown to use the GPI anchors as a sorting determinant for apical delivery. They are sorted by a lipid raft dependent mechanism. Depletion of cholesterol from Madin-Darby canine kidney cells (MDCK cells) results in missorting of the proteins to the basolateral compartment.

The putative activity of CEA at the apical and basolateral membranes prompted us to examine the impact of sorting of CEA in a polarized colonic cell line. This maneuver would shift its function from a molecule that binds and possibly promotes bacterial infection and inflammation to a molecule that may activate suppressor T cells and reduce the inflammatory response. In the present study, we were able to show that sorting of CEA to the apical and basolateral membranes can be altered by modifying the cholesterol content of the cell.

MATERIALS AND METHODS

Cells
Caco-2 cells were gifted by Professor Fricker, Institute for Molecular Biotechnology, University of Heidelberg. Caco-2 cells were cultured at 37°C in DMEM supplemented with 10% FCS, 1% non essential amino acid solution, 1% sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L L-glutamine (Invitrogen, USA) and grown to achieve polarization for 14 d on 2.5 cm, 0.4 µm pore size Transwell polycarbonate filters (Costar, Cambridge, USA). The formation of an intact, confluent monolayer was monitored by measuring the transepithelial electrical resistance (TEER) and in representative experiments by detecting the unidirectional flux of inulin. Cell monolayers in Transwells were incubated in the presence of 3.6 mmol/L [3H]-inulin (NEN) in the basal well and 10 mmol/L methyl-β-cyclodextrin (MβCD, Sigma, USA) for 30–60 min. Two hours after MβCD treatment, radioactivity of the apical medium and of the basolateral medium were measured as described below using a Beckman LS6000 Scintillation Counter (Beckman, USA). The flux into the apical well was calculated as the percent of total inulin administered into the basolateral well. The same quantity of cells were used for the corresponding experiments.

Cholesterol depletion, pulse/chase experiments
For the cholesterol depletion study, the cells were grown for 1 d in either DMEM supplemented with 2 mmol/L L-glutamine, 10% FCS, 2 µmol/L lovastatin (Calbiochem, USA), and 0.25 mmol/L mevalonate or in complete medium. The following day, the cells were treated for 30–60 min with 10 mmol/L MβCD in methionine-free medium (labeling medium), and subsequently metabolically labeled with 100 µCi/dish of [35S]-methionine (NEN). The cells were chased for 2 h in labeling medium containing an excess of 150 µg/mL methionine and 20 µg/mL cycloheximide in order to inhibit protein synthesis. Cholesterol determinations were performed using the Amplex Red Cholesterol Assay kit (Molecular Probes). Proteins from the apical and basolateral medium were precipitated with trichloroacetic acid and radioactivity from the precipitate was quantified in 5 mL of Ultima Gold™ liquid scintillation fluid (Packard Bioscience) using a Beckman LS6000 Scintillation Counter (Beckman Instruments).

Western blot analysis, CEA quantification
Media from the apical and basolateral chamber were separated on 10% polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. The blot was probed with polyclonal CEA antibody (1:300, A0115, Dako, USA), and protein visualized using an HRP-conjugated secondary antibody and an enhanced chemiluminescence detection kit (Amersham, USA). CEA was quantified using an electrochemiluminescence immunoassay (ECLIA) from Roche Diagnostics that specifically recognizes CEA and not other CEA-like proteins.

Immunofluorescence
Filter grown Caco-2 cells were treated with lovastatin/mevalonate and MβCD as described above. The cells were fixed for 10 min with 4% PFA at 8°C followed by incubation with 0.1% Triton X-100. The fixed cells were incubated for 1 h at room temperature with the polyclonal antibody against CEA (1:200, Dako) or the monoclonal antibody against Na+/K+-ATPase (1:100, Affinity BioReagents, Golden, USA) in PBS containing 0.2% gelatine. After three washes with PBS containing 0.2% gelatine, the cells were incubated with the respective Cy3- or Cy2-labeled secondary antibodies (1:600 and 1:100, respectively) in PBS containing 0.2% gelatine for 1 h at room temperature. Confocal image acquisition were done on a Leica TCS SP2 system and pictures arranged with Adobe Photoshop.

