Immunocytochemistry of *In vitro* produced mucin from HT29-MTX cell line

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Abstract

Mucus represents the first line of defense against pathogens and damage in the human intestine. the major components of mucus are mucins which are high molecular weight glycoproteins providing potential binding sites for both pathogenic and commensal microorganisms. To study host-enteric pathogen interactions, human intestinal cell models are widely used. Epithelial cell lines derived from human colon carcinomas providing useful experimental alternatives as getting normal intestinal cells is not easily applicable.

In the current study, we described a condition enhances HT29-MTX cells to produce continuous mucus layer. Immunocytochemistry was used to visualize Muc 2, the most secreted mucin from HT29-MTX cells using anti Muc2 antibody.

The results showed that incubation of HT-29 MTX cells with shaking and providing cells with fresh medium once to twice daily for three weeks can be the best way to mimic the natural behavior of the intestine which enhanced the seeded cells to produce the mucin *in vitro*.

Keywords: *HT29-MTX cells, Mucus, Mucin, Immunocytochemistry.*

Introduction

Goblet cells are simple columnar epithelial cells found in the lining epithelium of the stomach, intestine (with highest density in the colon), respiratory tract and genital tract. They secret the mucin which is forming a gel covering layer protecting the mucosa[1-6]. The slow, continuous liberation of mucin from a certain goblet cells over the epithelium is renewing and maintaining the mucus blanket under the normal conditions [1,6,7]. However, different irritants are known to induce the mucus secretin from the goblet cells, which built a protecting layer to the affected surface [1,2,6]. These irritants found to increase the activity of goblet cells mucus secretion. They include chemical substances such as ammonia, sulfur dioxide, smoke [1], or biological such as proteases, bacterial toxins [2,6-9], parasitic and viral infections [10], or mechanical insults or traumas[1]. In addition, acetylcholine found to be induce mucus secretion in most studies of large and small intestines in laboratory animals and human colon [5,11].

The continuous slow secretion of mucus in the normal mucosa sustain the mucus blanket which has a significant biological importance in protecting the lining epithelium against different challenges, such as some dangerous particulates [1,12], binds toxins [1,13], microorganisms [1,10,14] and antigenic macromolecules [1,13]. Generally, mucus is controlling the invasion and adhesion of different pathogens on the surface of the epithelial cells [4].

Goblet cells observed to show some variations when response to some infections. including its mucin secretion. Changes like goblet cells hyperplasia and increased mucin secretion in addition to some changes in mucin glycosylation are the main changes have been

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observed [10].

The main component of goblet cell mucus is the mucin, the viscoelastic and protective components of mucus [6,15-18]. Mucus also includes other components such as water, ions, some immune system components like the IgA and anti-microbial peptides which serve in removing the infected pathogens and providing a physical barrier protecting epithelial cell surfaces from the insults [7,10,12,17,19,20].

To study the host-enteric pathogen interactions, human intestinal cell models are widely used for this reason as. Epithelial cell lines derived from human colon carcinomas providing useful experimental alternatives as getting normal intestinal cells are not available [4,21]. Different cell lines were used to study the functional and specific characteristics of gut epithelium [4]. The Caco-2 and HT-29 human intestinal cell lines which are isolated from colon adenocarcinomas are the most widely used cell lines for *in vitro* attachment and mechanistic studies [4,22-24]. Caco-2 cell line grow as monolayers in culture and differentiates into highly similar cells to that of the normal enterocytes in the intestinal epithelium [4,24]. Whereas, HT29-MTX cells differentiates to a goblet cell-like phenotype and are able of secreting low amounts of MUC2 mucins which is normally secreted in the small and large intestine [3,4,7,20,24,25]. Therefore, HT29-MTX providing a model system for the study of human goblet cell differentiation and mucin secretion [21,24]. Accordingly, this study aimed to find a new method and a suitable condition to induce the HT29-MTX cells to produce mucus layer and visualize this layer using immunocytochemistry (ICC) technique as visualization of mucin helps in studying host-pathogen interactions at the mucosal surface.

Methods

-Cell lines

Caco-2 cells and HT29-MTX cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (HIFBS), 5 mM L-glutamine, 50 µg/ml streptomycin and penicillin in an atmosphere containing 5% CO_2 at 37˚C.

