The influence of luteolin on expression of epithelial MUC1 mucin in human skin fibroblasts

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ABSTRACT

Purpose: The membrane-anchored MUC1 mucin is typically expressed on normal and cancerous epithelial cells. Non-epithelial localization of this mucin is rare. However, the presence of MUC1 in human skin fibroblasts has been recently unexpectedly revealed. The aim of the study was to prove the expression of MUC1 mucin in human skin fibroblasts and the examine of the influence of luteolin on its expression.

Materials and methods: ELISA tests and real-time PCR analysis were used to assess the expression of MUC1 mucin in fibroblast cells cocultured with 30 μM concentration of luteolin.

Results: The expression of MUC1 was revealed in human skin fibroblasts. Luteolin decreased the relative level of mucin in cell lysates and media. Statistically significant decreased expression of *MUC1* gene after luteolin treatment of fibroblasts cells was also revealed.

Conclusion: Our results prove non-epithelial localization of MUC1 mucin. Luteolin inhibits the expression of MUC1 mucin in healthy human skin fibroblasts.

Key words: fibroblasts, luteolin, MUC1

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INTRODUCTION

MUC1 mucin (episialin, PEM, CA15-3) is transmembrane protein with a heavy glycosylated extracellular domain, extending up to 200-500 nm from the cell surface, transmembrane domain and cytoplasmic tail [1,2]. MUC1 is typically expressed by the luminal or glandular epithelial cells of the mammary gland, esophagus, stomach, duodenum, colon, pancreas, uterus, lungs, and to a much lesser extent, in hematopoietic cells [3,4]. It has been stated so far as negative in the skin epithelium and in mesenchymal cells [5]. However, unexpectedly, it has been recently observed that MUC1 mucin was expressed by normal human skin fibroblasts, which were assumed as negative control during authors' experiments [6].

The main role of MUC1 in healthy tissues is providing protection to underlying epithelia. Because of extended extracellular domain, the mucin creates a physical barrier limiting accessibility of pathological agents to epithelium [7]. Apart from that MUC1 takes part in regulation of cell signaling. It is postulated to serve as sensor of the external environment, as signals can be transmitted to the nucleus by association of MUC1 cytoplasmic tail with agents of signal transduction [8,9].

Luteolin is a flavone, universally present as a constituent of many herbs, species, vegetables, and fruit. It has been demonstrated to exhibit inflammatory effects, antioxidant properties and others. It has been used as a traditional medicine to treat various inflammatory diseases [10,11].

As there are not many studies on non-epithelial localization of MUC1 and its function we decided to check the expression of this mucin in human skin fibroblasts. We also examined the influence of luteolin on the mucin expression as MUC1 can be potentially involved in processes on which the flavone can effect.

MATERIALS AND METHODS

Fibroblast culture

The normal human skin fibroblasts CRL-1474 (American Type Culture Collection, USA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma, USA). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells reached confluency were used for assays. Cells (1×10^6 cells/ml) were suspended in 6 ml DMEM and incubated with or without 30 μM concentration of luteolin in six-well cell culture plates. After 24 h cells were washed with PBS and lysed at 4ºC with RIPA buffer (Sigma, USA) supplemented with protease inhibitors (Sigma, USA), diluted 1: 200 in RIPA buffer. The lysates and collected culture media were centrifuged at 1000 x g for 5 min at 4ºC. The supernatants were frozen at -70 ºC and then used for ELISA tests. For real time PCR determinations the monolayers were washed three times with sterile 10 mM PBS pH 7.4, and cell membranes were disrupted using sonicator (Sonics Vibra cell). Aliquots of the homogenate were used for RNA isolation. Cells without addition of luteolin were treated as control.

A BCA Protein Assay Kit (Pierce, USA) was used for a protein concentration measurement [12].

