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A simplified cell culture model for research on intestinal inflammation

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Background/aim: The aim of this study was to combine some easy and economical defined methods and constitute a comprehensive cell culture model to use in the bowel diseases characterized by inflammation.

Materials and methods: Induction of inflammation was performed using lipopolysaccharide (LPS) or a cytokine mixture (TNF-α: 10 ng/mL; IFN-γ: 100 ng/mL; IL-1β: 1 ng/mL). IEC-6 cells were grown to confluence and serum-starved, wounds were constituted, and progress of cell migration into the wounds was photographed at 0, 2, 4, 6, 8, 10, 12, and 24 h in the presence or absence of an inflammatory environment. Cells were then grown and multiple scratches were performed to replicate the conditions of migration assay. Nitric oxide synthase-2 (iNOS) and cyclooxygenase-2 (COX-2) protein expressions were assessed.

Results: Cells covered 88% of the initial wound at 24 h in the control group, 54% in the LPS group, and 35% in the cytokine mixture group. LPS and the cytokine mixture were also found to independently increase iNOS and COX-2 expressions.

Conclusion: Our study, being inexpensive and practical, describes a model that integrates some methods to constitute a basic model for bowel diseases characterized by inflammation. It can be integrated as a preliminary experiment for etiopathogenesis and drug research studies.

Key words: Cell culture, inflammation, cell migration, nitric oxide synthase, cyclooxygenase

1. Introduction

Bowel diseases such as Crohn disease, ulcerative colitis, Hirschsprung enterocolitis, celiac disease, and necrotizing enterocolitis are characterized by both local and systemic excessive inflammatory response. However, the exact etiopathogenesis underlying these pathologies is still obscure and effective treatment strategies are not yet well established. Since ethical considerations limit both clinical and animal studies, in vitro cell culture studies are of importance for the investigation of the mechanisms underlying the pathogenesis of these complicated diseases and to develop effective management strategies. This preliminary report is the product of our research for an efficient and inexpensive model for evaluating a candidate drug for necrotizing enterocolitis.

Bowel diseases characterized by inflammation have some major common properties. Impairment in bowel mucosal wall integrity, excessive inflammatory response, and a delay in epithelial restitution are observed in all these diseases (1). Thus, we aimed to constitute a model in order to evaluate mucosal injury, inflammation, and restitution.

2. Materials and methods

2.1. Cell culture

IEC-6 cells were normal rat epithelial cells from the small intestine, and obtained from DSMZ (ACC 111). IEC-6 cells were maintained in tissue culture medium consisting of 45% Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 45% Roswell Park Memorial Institute medium, 10% heat-inactivated fetal bovine serum, 0.1 U/mL insulin, 4 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2 (all from Sigma). In this study, we used passages between 15 and 20. Cells were grown on 75-cm^2 plates for protein extraction and six-well plates for migration assay. They were cultured and grown to confluence in the standard until the study day and then serum-starved for 12 h before the experiments to stop cell proliferation (2).

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2.2. Treatment

Application of lipopolysaccharide (LPS, 50 μg/mL) was used for an indirect evaluation of the effect of the gram-negative bacteria. In another set of experiments, cells were treated using a mixture of cytokines (Cytomix: TNF-α, 10 ng/mL; IFN-γ, 100 ng/mL; IL-1β, 1 ng/mL) (Sigma Chemicals, USA) to constitute a basic model of the inflammatory cascade (3,4). The cells treated with only serum-free medium were used a control group. Both treatments were applied after serum-starving the cells for 12 h and wounds were created just before the treatments. Photographing of the cells for migration assay started following the application of LPS/Cytomix. Sample collection for western blot analysis was performed 12 h after the treatments in separate sets of experiments.

2.3. Migration assay

Six-well plates were marked as quadrants with an indelible pen at the outer bottom before passaging of the cells to the plates. Cells (5×10^5) were incubated in each six-well plate and grown to confluence. In previous preparatory experiments, 2×10^5, 3×10^5, 4×10^5, and 5×10^5 cells were seeded into 6-well plates to set the number of cells needed for the initial number of cells for 95%–100% cell confluency at 24 h. Cells at 5×10^5 were found to be optimal in repetitive trials. At the 24th hour after seeding, in order to remove the rest of the serum, cells were washed with Ca^{+2}- and Mg^{+2}-free PBS. Then the cells were incubated for 12 h in serum-free medium. After starvation, cells were scratch-wounded with a sterile 200-μL pipette tip using the markings on the bottom of the well as reference points and washed twice with lukewarm phosphate-buffered saline to prevent reattachment of the cells, and serum-free medium in the absence or presence of treatment was renewed. The progress of cell migration into the wounds was photographed at 0, 2, 4, 6, 8, 10, 12, and 24 h using an inverted microscope hooked up to a camera (Olympus Optical, Tokyo, Japan).