-Growth conditions

1. HT-29MTX cells at a concentration of 2×10^4 cells/ml were seeded on coverslips in a 24 well plate and incubated for three weeks. Cells were fed with fresh medium every two days.

2. HT-29MTX cells at a concentration of 2×10^4 cells/ml were mixed with Caco2 cells at a concentration of 7.5 x 10^4 cell/well in a ratio of 1:1 and 1:3 and seeded on coverslips in a 24 well plate and incubated for three weeks**.** Cells were fed with fresh medium every two days.

3. HT-29MTX cells at a concentration of 2×10^4 cells/ml were seeded on coverslips in a 24 well plate and incubated for three weeks fed with fresh medium every two days. Cells were infected with Enteropathogenic *E. coli* (EPEC) for an hour.

4. HT-29MTX cells at a concentration of 2×104 cells/ml were seeded on coverslips in a 24 well plate and incubated for three weeks fed with fresh medium every two days. Cells then treated with Capsicum extract in DMEM medium for an hour.

5. HT-29 MTX at a concentration of 2×10^4 cells/ml were seeded on coverslips in a 24 well plate and incubated for three weeks with shaking 80-100 rpm/min and fed with fresh medium once to twice daily.

-Immunocytochemistry (ICC)

The fixed HT-29 MTX cells were treated with 0.1% Triton X-100 for 15 minutes, then 1 ml of 1% bovine serum albumin (BSA) was added for an hour. Anti muc 2 antibodies produced in mouse was added in blocking buffer 1:400 for an hour, then cells were washed three times with blocking buffer. In dark, the secondary antibody anti-mouse IgG FITC 1:1000 was added for 1 hour. Cells then washed with PBS three times, followed by adding concanavalin A 25µg/ml for 20 minutes. Lastly, cells were washed, and coverslips were mounted using an anti-fade medium.

-Infection of HT29 MTX cells with pathogenic bacteria

Enteropathogenic *Escherichia coli* (EPEC) was grown in LB broth containing ampicillin at a concentration of 100µg/ml the day before the experiment. HT29 MTX cells at a concentration of 2×10^4 cells /ml were seeded

on coverslips in a 24 well plate and incubated for three weeks**.** Bacteria were added at 100 MOI and the plates were incubated at 37℃ for 1 hour. After bacterial infection, DMEM medium was removed and HT29- MTX cells were washed with PBS and stained using ICC staining as mentioned above.

-Preparation of Capsicum extract

Capsicum dried fruit powder was extracted using hot water. A 250 ml distilled water was added to 50gm capsicum powder and heated to 120-150ºC for 30 minutes. The extract was filtered using Whitman filter paper and sterilized using 0.2µm Millipore filter.

Results

The results showed that using agitation during cells incubation period had significantly increased the mucus secretion in the seeded cells as it can be seen in figure 5. However, incubation of HT-29 MTX cells with and without Caco2 cells, infection of HT29-MTX cells with Enteropathogenic *E. coli* (EPEC) and treatment of HT29-MTX cells with Capsicum spp (chili) did not show a clear enhancement in mucus production (Figures 1, 2, 3 and 4).

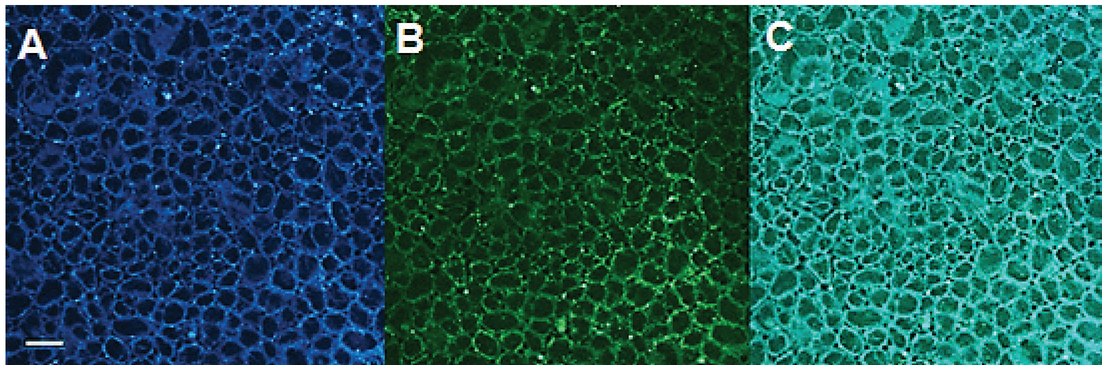

Figure (1): Immunocytochemistry staining of HT-29MTX mucin. The image displays: (A) cell membrane, (B) mucin and (C) merged image, scale bar 20 µm.

