Differential expression of complement proteins and regulatory decay accelerating factor in relation to differentiation of cultured human colon adenocarcinoma cell lines

M-F Bernet-Camard, M-H Coconnier, S Hudault, A L Servin

Abstract
Self protection of host cells against inadvertent injury resulting from attack by autologous complement proteins is well reported for vascular epithelium. In intestinal epithelium, the expression of C complement proteins and regulatory proteins remains currently poorly reported. This study looked at the distribution of C complement proteins and regulatory decay accelerating factor (DAF) in four cultured human intestinal cell lines of embryogenic or colon cancer origins. C3 and C4 proteins and DAF were widely present in human colon adenocarcinoma T84, HT-29 glc-/+ cells compared with human embryonic INT407 cells. In contrast, no expression of C5, C5b-9, and CR1 was seen for any of the cell lines. Taking advantage of the Caco-2 cells, which spontaneously differentiate in culture, it was seen that the C3, C4, and DAF were present in undifferentiated cells and that their expression increased as a function of the cell differentiation. These results, taken together with other reports on the presence of C complement proteins and DAF in the intestinal cells infer that the expression of regulatory C complement proteins develops in parallel with the expression of C proteins to protect these cells against the potential injury resulting from the activation of these local C proteins. Moreover, the finding that the pathogenic C1845 Escherichia coli binds to the membrane bound DAF in the cultured human intestinal cells synthesising locally C proteins and regulatory C proteins supports the hypothesis that E coli could promote inflammatory disorders by blocking local regulatory protein function.

Keywords: inflammatory disorders, enterocytic differentiation, decay accelerating factor, human intestinal cells, diffusely adhering E coli.

It has been well reported that the activation of the complement system with and without the loss of the complement regulatory proteins would augment inflammation by allowing host complement to damage host cells. Human cells are protected from homologous C attack by expressing C regulatory proteins. In particular, decay accelerating factor (DAF – CD55), a glycosylphosphatidylinositol anchored glycoprotein (GPI) present on epithelial cells lining the vascular compartment, protects these cells from potential injury arising from attacks by autologous complement proteins by regulating the activity of both the classic and alternative complement pathways C3 and C5 convertases.1

DAF is present in extravascular cells and in these cells its function remains unknown. Expression of the C3 and C4 complement proteins has been reported in cultured human adenocarcinoma Caco-2 cells2,3 and their synthesis is regulated by cytokines.2 Very few studies have shown the presence of DAF in human intestinal cells.4 Our previous studies suggest, however, that this regulatory protein could be expressed by several cultured human colon adenocarcinoma cells. Indeed, we have seen that the cultured human adenocarcinoma HT-29 and Caco-2 cells can be infected by Escherichia coli bearing the fimbrial F1845 adhesin, Dr haemagglutinin or afimbrial AFA-I adhesin,5,6 which recognise in erythrocytes the Dr receptor,7,8 a part of the DAF.9

The aim of this study was to examine the comparative expression of several C complement proteins and DAF by the cultured human intestinal cells. As cells in the intestine differentiate along the crypt-villus axis,10 we used a subset of cultured human intestinal cell lines expressing different states of differentiation11,12 to approach in vitro this dynamic situation.

Methods

Cell culture
HT-29 glc-/+ and Caco-2 cells were a gift from A Zweibaum (INSERM Unité 178, Villejuif, France). The enterocytic Caco-2 cells13 spontaneously differentiate in culture under standard conditions – that is, in a standard glucose containing medium. The enterocytic HT-29 glc-/+ cells14 are a subpopulation obtained from the parental, mainly undifferentiated HT-29 standard cell line15 by selection through glucose deprivation16 reversion back to a standard glucose containing medium. HT-29 glc-/+ and Caco-2 cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (DMEM) (25 mM glucose) (Eurobio, Paris, France), supplemented with 10% (HT-29 glc-/+ ) or 20% (Caco-2)
inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Germany) and 1% non-essential amino acids (Caco-2) at 37°C in a 10% CO₂/90% air atmosphere. Cells were seeded at a concentration of 10³ (HT-29 glc⁻/⁻) and 7.4x10⁴ (Caco-2) cells per cm². For maintenance purposes, cells were passaged weekly using 0.25% trypsin in Ca²⁺/Mg²⁺ free phosphate buffered saline (PBS) containing 0.53 mM EDTA. Unless otherwise stated, cells were used for adherence assays at late post-confluence – that is, after 15 days (Caco-2) and 20 days (HT-29 glc⁻/⁻) in culture. For time course, Caco-2 cells were used after 5 days, 10 days, and 15 days in culture.