Preparation of detergent resistant membranes (DRMs)
Detergent extraction of Caco-2 cells with Triton X-100 was performed as described. The cells were grown on filters, washed once with PBS and scraped on ice into 300 µL 25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 3 mmol/L EDTA (TNE) buffer containing 25 g/mL each of chymostatin, leupeptin, antipain and pepstatin A. The cells were homogenized through a 25 G needle and centrifuged for 5 min at 3000 r/min. The postnuclear supernatant was subjected to centrifugation for 30 min at 4°C in 2% Triton X-100/TNE. The extracts were adjusted to 40% OptiPrep (Nycomed, Oslo) and overlayed in a TLS 55 centrifugation tube with 30% OptiPrep/TNE, and TNE. After centrifugation for 2 h at 55000 r/min, six fractions were collected from the top, and Western blots were performed.

Statistical analysis
All values are reported as mean and standard error of the mean (mean ± SE). The Kruskal-Wallis test was used to test for statistical significance. Probability values of P < 0.05 were set as threshold for statistical significance.

RESULTS

Depletion of cholesterol from polarized Caco-2 cells
Cholesterol depletion was achieved by a combination of lovastatin/mevalonate treatment and MβCD extraction. Lovastatin decreases the de-novo synthesis of cholesterol.
Cholesterol can be depleted from Caco-2 cells by a combination of Lovastatin/Mevalonate. In polarized Caco-2 cells, there was no significant staining of CEA at the basolateral membrane (Figure 3A). In addition, no significant basolateral secretion of CEA could be measured by Western blot analyses. However, in cholesterol depleted cells, there was significant secretion of CEA from the basolateral membrane (Figure 3B). Immunofluorescent analyses of CEA in cholesterol depleted cells revealed still an apical staining of CEA, however the fluorescent band was much weaker than in the control cells, and no basolateral staining was detected. We believe that cleavage of CEA at the basolateral side may explain the difference between the staining and the amount of CEA present in the basolateral medium. Quantification of secreted CEA within 2 h after cholesterol depletion using a CEA specific electrochemoluminescence immunoassay revealed that apical secretion was reduced by 39% ± 15% (Figure 3C). In non-depleted cells, no significant secretion of CEA was detected in the basolateral medium after 2 h (i.e., still below the detection level), whereas in depleted cells a concentration of 0.26 ± 0.06 µg/L was obtained.

**Effect of cholesterol depletion on association of CEA to DRMs**

One way to analyze if a protein is found is to isolate DRMs. Association of a protein with DRMs is shown by its insolubility in detergents such as Triton X-100. In order to determine the cholesterol depleted Caco-2 cells were still fully polarized, the permeability of the commonly used paracellular marker [3H]-inulin was analyzed. Only small amounts of [3H]-inulin are able to move across polarized monolayers unless the tight junctions are altered or cell injury occurs. In representative experiments, no increased translocation from basal to apical location was observed after cholesterol depletion (Figure 1B). TEER was measured before and after cholesterol removal. In representative experiments again, there was no significant difference between cholesterol depleted and non-depleted cells (99.0 ± 8.1 Ω/cm² vs 106.9 ± 9.8 Ω/cm²; mean and SEM of 3 representative experiments). In addition, polarization was controlled by immunofluorescence of filter grown Caco-2 cells, analyzing the basolateral distribution of Na+/K+-ATPase. The distribution of this protein did not change after cholesterol extraction.

**Effect of cholesterol on the overall transport capacity of newly synthesized proteins in the direction of the apical plasma membrane**

To obtain a quantitative assessment of the effect of cholesterol depletion on apical and basolateral protein secretion and to analyze the role of cholesterol in the sorting of newly synthesized proteins in general, pulse/chase experiments were performed. Caco-2 cells were metabolically labeled for 1 h and the total counts of labeled proteins secreted within 2 h into the apical and basolateral medium were analyzed. The chase period was performed in the presence of cycloheximide to inhibit further protein synthesis. In non-depleted cells, the total secreted radioactivity in the apical chamber was 11.1% ± 3.4%. After cholesterol depletion, apical secretion was reduced to 4.7% ± 1.1%. This change was not due to a decrease in protein secretion in general. Whereas cholesterol depletion reduced apical secretion by 35%, at the same time, the basolateral secretion increased by 39% (Figure 2).