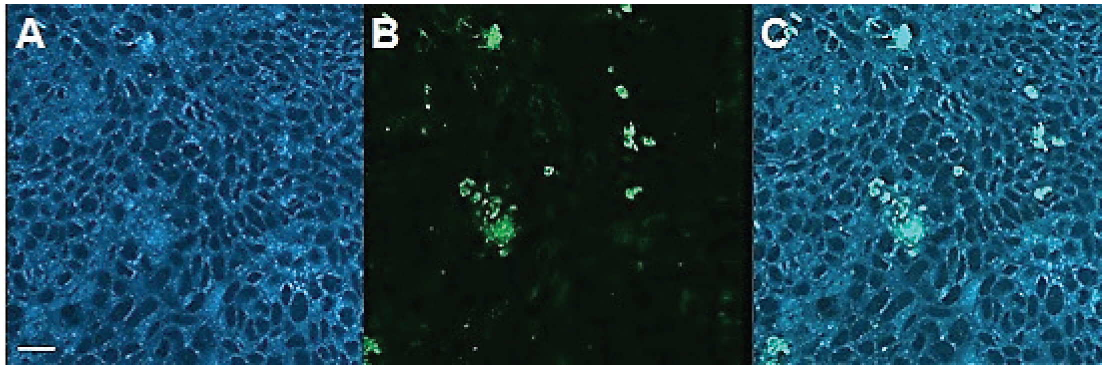

Figure (2): HT-29MTX cells and Caco2 cells cultured together for three weeks. The image displays: (A) cell membrane, (B) mucin and (C) merged image, scale bar 20 µm.

Figure (3): HT-29MTX cells infected with EPEC. Cells were infected with EPEC expressing mCherry for 1 hour. The image displays: (A) cell membrane, (B) mucin, (C) EPEC1 expressing m Cherry (Red) and (D) merged image, scale bar 20 µm.

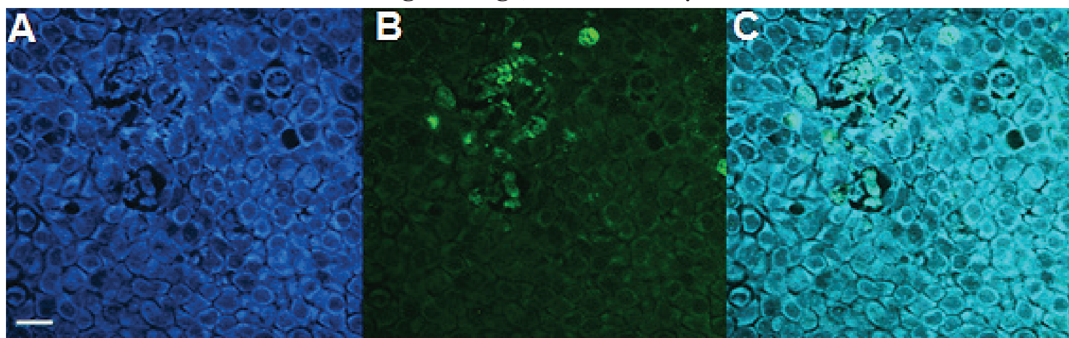

Figure (4): HT29-MTX cells after treatment with capsicum extract. The image displays: (A) cell membrane, (B) mucin and (C) merged image, scale bar 20 µm.