T₈₄ cells were from K Dharmasathaphorn (University of California, San Diego, CA). Cells were routinely grown in DMEM (50%) and Ham's F12 (50%) supplemented with 2 mM glutamine, 50 mM HEPES, 1% non-essential amino acids, and 10% inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Germany) at 37°C in a 10% CO₂/90% air atmosphere. Cells were seeded at a concentration of 10⁴ cells per cm². Cells were used for adherence assays at late post-confluence – that is, after 10 days.

INT407 cells (human embryonic intestine; ATCC CCL 6) were from a stock culture of the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with 1% non-essential amino acids and 10% inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Germany) at 37°C in a 10% CO₂/90% air atmosphere. Cells were used for adherence assays at confluence – that is, after four days.

Antibodies
The mouse monoclonal Cy-DAF antibody raised against human DAF was from Valbiotech (France). The rabbit polyclonal anti-human C5 fluorescein conjugated antibody F255, the anti-human C5b-9 mAb M777, and the anti-human CR1 (CD35) mAb M710 were purchased from Dako (Denmark). The rabbit polyclonal C-7761 anti-human complement C3 antibody and the rabbit polyclonal C-3402 anti-human complement C4 antibody were from Sigma Chemical Company (St Louis, USA). The ascites fluid containing antibody HBB 2/614/88 against human sucrose-isomaltase (SI) was a gift from Haufr HP (Biocentre of the University of Basle, Switzerland). The ascites fluid containing antibody 4H3 against human dipeptidylpeptidase IV (DPP IV) was obtained from Maroux S (Centre de Biochimie et de Biologie Moléculaire, Marseilles, France). The ascites fluid containing antibody HBB2/45 against aminopeptidase N (ApN) was from Quarori A (Cornell University, Ithaca, USA). Anticarcinoembryogenic antigen (CEA) mAb 517 was obtained from Le Bivic A (URA 179 CNRS, Marseilles).

Immunofluorescence
Indirect immunofluorescence was performed on unpermeabilised cell layers as previously reported. Preparations were fixed for 10 minutes at room temperature in 3:5% paraformaldehyde in PBS. Cell monolayers were incubated with specific primary antibody for 30 minutes at room temperature, washed, and then incubated with their respective secondary fluorescein conjugated antibody. Primary antibodies were diluted 1:50-200 in PBS (Cy-DAF, 1:50; anti-C5 F-255, 1:50; anti-C3 C-7761, 1:50; anti-C4 C-3402, 1:50; anti-C5b-9 M777, 1:50; anti-human CR1 M710, 1:50; anti-SI HBB 2/614/88, 1:200; anti-DPP IV 4H3, 1:50, anti-ApN HBB2/45, 1:50, and anti-CEA 517, 1:50) in 2% bovine serum albumin (BSA) PBS. Secondary antibodies were either fluorescein or rhodamine conjugated goat anti-mouse IgG from Immunotech (Luminy, France), and fluorescein conjugated goat antirabbit IgG from Institut Pasteur Productions (Paris, France), used at a dilution of 1:20 in 2% BSA-PBS. No fluorescent staining was seen when non-immune serum was used and when the primary antibody was omitted. Immunolabelling was examined using a Leitz Aristoplan microscope with epifluorescence. All photographs were taken on Kodak T-MAX 400 black and white film (Eastman Kodak, Rochester, NY).

Scanning and electron microscopy
For scanning electron microscopy, the intestinal cells were grown on glass coverslips. Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 24°C for one hour. After five washes with phosphate buffer, cells were post-fixed for 30 minutes with 0.5% osmium tetroxide and washed three times with the 0.1 M phosphate buffer. Cells were dried after substitution of buffer with liquid carbon dioxide (Balzers CPD030, Hudson, NH) and coated with gold. The specimens were then examined with a Jeol JSM 25S scanning electron microscope.

Bacterial strains, growth conditions, and radiolabelling
The diffusely adhering E coli (DAEC) C1845 harbouring the fimbrial F1845 adhesin was grown on CFA-agar containing 1% Casamino Acids (Difco Laboratories, Detroit, MI), 0.15% yeast extract, 0.005% magnesium sulphate, and 0.005% manganese chloride in 2% agar for 18 hours at 37°C.