The closure and hence the healing of the denuded area of the wound was then measured using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

Photographs were taken with a magnification of 40× from the same area at the center of each well using the quadrant markings at the bottom of the plate. Then they were transferred to ImageJ software. They were zoomed in to 75% to increase the accuracy of area measurement. A rectangle of the same width and length for each well was drawn with one edge on the marking. It was overlaid on the photo. The area of the denuded area in this rectangle was marked using a freehand tool and its area was measured using the area measurement tool (Figure 1).

2.4. Western analysis

Nitric oxide synthase-2 (iNOS) and cyclooxygenase-2 (COX-2) protein expressions were assessed to confirm the activation of the inflammatory cascade. Cells were

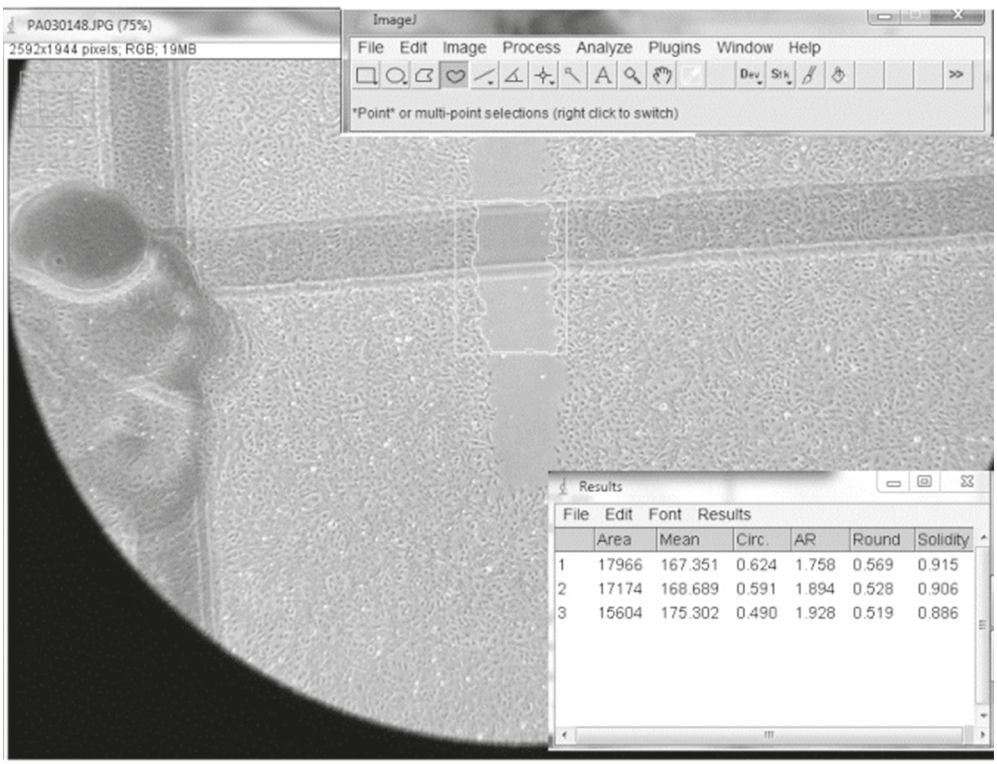

Figure 1. Representative image of area measurement in ImageJ software.

grown to confluence in 75-cm^2 flasks. After serum-starving for 12 h, flasks were used in the multiple scrape model as previously described to mimic the conditions of migration assay (5). After scratching, cells were incubated for 12 h with either LPS or the cytokine mixture. The 12-h incubation period was chosen as significant after statistical analysis of migration assays as seen in Figure 2. After incubation, the medium was removed and flasks were placed on ice. The cells were washed twice with ice-cold phosphate-buffered saline and were then lysed on ice in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate (Na_3VO_4), 50 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate, 1% Triton X-100, 10% glycerol, 1% deoxycholic acid, 0.1% SDS, 1 μg/mL aprotinin, and 1 μg/mL leupeptin. Lysates were centrifuged at 14,000 rpm for 30 min at 4 °C and aliquots of supernatants were stored at –80 °C. Protein concentration of the supernatant was measured with a spectrophotometer using the Lowry method (6).

