TECHNIQUES AND PROCEDURES

Expansion of Primary Bronchial Epithelial Cell Cultures

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OBJECTIVE: Cell cultures provide a good model for studying lung diseases but they are difficult to reproduce and the number of cells obtained is limited. The aim of this study was to develop a way to increase the production of human bronchial epithelial cells (BEC) in primary cultures.

MATERIAL AND METHODS: A total of 12 samples (9 from surgical specimens and 3 from endoscopic biopsies) were processed on plates coated with type I collagen with growth medium supplemented for BEC. When cell proliferation started, the explants were removed for successive subculturing. The remaining cells were left to proliferate and were trypsinized after 50% confluence. We recorded the number of cells obtained, cell viability, and the percentage positive for cytokeratin 7.

RESULTS: The total number of cells obtained by this method was 3-fold the number of human BEC obtained with simple primary cultures. The maximum number of subcultures was 5, mean (SD) cell viability was 91.9% (11.7%), and the percentage of cells positive for cytokeratin 7 was 30.71% (10.68%).

CONCLUSIONS: The described method for expanding primary BEC cultures increases cell production.

Key words: *Primary cultures. Bronchial epithelial cells. Culture expansion. Explants. Cytokeratin 7.*

Método para amplificar cultivos primarios de células epiteliales bronquiales

OBJETIVO: Los cultivos celulares son un buen modelo para el estudio de las enfermedades pulmonares, pero son difíciles de reproducir y producen un número limitado de células. El objetivo de este estudio ha sido desarrollar un método que incrementase la producción de células epiteliales bronquiales (CEB) humanas en cultivos primarios.

MATERIAL Y MÉTODOS: Se procesó un total de 12 muestras (9 procedentes de muestras quirúrgicas y 3 de biopsias endoscópicas) en placas recubiertas de colágeno tipo I con medio suplementado para CEB. Al iniciarse la proliferación celular a su alrededor, los explantes se extrajeron y subcultivaron sucesivamente. Las células restantes se dejaron proliferar y se tripsinizaron tras alcanzar más del 50% de confluencia. Se valoraron el número de células obtenidas, la viabilidad y la citoqueratina 7.

RESULTADOS: El número total de células obtenidas con este método superó en una media de 3 veces el número de CEB humanas obtenidas en cultivos primarios simples. El número máximo de subcultivos fue de 5, la viabilidad media (± desviación estándar) fue de 91,9 ± 11,7% y el porcentaje de células positivas para la citoqueratina 7 del 30,71 ± 10,68%.

CONCLUSIONES: El método descrito para amplificar cultivos primarios de CEB permite incrementar la producción de células obtenidas.

Palabras clave: *Cultivos primarios. Células epiteliales bronquiales. Amplificación. Explantes. Citoqueratina 7.*

Introduction

Recent studies demonstrate that the bronchial epithelium is more than simply a structural barrier. It helps initiate and sustain the process of inflammatory infiltration and induces structural changes in the airway walls themselves.[1] Therefore, studying the epithelium furthers our understanding of how a variety of respiratory diseases are triggered or sustained. Moreover, the epithelium may have yet unknown functions.

In vitro models based on primary cultures of human bronchial epithelial cells (BEC) are difficult to develop. A primary culture is established from explants of samples taken from operated patients or autopsies or it may be started from fragments obtained from bronchoscopic biopsies,[2] although such fragments are small and, therefore, yield few BEC. In response to the difficulty of obtaining a sufficient number of cells for more complex experiments, commercial BEC lines have been developed, but such cell lines do not always provide the genotype needed for the disease under study.[3]

The aim of the present work was to develop a method for increasing the production of primary cultures of human BEC. The method sought would allow repeated performance of complex studies on explants from samples obtained from a single patient by bronchoscopy or thoracic surgery.

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Material and Methods

Sample Harvesting

Samples came from patients who underwent surgical resection or bronchoscopy under normal conditions according to standard protocols for their diseases. The patients were informed about the type of procedure to be performed and gave their signed consent to the study, which was approved by the hospital ethics committee.

Thoracic Surgery

Nine patients undergoing thoracic surgery to remove tumors were included. A small fragment of uninvolved tissue was removed from the resected material, distal to the tumor.

Bronchoscopy

Three patients who underwent bronchoscopy were included. A bronchoscopy forceps was used to biopsy the main carina of each patient under the assumption that that site would be unaffected by disease.