Cell infection conditions
Quantitative binding assay involving the incubation of a fixed concentration of E coli (10⁸ CFU/ml), metabolically labelled by the addition of ¹⁴C-acetic acid (Amersham, 94 mCi/mmol; 100 µCi/10 ml tube) with cultured cells, was performed as previously described. Briefly, the cell monolayers were washed twice with PBS. Radiolabelled E coli were suspended in the culture medium without antibiotic, and a total of 1 ml (10⁸ ¹⁴C-labelled cells/ml) of this suspension was added to each well of the
Results

Expression of complement proteins and DAF in cultured human intestinal cell lines

We examined the expression of C complement proteins and regulatory DAF in INT407, T84, and HT-29 glc-/-, in parallel with the expression of differentiation associated intestinal markers; SI, DPP IV, ApN, and carcinoembryogenic antigen CEA.

Currently, the morphological and functional characteristics of the human embryonic intestinal INT407 cells are not reported. Observation by scanning electron microscopy of the cell surface showed no brush border, which is the principal morphological characteristic of differentiated intestinal cells. These cells exhibit an absence of expression of differentiation marker DPP IV (Fig 1), SI, and ApN, whereas expression of CEA was seen (Table I). These results show that INT407 cells are undifferentiated. C3 and C4 complement proteins were expressed at a low level by the INT407 cells, and the faint labelling seemed localised to the cell to cell contacts. No expression of C5 and C5b-9 proteins was seen. In contrast, a pronounced expression of DAF was found.

The T84 cells do not differentiate toward the villus-like state; these cells express functional properties of colon crypt cells. Observation of the T84 cells by scanning electron microscopy showed rudimentary microvilli sparsely distributed at their apical domain (Fig 2). Staining of DPP IV and CEA in T84 cells showed a high level of expression of these proteins, and the mosaic pattern showed variable intensities in expression among the cells (Fig 2). In contrast, ApN was sparsely expressed, whereas SI was not expressed at all. DAF was intensely expressed in a mosaic pattern, whereas the C3 and C4 complement proteins were highly expressed in a diffuse pattern with randomly distributed pronounced patches. No C5, C5b-9, and CRI expression occurred in T84 cells.

The HT-29 glc-/- cell population expressed structural and functional differentiation characteristics similar to those of the mature enterocyte of the small intestine. Scanning electron microscopy of the HT-29 glc-/- cells show the presence of tall and regular microvilli uniformly distributed and forming a dense and well organised brush border, which carpets the apical surface (Fig 3). These cells widely expressed the DPP IV and CEA, expressed little ApN, and lacked SI, the last because in these cells the SI expression is inhibited by the presence of glucose in the culture medium. The C3 complement protein and the DAF were intensely expressed by HT-29 glc-/- cells. In contrast, low expression of C4 protein was evident. As for T84 cells, no C5, C5b-9, and CRI positive immunoreactivity was detected.

As summarised in Table I, the undifferentiated INT407 cells expressed a low level of C complement proteins and regulatory DAF. In contrast, such proteins were widely expressed in the T84 colonic crypt cells and in the fully

Figure 1: Expression of C3 and C4 complement proteins, and DAF in human cultured intestinal INT407 cells. (A) High magnification scanning electron micrograph of confluent cells showing the disorganised cell surface with long bulges. Magnification × 10 000. (B) Interference contrast micrograph of cells, magnification × 40. (C to F) Showing indirect immunofluorescence labelling of DPP IV (C), C3 (D), C4 (E), and DAF (F). Note the absence of immunolabelling for DPP IV and the low level and faint immunofluorescence labelling localised to the cell to cell contact for C3 and C4. In contrast, the immunofluorescence labelling of DAF at the cell to cell contact was considerably pronounced.

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Expression of C proteins, CR1, DAF, and differentiation associated markers by human cultured intestinal INT407, T84, HT-29 glc-/-, and Caco-2 cells</th>
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<tbody>
<tr>
<td>INT407</td>
<td>T84</td>
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<tr>
<td>----------</td>
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</tr>
<tr>
<td>Sucrase-isomaltase</td>
<td>Negative</td>
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<tr>
<td>Dipeptidylpeptidase IV</td>
<td>Negative</td>
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<tr>
<td>Aminopeptidase neuter</td>
<td>Negative</td>
</tr>
<tr>
<td>CEA</td>
<td>Positive</td>
</tr>
<tr>
<td>Complement C3</td>
<td>Low positive</td>
</tr>
<tr>
<td>Complement C4</td>
<td>Low positive</td>
</tr>
<tr>
<td>Complement C5</td>
<td>Negative</td>
</tr>
<tr>
<td>Complement C5b-9</td>
<td>Negative</td>
</tr>
<tr>
<td>CRI</td>
<td>Negative</td>
</tr>
<tr>
<td>DAF</td>
<td>Low positive</td>
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</table>

HT-29 glc-/- cells after 20 days in culture; †: Caco-2 cells after 15 days in culture.