Equal amounts of protein (40 μg) were separated by SDS-PAGE using 8% separating gel followed by transfer to nitrocellulose membrane (Hybond-ECL, Amersham, GE Healthcare, Piscataway, NJ, USA). After transfer, membranes were blocked in 5% nonfat dried milk for 1 h before incubation with primary antibody (iNOS, 1:1000; COX-2, 1:2000; or β-actin, 1:20,000) overnight at 4 °C. The membranes were washed three times with wash buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (for iNOS and COX-2, 1:5000; for β-actin, 1:10,000) for 1 h at room temperature. After washing, immunoreactive bands were visualized using an a chemiluminescence detection kit (ECL Plus; Amersham, GE Healthcare) and exposed to X-ray film. The autoradiographs were scanned and the bands were quantified using ImageJ software. Data are given as the ratio of specific protein (iNOS or COX-2) versus β-actin band density. Rabbit polyclonal iNOS antibody, monoclonal anti-β-actin, and secondary antibodies (antimouse IgG for β-actin and antirabbit IgG in goat for the others) were from Sigma Chemicals (St. Louis, MO, USA). Rabbit polyclonal COX-2 antibody was from Abcam (Cambridge, UK).

2.5. Statistical analysis

Results are expressed as mean ± SEM from at least 3 separate experiments. The Shapiro–Wilk test was used to assess data normality. The cell migration assay results and protein expression results were analyzed using one-way ANOVA with post hoc analysis by Dunnett test. $P < 0.05$ was considered significant.

3. Results

3.1. Migration assay

There was a significant delay in migration both with the cytokine mixture and LPS application. Cells covered 88% of the initial wound at 24 h in the control group, 54% in the LPS group, and 35% in the cytokine mixture group. Both LPS and the cytokine mixture impaired migration. Repeated images of the wounded areas were taken at 0, 2, 4, 6, 8, 10, 12, and 24 h and then analyzed. Significant differences from the control group were observed beginning at the 12th hour for LPS and at the 6th hour for cytokine application ($P < 0.05$, Figure 2). The total percentages of the wound covered by the cells in LPS groups were always ahead of those of the Cytomix groups in all experiments. Even though statistically nonsignificant, the cytokine mixture seemed to impair migration more efficiently, both regarding wound closure changes and overall results.

3.2. Western blot analysis

As a statistically significant difference was demonstrated beginning at the 12th hour for both groups during the scratch wound healing assays, samples for western analysis were collected after a 12-h application of LPS or the cytokine mixture. There was a significant increase in the expression of iNOS both with LPS and Cytomix (Figure 3). The induction of COX-2 showed a similar pattern with more striking band intensities (Figure 3). As expected from the migration experiments, both LPS and the cytokine mixture were found to independently increase

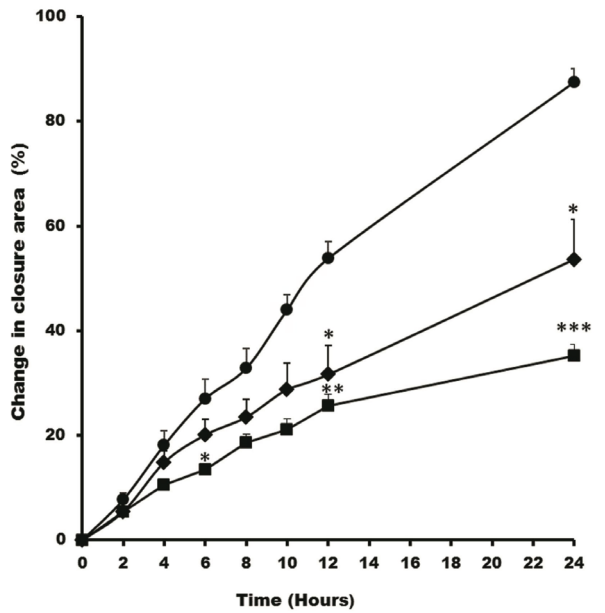

Figure 2. Time-response curves of wound closure (%) in control (●), lipopolysaccharide (LPS, ◆), and cytokine mixture (Cytomix, ■) groups. Data are expressed as mean ± SEM; n = 5 in each group.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control.