**Effect of cholesterol depletion on polarized CEA secretion.**

Under normal cell culture conditions, CEA family proteins are sorted predominately to the apical membrane. In polarized Caco-2 cells, there was no significant staining of CEA at the basolateral membrane (Figure 3A). In addition, no significant basolateral secretion of CEA could be measured by Western blot analyses. However, in cholesterol depleted cells, there was significant secretion of CEA from the basolateral membrane (Figure 3B). Immunofluorescent analyses of CEA in cholesterol depleted cells revealed still an apical staining of CEA, however the fluorescent band was much weaker than in the control cells, and no basolateral staining was detected. We believe that cleavage of CEA at the basolateral side may explain the difference between the staining and the amount of CEA present in the basolateral medium. Quantification of secreted CEA within 2 h after cholesterol depletion using a CEA specific electrochemoluminescence immunoassay revealed that apical secretion was reduced by 39% ± 15% (Figure 3C). In non-depleted cells, no significant secretion of CEA was detected in the basolateral medium after 2 h (i.e., still below the detection level), whereas in depleted cells a concentration of 0.26 ± 0.06 µg/L was obtained.
gradients. After isolation of cellular membranes our experiments revealed that CEA is found in two membrane compartments within DRMs as well as in detergent soluble parts. Cholesterol depletion reduced significantly the association of CEA with DRMs (Figure 4).

DISCUSSION

The present data shows that the transport and secretion of CEA to either the apical or the basolateral membrane of polarized Caco-2 cells can be influenced by changing the cholesterol level of the cells. Under normal cell culture conditions, CEA is predominantly sorted and partially released at the apical membrane. Cholesterol depletion results in a significant shift to basolateral secretion.

These findings are in line with previous reports by Keller et al and Prydz et al who used the model of polarized MDCK[15,16]. These workers showed that the overall transport capacity in the direction of the apical membrane is reduced and that the sorting of proteins with a high affinity for lipid rafts is influenced by cholesterol depletion. Therefore, these workers suggested the presence of two constitutive exit routes from the TGN towards the plasma membrane in polarized cells: An apical route, which is lipid raft-dependent, and a raft-independent basolateral route[15,16]. Lipid rafts are small platforms, composed of sphingolipids and cholesterol in the outer exoplasmic leaflet, connected to phospholipids and cholesterol in the inner cytoplasmic leaflet of the cellular membranes. In mammalian cells, these are first assembled at the Golgi complex. From there, they move to the plasma membrane and spread into the endocytic pathway. Cholesterol is believed to serve as a spacer between the hydrocarbon chains of the sphingolipids and to function as a dynamic glue that keeps the raft assembly together. Removal of raft cholesterol, leads to the dissociation of lipid rafts and affects its putative functions[24]. Under normal cell culture...
conditions, the majority of raft proteins are found at the apical membrane where in the presence of cholesterol depletion, these proteins are missorted to the basolateral membrane\cite{9,10}. Typical raft proteins are doubly acylated such as tyrosine kinases of the Src family, cholesterol-linked and palmitate-anchored proteins like hedgehog and GPI-anchored proteins to which CEA belongs\cite{13}. Therefore, under normal cell culture conditions, most of the CEA is sorted to the apical membrane and secreted into the apical medium (Figure 3). This picture changes following cholesterol depletion. CEA is sorted and secreted in the basolateral membrane, suggesting that it is no longer associated with lipid rafts. Our results on DRM association support this hypothesis (Figure 4). However, lowering the cholesterol levels is not only a specific treatment to influence lipid raft function, but it also has an impact on several other cellular processes such as lipid metabolism, endocytosis and enzyme activity\cite{25,26}. CEA release from the membrane is dependent upon the action of phospholipase D. It has been shown recently that its activity is increased after removal of cholesterol\cite{6}. How this phenomenon impacts the polarized secretion of CEA under low cholesterol conditions needs further evaluation.