ELISA Tests

Aliquots (50 μl; 200 μg of protein/mL of cell lysates and 50 μL of undiluted medium) of the samples were coated onto microtiter plates (NUNC F96; Maxisorp, Denmark) at room temperature (RT) overnight. The plates were washed 3 times (100 μl) in the washing buffer - PBS-T (PBS, 0.05% Tween) between all ensuing steps. Unbound sites were blocked with 100 μl of 1% blocking reagent for ELISA (Roche Diagnostics, Germany) for 1 h. Then the plates were incubated (2 h at RT) with 100 μl of anti-MUC1 (BC2, Thermo Scientific,USA), diluted 1 : 400 in PBS-T-BSA (1%) (Sigma, USA). Then the plates were incubated (2 h at RT) with 100 μl of secondary, rabbit anti-mouse IgG horseradish peroxidase conjugated (Sigma, USA), diluted (1 : 2,000) in PBS-T-BSA (1%). Next, after washing five times in PBS, the colored reaction was developed by incubation with 100 μl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) – the liquid substrate for horseradish peroxidase (Sigma, USA). Absorbance at 405 nm was measured after about 30 – 40 min. As negative controls wells with BSA instead of proper samples were used. As positive control for MUC1 lysates and media of gastric cancer cells (CRL-1739, American Type Culture Collection, USA) cultured according to standard procedure were used. The test was performed twice with three replicates of each sample.

Real-Time PCR

Total RNA was isolated using the *EXTRACTME* TOTAL RNA purification kit according to the manufacturer's instruction. Concentration and purity of RNA was determined spectrophotometrically (Nanodrop 2000, ThermoScientific). First-strand cDNA was synthesized from 1 μg of total RNA using Tetro cDNA Synthesis Kit. The reaction mixture containing in the volume of 20 μl 1 μl oligo$(dT)_{18}$ primer, 1 μl of dNTP mixture (10 mM each), 5 μl of 5 x RT Buffer, 1 μl of RiboSafe RNase Inhibitor (10u/μl), 1 μl of Tetro Reverse Transcriptase (200u/μl) and DEPC-treated water was incubated for 30 min at 45°C and then inactivated at 85°C for 5 min. Real-time PCR assay was performed in CFX96 Real-time system (Bio-Rad) using SensiFAST™

SYBR Kit. The reactions contained 2 µl of diluted twice cDNA template, 0.8 µl of each primer (10 µmol/L), 10 µl 2x SensiFAST SYBR Mix and nuclease-free water in a final volume of 20 µl. Forward and reverse primer sequences are listed in Table 1. The gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was evaluated as housekeeping. Cycling parameters were: 95°C for 1 min to activate the DNA polymerase, then 40 cycles of denaturation for 10 s at 95°C, annealing for 15 s at 60°C, and extension for 20 s at 72°C. The reaction was then subjected to a melting protocol from 55ºC to 95°C with a 0.2°C increment and 1 s holding at each increment to check the specificity of the amplified products. Single product formation was confirmed by melting point analysis and agarose gel electrophoresis. For negative control, water instead of mRNA samples was used. Samples were run in triplicate and the ΔΔCT method was applied for statistical analysis of the CT-values. The relative gene expression levels were standardized with those measured in the untreated control.

The study was approved by the institutional ethics committee with the principles of the Declaration of Helsinki.

Table 1. Sequences of primers used for real-time quantitative RT-PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	References
MUC1	TGCCTTGGCTGTCTGTCAGT	GTAGGTATCCCGGGCTGGAA	[19]
GAPDH	GTGAACCATGAGAAGTATGACAA	CATGAGTCCTTCCACGATAC	[20]

Statistical Analysis

Experimental data were presented as mean ± standard deviation SD from at least three independent experiments. Statistical differences were determined by one-way ANOVA followed by the Duncan's multiple range post hock test, $p < 0.05$ was considered statistically significant.

RESULTS

BC2 anti MUC1 monoclonal antibody was used in ELISA test to assess the presence of MUC1 mucin in lysates and media of human skin fibroblasts. The antibody is directed against extracellular domain of examined mucin and it recognizes sequence of certain amino acids in polypeptide chain regardless of glycosylation. Figure 1 shows the results of the test for lysates of the culture cells. We can observe a decreased relative level (by 15 %) of MUC1 in fibroblasts luteolin treated (line 1 and 2). In line 3 we see much higher absorbance for MUC1 detected in gastric cancer cells CRL-1739 which were used as positive control as gastric cells represent epithelial cells typically expressing MUC1 mucin.