tissue culture plate. Adherence assays were performed in the presence of 1% mannose to block the non-specific attachment of the bacteria by type I fimbrae to the cells. The plates were incubated at 37°C in 10% carbon dioxide/90% air for one hour. The monolayers were then washed three times with sterile PBS. Adhering bacteria and intestinal cells were dissolved in 0.2 N NaOH solution. The level of bacterial adhesion was evaluated by liquid scintillation counting. Inhibition of adhesion was conducted using anti-DAF mAb CY-DAF (diluted 1:20 in PBS). Cells were treated with 4 mg/ml phosphatidylinositol specific phospholipase C (PIPLC, Sigma Chemical Company, St Louis, USA) for 60 minutes at 37°C before incubating with E.coli. Each adherence assay was conducted in duplicate with three successive cell passages.
differentiated colon carcinoma HT-29 glc\(^{-+}\) cells. These results suggested that the expression of C3, C4, and DAF could evolve as a function of cell differentiation.

Expression of C3 complement protein and DAF as a function of the intestinal cell differentiation

In the intestine a rapid epithelial cell renewal occurs and the cells differentiate along the crypt-villus axis.\(^{11,12}\) To approach in vitro the dynamic situation of the intestine, we took advantage of the Caco-2 cells, which spontaneously differentiate in culture.\(^{13}\) Time course of appearance of C3 and DAF was determined in Caco-2 cells as a function of the days in culture together with the differentiation marker SI (Fig 4). The proportion of SI positive cells increased progressively with the age of the culture, consistent with the well reported growth related expression of intestinal brush border hydrolases.\(^{11}\) The C3 complement protein and DAF were expressed by Caco-2 cells perpetually in culture in a diffuse pattern. When examined in relation to the time course of differentiation, we found that the level of expression of C3 complement protein and DAF increased in parallel with differentiation. The same result was obtained for the C4 protein (not shown). As for HT-29 glc\(^{-+}\) cells and T\(_{84}\) cells, no C5, C5b-9, and CR1 positive immunoreactivity was detected in Caco-2 cells at any period of culture.

**DAF acts as receptor for the diarrhoeagenic C1845 E coli in cultured human intestinal cells**

The diarrhoeagenic C1845 E coli bearing the fimbrial F1845 adhesin binds equally to the undifferentiated INT407, the poorly differentiated T\(_{84}\), and the fully differentiated Caco-2 cells (Table II). Significant inhibition of adhesion was obtained by incubating the cells and the bacteria with the anti-DAF mAb CY-DAF, although the effect was comparatively less prominent in T\(_{84}\) and Caco-2 cells than in INT407 cells. In Caco-2 cells, the inhibition of the C1845 binding by anti-DAF antibody is not significantly different from the inhibition obtained with anti-F1845 antibody. To analyse how the DAF is anchored to the cell membrane of cultured human intestinal cells, we treated the cells with PIPLC. A high level of inhibition of the E coli binding was seen in INT407 cells after PIPLC treatment, whereas in T\(_{84}\) and Caco-2 cells the inhibition was less pronounced.

**Discussion**

Defect of C complement regulatory proteins and blockage of their regulatory functions renders the host cells sensitive to inadvertent local complement mediated damage.\(^{20-22}\) Human cells exposed to abundant plasma C sources are protected from homologous C attack by expressing C regulatory proteins.\(^{1} 23 24\) Among these, membrane DAF and MCP, and plasma factors are responsible for self protection against classic and alternative pathways of homologous C.\(^{1,2} 23\) A local synthesis of C components occurs in extravascular non-intestinal epithelial cells.\(^{25-28}\) In the case of the intestine, regulatory DAF is present in cells lining the epithelium,\(^{9}\) and in cultured human intestinal cells synthesis of C3 and C4 proteins has been
Figure 4: Expression of C3 complement protein and DAF as a function of the cell differentiation of the human cultured intestinal Caco-2 cells. (A, D, G, J) Caco-2 cells at day 5 in culture; (B, E, H, K) Caco-2 cells at day 10 in culture; (C, F, I, L) Caco-2 cells at day 15 in culture. (A to C) Interference contrast micrograph of confluent cells. Indirect immunofluorescence labelling of sucrase-isomaltase (D–F); complement C3 protein (G–I); and DAF (J–L). Note the growth related expression of SI. Complement C3 and DAF were poorly expressed by the undifferentiated Caco-2 cells; the level of expression increases as a function of the days in culture.