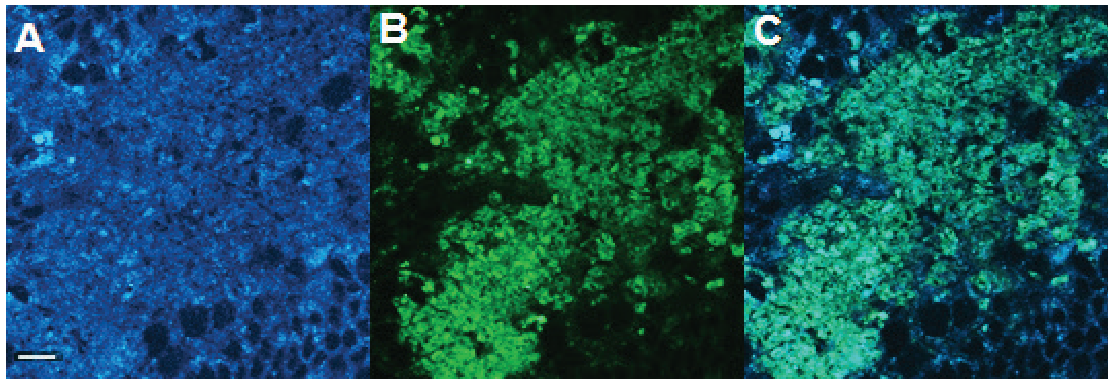

Figure (5): HT29-MTX cells cultured for three weeks under Agitation. The image displays: (A) cell membrane, (B) mucin and (C) merged image, scale bar 20 µm.

Discussion

Mucus represents the first line of defense against pathogens and damage. Major components of mucus are mucins, which are high molecular weight glycoproteins that provide potential binding sites for both pathogenic and commensal microorganisms. MUC2 and MUC5AC are the main constituents of the mucus in the intestine and stomach, respectively which are both produced by the goblet cells [6,7].

Here, we described a condition to induce a mucus layer production from HT29-MTX cells, then we stained the produced mucin using the ICC technique to visualize this layer. The anti muc 2 antibodies produced in the mouse were used in the ICC which binds to mucin. Whereas the secondary antibody (anti-mouse IgG FITC) binds to the primary antibody that allows the visualization of the mucin under the fluorescence microscope. Concanavalin A was used to visualize the cell membrane. Concanavalin A interacts with the glucosyl and mannosyl groups on the cell membrane [26].

Incubation of HT29-MTX cells for three weeks with feeding every two days results in the production of a few spots of mucus layer on cells (Figure 1). Thus, a variety of treatments were used to enhance mucus production.

Firstly, a co-culture of human Enterocytes (Caco-2) and goblet cells (HT29-MTX) were seeded at a concentration of 1:1 and 1:3. It has been reported that the proportion of goblet cells in the human colon is 25-55% and the mixed culture of Caco-2 and HT29 leads to a morphology similar to the small intestine [27,24]. Herein, HT-29MTX cells and Caco2 cells were mixed, seeded and incubated for three weeks with feeding every two days. Yet, the observed result revealed that no continues mucus layer was seen (Figure 2).

Kim *et al.* (2003) found that encounter with pathogens and their products can result in mucin production. In addition, Linden *et al*. (2007) reported that co-cultures consist of pathogen-mammalian cells were used to study induction of pathogens adhesion, invasion and host signaling. But cell lines usually used for host-pathogens studies were variable in mucins expression with low mucin production. Therefore, the second condition in the current study involved infection of HT29-MTX cells with Enteropathogenic *E. coli* (EPEC). Despite the previously reported data, it has been found that this treatment did not improve mucus production, Figure 3.

Moreover, it has been reached that treatment of rat's lung by capsaicin can result in mucus secretion [30,31]. Thus, the third condition in this study involved the treatment of HT29-MTX cells with Capsicum spp. extract. Yet, the result revealed that no continues mucus layer was seen (Figure 4), which may indicate that Capsicum seems to induce mucus production only *in vivo*. This may be because Capsicum activates sensory nerves in the lungs, which leads to mucus release [30,31].

Alternatively, HT-29 MTX cells were incubated under shaking for three weeks and fed with fresh medium once to twice daily, which was the fourth condition in the current study. This condition can mimic the behavior of the intestine condition. Interestingly, the result of this condition clearly showed that shaking cells with continuous feeding provides a good condition to enhance mucin production *in vitro* (Figure 5).

Conclusion

Incubation of HT-29 MTX cells with shaking and providing cells with fresh medium once to twice daily for a period of three weeks can be the best way to mimic the natural behavior of the intestine and lead to enhance the seeded cells to produce the mucin *in vitro*.

Acknowledgement: The authors would like to thank the University of Mustansiriyah for the encouragement to its researchers.

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Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: sNon

Funding: Self-funding

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