Influencing the sorting of CEA may be relevant, since the compartmentation of the protein may have distinct functions. Whereas its apical expression may serve to bind mucus and microorganisms\cite{8,9}, its basolateral expression influences the activation of anti-inflammatory suppressor T-cells that is important in controlling the inflammatory response of the gut\cite{28}. Therefore, an increased basolateral expression of CEA may be beneficial in the clinical course of IBD. IBD is thought to be the result of an uncontrolled intestinal inflammatory response. While the exact pathogenesis of the disease is not understood, it is likely that both the immune system as well as luminal agents (e.g. ingested nutrients or microbial agents) are important. Therefore, factors that are critical in permitting signals from the lumen to the mucosal immune systems may be important targets for therapy. It has been shown that intestinal epithelial cells from patients with IBD have reduced ability to activate suppressor T-cells and this correlates with the level of expression of CEA\cite{13}. It is interesting to note that whereas in normal intestinal tissue faint basolateral staining of CEA is detected, in patients with ulcerative colitis the basolateral staining is lost\cite{13}. Therefore, it is intriguing to speculate that cholesterol depletion of enterocytes in these patients may enhance the basolateral expression and therefore increase the reduced ability to activate CD8+ T-cells.

One therapeutic approach that has already been used in clinical studies in IBD is to alter the lipid composition of the cellular membranes. Lipid-based therapies such as the use of fish oil and phospholipids have been found to be successful\cite{24,25}. In addition, animal experiments employing ganglioside enriched diets and short chain fatty acids have shown reduction in intestinal inflammation\cite{11,12}. Interestingly, reducing cholesterol by systemic inhibition of HMG-CoA with pravastatin has also been found to reduce inflammation of DDS-induced colitis in rats\cite{31,32}. The authors noted that the effect of pravastatin was due to a decrease in the activity and expression of endothelial nitric-oxide synthase (eNOS). Emerging data suggests, that even small changes in cellular cholesterol levels is successful in influencing lipid raft processes\cite{24,34}. However, whether different compartmentation of apical and basolateral expressed proteins may, in part, have contributed to the positive effect of statins, remains speculative. Therefore, whether the lipid based therapeutic approach in IBD should include reduction in the cholesterol content of enterocytes, remains an open question. Further studies are needed to determine the clinical significance of our findings.

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**COMMENTS**

**Background**

Carcinoembryonic antigen (CEA) is a GPI-anchored glycoprotein that is believed to have distinct functions depending upon its expression at the apical or basolateral plasma membrane. Whereas it is possible to interact with bacteria with the apical location, basolateral expression may be involved in the activation of suppressor T-cells and thus have an anti-inflammatory effect. A reduced expression at the basolateral compartment has been implicated in the pathogenesis of inflammatory bowel disease (IBD). Thus influencing the apical to basolateral transport ratio may be a potential tool in the treatment of IBD.

**Research frontiers**

Sorting of proteins to the apical or basolateral membrane is a precisely regulated mechanism. One part of this sorting is regulated by the dynamic interaction of protein with lipid rafts. Influencing lipid raft dependent mechanisms by changing the lipid content of cells may provide a new therapeutic approach to human diseases.

**Innovations and breakthroughs**

Apical sorting of CEA is cholesterol and presumably lipid raft dependent. After cholesterol depletion, basolateral sorting is increased, thus increasing the presumed basolateral anti-inflammatory properties. These results are in line with previous reports on apical sorting of lipid raft associated proteins.

**Applications**

Local depletion of cholesterol from the luminal side of the gut may be a therapeutic approach for inflammatory intestinal disorders. This can possibly be integrated in a lipid based approach in the treatment of IBD, as previously shown with the use of phospholipids and omega-3 fatty acids.

**Peer review**

The manuscript is very innovative. Changing the intracellular transport of a protein towards an anti-inflammatory route is interesting. However, the clinical relevance remains unclear.

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