In the human intestine, cells differentiate during their migration along the crypt-villus axis. In an attempt to record the comparative expression of C complement proteins and regulatory C proteins in human intestinal cells as a function of the intestinal cell differentiation, we used a subset of cultured human colon adenocarcinoma cells. Indeed, there are similarities between differentiated adenocarcinoma cells and the small intestine. Taking into account the origin of these cells – that is, malignant cells originating from the colon15 and expressing the characteristics of fetal colon at midgestation – they seem to express features of the fetal colon, as the fetal colon expresses transient small intestinal differentiation during the mammalian development. Regardless of the limitations of these cellular models, they have provided useful tools to study the functions of mature enterocytes and mucus secreting cells of the small intestine,11 and to analyse the intestinal cell differentiation process.12

Activation of the complement system participates in the pathogenesis of intestinal disorders.29–31 Local activation of the C system has been seen in ulcerative colitis32 and Crohn’s disease.33 Results presented here show that a panel of cultured human intestinal cells, such as INT407, T84, and HT-29 glc-/+ cells, expressing different degrees of cell differentiation, synthesise the C3 and C4 complement proteins and the regulatory DAF. There were some differences, however, in the pattern of expression and staining intensity among the four cell lines. Indeed, the presence of DAF, C3, and C4 proteins was especially noticeable in cases of carcinoma cells, whereas it was lower in embryonic undifferentiated intestinal cells. In contrast, all these cells did not express the C5 and C5b-9 complement proteins and the CR1 receptor. As the cells lining the vascular compartment1 and extravascular cells,21 22 24 the intestinal cells develop a C complement regulatory system. It was noticed that the expression of C complement proteins in the colon adenocarcinoma cells was somewhat different from that seen in thyroid cancer cells, which expressed C5b-9 and CR1 together with C3d, S-protein, DAF, MCP, CR2, and protectin.24 We report that C3 and C4 expression increases as a function of the cell differentiation. It was particularly interesting to note that the expression of the regulatory DAF increases in parallel. As the Caco-2 cells mimics in vitro the differentiation of the enterocytes during their migration along the crypt-villus axis,11 12 this suggests that the intestinal cells in the tips of the villi could be more protected against inadvertent C complement damage than the cells at the base of the crypt.

Our results are also of interest with regard to intestinal microbial pathogenesis. Indeed, several findings suggest that microbial infections could be an inducing factor of inflammatory bowel disease. For example, this hypothesis is supported by the finding of an association of viruses, such as the Epstein-Barr virus interacting with a membrane cofactor regulating the complement activity, with acute episodes of exacerbations of chronic inflammatory bowel disease.34 Moreover, gut mucosal adherent pathogenic E. coli have been seen in patients with colitis.35 Involvement of adherent pathogenic E. coli in gut inflammatory disease was also suggested by Bilge et al.,56 who saw inflamed mucosa in a pig model infected with the E. coli C1845 strain. This diarrheagenic E. coli belongs to a family of uropathogenic and diarrheagenic E. coli7 8 colonising different parts of the human urinary tract37 38 and the intestine.38 Considering the C regulatory function of DAF, Nowicki et al.9 postulated that interaction of these pathogenic E. coli with the cell membrane associated DAF could lead to an immunopathological lesion in the target cells. Our results are consistent with

<table>
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<th>TABLE II</th>
<th>DAF acts as receptor for the C1845 E. coli bearing the F1845 fimbrial adhesin in human cultured intestinal INT407, T84, and Caco-2 cells</th>
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<tbody>
<tr>
<td>INT407</td>
<td>T84</td>
</tr>
<tr>
<td>C1845 E. coli</td>
<td>5·89 (0·5)</td>
</tr>
<tr>
<td>Incubation with anti-F1845 antibody</td>
<td>ND</td>
</tr>
<tr>
<td>Incubation with anti-DAF antibody</td>
<td>1·33 (0·4)***</td>
</tr>
<tr>
<td>Incubation after PIPLC cell treatment</td>
<td>2·47 (0·2)***</td>
</tr>
</tbody>
</table>

* Caco-2 cells after 15 days in culture. ND = not determined. Each experiment was conducted in triplicate. The data represent mean (SEM) values of experiments from two or three successive passages of cells. **Significant difference (p<0·01); ***highly significant difference (p<0·001).
C proteins and DAF in cultured human intestinal cells

this hypothesis. Indeed, we show that E coli harbouring the fimbrial F1845 adhesin interacts in part with the membrane bound DAF to colonise the apical surface of undifferentiated, poorly differentiated, and fully differentiated human cultured intestinal cells in which C components were expressed. In consequence, it is possible that the colonisation of the intestine by this pathogenic E coli could promote inflammation of mucosa through a local C system, resulting from the blockage of the regulatory function of DAF by adhesin-DAF interaction.

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