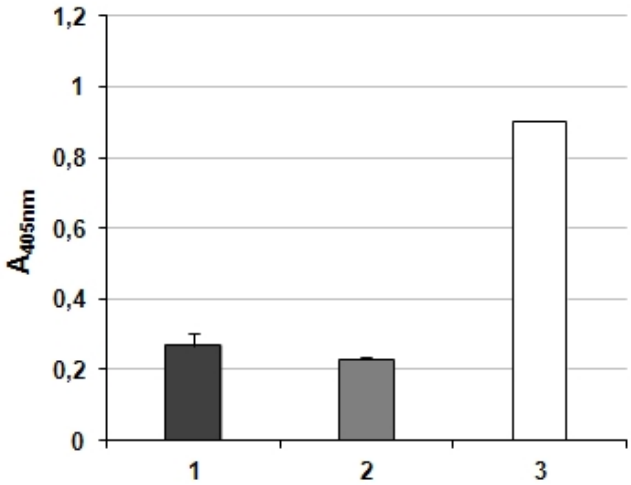

Figure 1. Relative levels of MUC1 mucin in fibroblasts lysates
The results are shown as absorbance at 405 nm after reactivity with monoclonal anti-MUC1 in ELISA test. 1 – lysates without luteolin treatment; 2 – lysates luteolin treated (30 µM); 3 – lysates of gastric cancer cells CRL-1739 as positive control of MUC1. Bars represent ± SD.

In Fig. 2 there are results of MUC1 assessment in culture media. Luteolin decreased by 15% the relative level of examined mucin (lines 1 and 2). In lane 3 higher absorbance assessed for gastric cancer cells is also seen.

To evaluate the expression of *MUC1* gen quantitative real-time PCR analysis was performed. Fig. 3 shows the expression of MUC1 mRNA in human skin fibroblasts and statistically significant inhibition effect of luteolin on *MUC1* gene expression (lane 2).

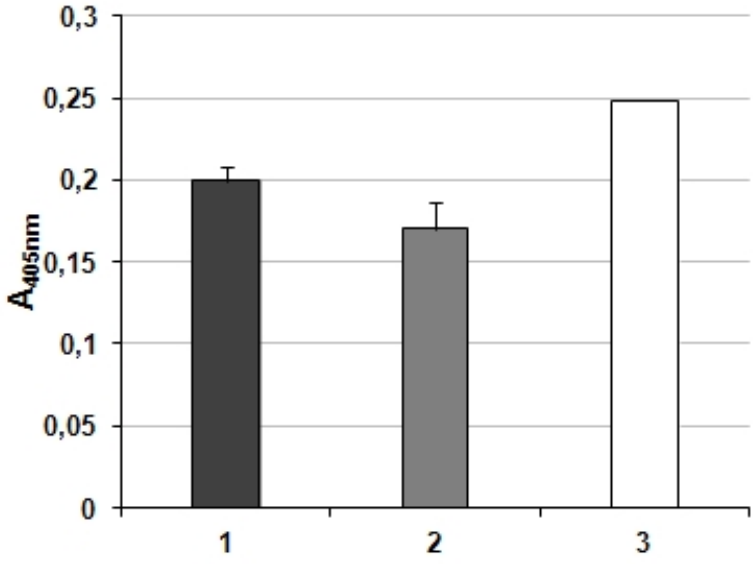

Figure 2. Relative levels of MUC1 mucin in fibroblasts media
The results are shown as absorbance at 405 nm after reactivity with monoclonal anti-MUC1 in ELISA test. 1 – media without luteolin treatment; 2 – media luteolin treated (30 μM); 3 – media of gastric cancer cells CRL-1739 as positive control of MUC1. Bars represent ± SD.