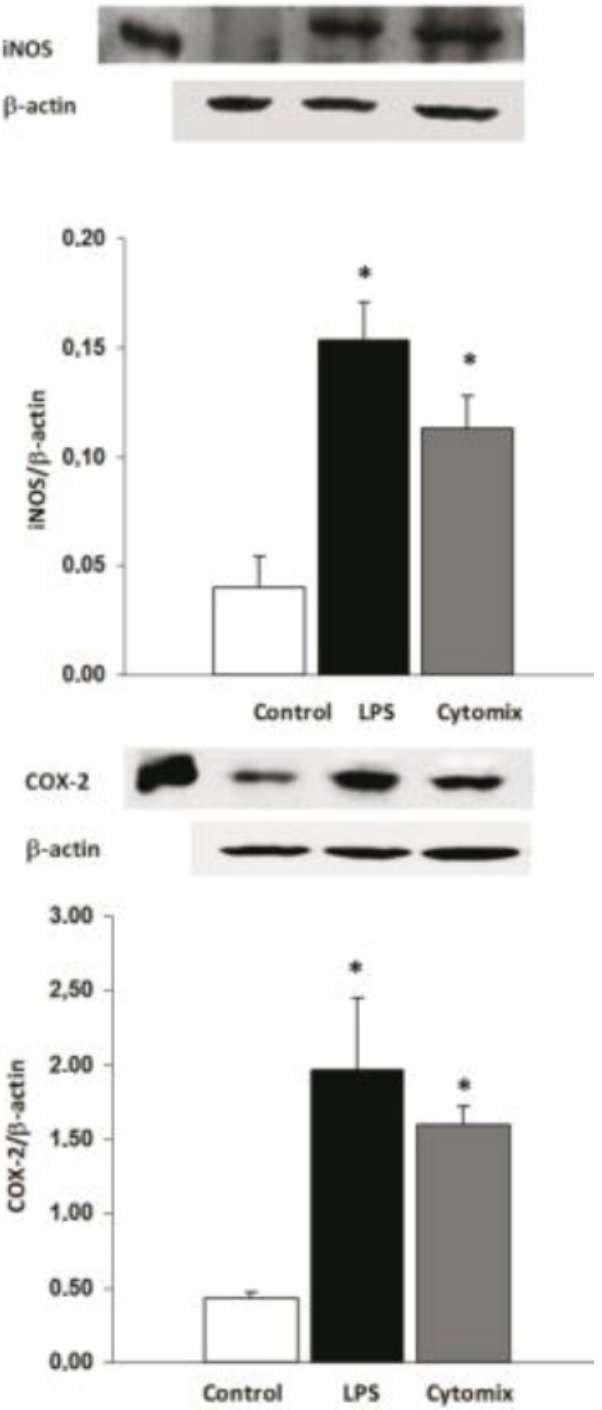

Figure 3. Representative blots and densitometric analysis for (A) inducible nitric oxide synthase (iNOS) and (B) cyclooxygenase-2 (COX-2) proteins. The graphs represent the mean ± SEM of 3 independent experiments. The left first lane in bands represents positive control for iNOS and COX-2. * $P < 0.05$ compared to the control.

iNOS and COX-2 expressions. On the other hand, despite the apparent discrepancy in migration experiments, there was no significant difference in iNOS and COX-2 levels between stimulations with LPS or Cytomix.

4. Discussion

Despite the lack of exact data about etiopathogenesis, all bowel diseases characterized by inflammation share some fundamental properties. Abnormal inflammatory response following increased transposition of toxic and immunogenic factors due to damage to the mucosal barrier and impaired restitution constitute some part of the etiopathogenesis in all (1).

Bacterial translocation and a basal level of inflammation are normal; however, factors like the presence of lipopolysaccharide induce gut barrier failure and excessive inflammatory response. This also leads to apoptosis and impairment in restitution, which in turn worsens mucosal barrier failure (7). Besides this common mechanism, some specific pathways are identified for some of these diseases. For example, necrotizing enterocolitis is known to be strongly associated with high levels of nitric oxide (8) and TNF-α has been shown to be relevant to celiac disease (7). There is even evidence suggesting that the TNF gene may also be implicated in the susceptibility to the disease (7).

Our knowledge continues to widen rapidly, revealing various mechanisms. To gain clinical benefits from this valuable information, we need studies that can carry the data bench to the bedside. Our focus was on necrotizing enterocolitis and we sought a method that was easy to use and reproducible to widen the research about bowel diseases characterized by inflammation, especially for the purpose of drug research and development.

Animal studies and clinical investigations are hard to perform and expensive and they entail significant ethical problems. They also suffer from representative quality of the study sample. Besides, at the beginning of the drug research and development process, we need to attain consistency and reproducible results. Understanding the pathology at the level of cells also contributes to finding new targets for new drugs. Cell culture studies are useful for both providing consistent, reproducible data at the cell level and being free of ethical issues. They are also appropriate to focus on a specific pathway while excluding possible unwanted factors. We therefore chose a cell culture model for our purpose for being goal-oriented and functional.