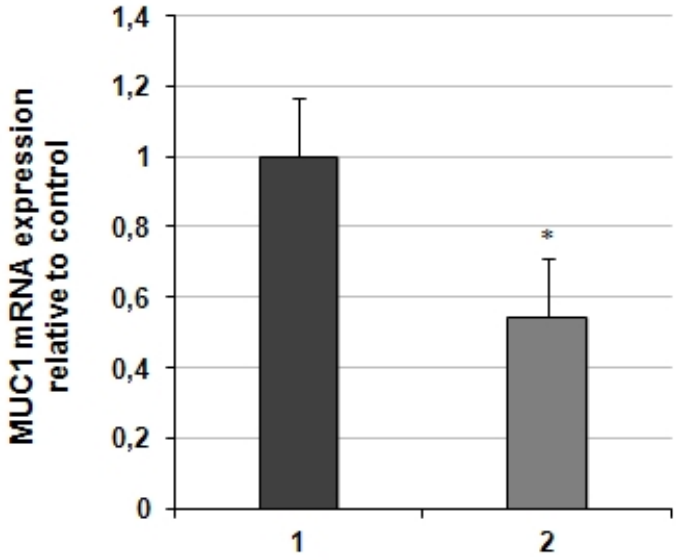

Figure 3. Real-time PCR analysis of MUC1 mRNA in skin fibroblasts
The results were expressed as a relative fold change in mRNA expression of *MUC1* gene in comparison to control where expression was set as 1. Values ± standard deviation (SD) are the mean of triplicate cultures. Statistically significant difference versus control was marked with asterisk ($p<0.05$).

DISCUSSION

Membrane-tethered MUC1 mucin is glycoprotein typically located on normal and cancerous epithelial cells [1,3,4]. However, this mucin can be also expressed by activated T cells, dendritic cells as well as some non-epithelial cancer cells and embryonic stem cells [13,14]. Because of its specific location MUC1 creates kind of mediator between extracellular and intracellular environment and plays roles in regulating adhesion, migration, cell signaling. In healthy tissues the expression of this mucin is restricted to the epical surface of the cells. In cancers, because of the loss of polarity, MUC1 is present on the whole surface of the cells and in this way can change cell-cell interactions [7].

Recently, the expression of MUC1 mucin has been unexpectedly observed in normal human skin fibroblasts (isolated from human neonatal foreskin and from adult truncal skin), which have been treated as negative control for this mucin so far. It is worth to emphasize that the authors of the study presented the first evidence of the functional role for MUC1 in fibroblasts. Upon their results they propose the participation of the mucin in the regulation of human skin fibroblasts adhesion and migration. They also showed that MUC1 regulates the expression of α_2-integrin which is said to be involved in fibroblasts motility [6,15].

As the subject of MUC1 expression in skin fibroblasts is new and because of our long lasting interest with MUC1 (especially in gastric epithelium) [16,17] we decided to prove or not the presence of this mucin in healthy human skin fibroblasts cell line. Our preliminary results revealed the expression of MUC1 in lysates and culture media. Simultaneously we also observed the expression of *MUC1* gene revealed by RT PCR analysis. So these results seem to confirm untypical localization of MUC1 in human skin fibroblasts.

Because of its special structure with extracellular, transmembrane and intracellular domains, MUC1 can be involved in many important processes, connecting, among others, to interactions between cells or inflammation process. It is said that MUC1 reveals an inflammatory role in cancer cells but has anti-inflammatory function during infectious diseases [7,18]. In our study, we showed inhibitory effect of luteolin, well known anti-inflammatory agent, on MUC1 expression in the examined healthy fibroblasts. Upon our results we can assume that MUC1 mucin may participate in processes influenced by luteolin.

We want to emphasize that our study should be treated as preliminary one. The subject is going to be explored more thoroughly. We are going to assess the participation of the mucin in interactions with some factors of extracellular matrix like integrins or collagen. We believe that the results of our future studies will be able to define the role of MUC1 in healthy and pathological human skin fibroblasts.

CONCLUSIONS

We proved non-epithelial localization of MUC1 mucin. We revealed an inhibitory effect of luteolin on MUC1 expression in human skin fibroblasts.

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Conflicts of interest

The authors declare that they have no conflicts interests.

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