The first step of the study was to choose an appropriate cell line. IEC-6 is a normal intestinal cell line belonging to *Rattus norvegicus*. It was chosen for its popularity among similar studies due to its functional similarities with human intestinal cells (9). This cell line is easy to handle and functionally similar to human intestinal cells.

Its doubling-time (about 50 h as stated in the datasheet by the DSMZ) is appropriate for our experiments for allowing us to comment that the closure of the wound was due to migration, not proliferation (8).

There are various models to evaluate migration including in vivo and in vitro techniques. In vitro techniques include the scratch wound model, individual cell tracking, phase-contrast microscopy, screening cell motility apparatus, live cell confocal microscopy, and transwell cell invasion model. We chose the scratch wound model for being easy, requiring the least technical support, and providing similar data as some more sophisticated models (2,10).

In our model, we basically induce inflammation and create wounds in the epithelium. We induce inflammation through two different phases: first with LPS, mimicking the presence of gram-negative bacteria, and then with a powerful cytokine mixture, mimicking triggered inflammatory responses, which have been shown to be efficient in these types of experiments (4,11,12). Monitoring the migration by timely evaluation of the wound, we collect information on restitution, and by evaluating some inflammatory proteins (iNOS, COX-2), we get informative and quantitative data of our functional results regarding inflammation.

Each component of our study focuses on a cornerstone phase of the etiopathogenesis of these diseases. LPS or endotoxin is a component of the cell wall of gram-negative bacteria. It is one of the most abundant proinflammatory stimuli in the gastrointestinal tract. It induces both local and systemic inflammatory responses and impairs restitution by increasing focal adhesion (13). Cytokines are used individually or as combinations in experimental inflammatory models. The cytokines in our mixture have pivotal roles in the inflammatory cascade and iNOS and COX-2 are key enzymes in the regulation of inflammation (4,11,12). iNOS, the inducible form of nitric oxide synthase (NOS), is known to be upregulated after insults such as endotoxemia and cause cellular injury and gut barrier failure (14,15). Cyclooxygenases are enzymes producing prostaglandins from arachidonic acid. Like iNOS, COX-2 is the inducible form. It is upregulated rapidly and transiently by proinflammatory mediators and mitogenic stimuli including cytokines, endotoxins, growth factors, oncogenes, and phorbol ester. Its upregulation induces inflammation, apoptosis, and angiogenesis (15).

Timely evaluation of the wound demonstrated impairment in migration both with LPS and the cytokine mixture. iNOS and COX-2 levels increased with both LPS and the cytokine mixture in a similar fashion. It is known that LPS promotes gut barrier injury in an autocrine fashion, which explains these outcomes in a single cell culture model (16). Autocrine and paracrine regulation of cell response provides inflammatory response and impairment in migration in the presence of LPS. Nonetheless, cytokines seem to impair more, which probably mimics the presence of inflammatory cells like T helper cells. These results demonstrate that enterocytes are capable of promoting inflammation that can impair migration, which can further be boosted by cytokines.

Our study mainly focused on early response of intestinal epithelial cells to the inflammatory state, namely delayed restitution, one of the common properties of bowel diseases characterized by aberrant inflammation. In order to evaluate the long-term effects of inflammatory environment on intestinal epithelial cells, different assays like proliferation assays and exposure times for LPS or cytokines need to be investigated.

Our study describes a model that integrates some well-known, easy, and practical methods to constitute a basic intestinal epithelial cell culture model for bowel diseases characterized by intestinal inflammation. Each part of our experimental model has been previously described as remarked with citations; however, none of these studies provided complete detailed information for researchers to constitute the model. We had to dedicate time and a significant portion of our grant for the preliminary studies. Therefore, we wanted to share our experience with researchers, especially those in developing countries who have limited grants and time for scientific studies. This simplified and practically applicable model starts from a point of functional results and combines this with changes in protein levels of intestinal epithelial cells as their responses in inflammatory environment. The study involves some important details of establishing a migration assay in an inflammatory state, like timing for quantification of some inflammatory proteins and easy calculation of wound closure that was not mentioned clearly or was scattered in previous papers, and it constitutes a basic inflammatory model with functional assay and quantitative changes of some inflammatory proteins. It can be used for studies about the etiopathogenesis of the diseases or drug research.

In conclusion, we believe that our paper can serve as a quick guide for researchers, especially those in developing countries with limited grants and time for scientific studies. Many drug candidates in the pipeline for bowel diseases characterized by aberrant inflammation can easily be evaluated using our model at some points of pharmacological screening. The area of interest can also be easily widened by the addition of more protein analysis and further studies including different techniques like immunohistochemistry.

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