Cell Cultures

The culture medium and final concentrations of solutions were prepared as described by Devalia et al,[2] as follows. Tissue culture medium 199 with L-glutamine was supplemented with insulin to a concentration of 2.5 µg/mL, with cortisone to a concentration of 0.361 µg/mL of hydrocortisone, and with apo-transferrin to 2.5 µg/mL (Sigma Chemical Co, St Louis, Missouri, USA). Nu-serum IV (Becton Dickinson, Bedford, Massachusetts, USA) was used at a concentration of 2.5%. The concentration of epidermal growth factor (Becton Dickinson) was 20 ng/mL. Also used were 1% penicillin-streptomycin (Life Technologies, Gibco BRL, Grand Island, New York, USA) and amphotericin B at a concentration of 2 µg/mL (Squibb Industria Farmacéutica SA, Esplugues de Llobregat, Barcelona, Spain).

Epithelial Cell Harvesting

The greatest number of explants that could be scraped from the internal bronchial wall of each surgically removed block were taken with the help of forceps and scalpel. Endoscopic biopsies were divided into fragments or explants. All explants measured 1 by 2 mm. Explants were washed with normal saline solution (sodium chloride 0.9%) with 1% penicillin-streptomycin and placed on petri dishes previously coated with type I collagen (Sigma Chemical Co) to stimulate growth. A drop of supplemented medium 199 was placed over each explant after its attachment to the dish had been checked. Each dish was incubated at 37ºC in a 5% CO_2 atmosphere at 90% humidity. The cultures were not moved for 3 days to avoid detaching the explants. After incubation, the medium in each plate was replaced by 3 mL of new medium per dish. On alternate days cell growth was checked under a magnifying glass and the culture medium was changed.

Explant Subculturing

The explants were passaged to another plate after 10 days, when a moderate halo of cell growth had been observed around each. Each explant was removed and transferred to another collagen-coated dish, leaving the BEC that had grown.

Detachment

When the cells covered more than half the growing surface, after 3 weeks, they were enzyme disaggregated. The medium was removed from the dishes and washed with sterile physiological saline solution with 1% penicillin-streptomycin. Two milliliters of a solution of trypsin and ethylenediamine tetraacetic acid was added per dish and they were incubated at 37ºC for 5 minutes. To inhibit trypsinization, 2 mL of fetal calf serum (Biological Industries, Beit Haemek Kibbutz, Israel) was added and the mixture was centrifuged at 345 g for 5 minutes. Slides were prepared in duplicate for immunocytochemistry. The rest of the mixture was resuspended in 2 mL of sterile physiological saline solution (sodium chloride 0.9%) with 1% penicillin-streptomycin. Cells were counted and checked for viability in 10 µL of the suspension by the trypan blue (0.4%) exclusion method (Sigma Chemical Co) in a Neubauer chamber.

Immunocytochemical Staining for Cytokeratin 7

The cells were stained with primary monoclonal mouse anti-human cytokeratin 7 antibody and secondary peroxidase-labeled goat anti-mouse-immunoglobulin G antibody. The chemical reaction was started with 3,3'-diaminobenzidine substrate for peroxidase and stained with Harris's hematoxylin for contrast. The material for immunocytochemistry was supplied by DAKO Corp (Carpinteria, California, USA). The percentage of cells positive for anticytokeratin 7 was calculated. At the same time, a paraffin block containing healthy bronchial cells was prepared and slices were stained by immunohistochemistry to calculate the percentage of cytokeratin 7 positive cells. Fibroblast cultures were the negative controls.

Statistical Analysis

Analyses were carried out with the Statistical Package for the Social Sciences (SPSS) version 10.0 from 1999. Mean (SD) values for each variable were calculated.

Results

Twelve samples were harvested: 9 from thoracic surgical resections and 3 from bronchoscopic procedures. The 11 men and 1 woman had a mean age of 65 (12) years. All patients were smokers or ex-smokers. Mean forced expiratory volume in the first second was 70% (13%) of the theoretical value and 5 patients had received chemotherapy. Results are shown case by case in Table 1.

The mean number of explants obtained was 18.1 (14) from surgical resections and 6 (1.2) from bronchoscopic biopsies. The explants from cultivated samples were passaged a mean 3.4 times (range, 2–5 subcultures). Fragments from surgical resections gave better results than those from bronchoscopic procedures. Table 2 shows the mean yield from each passage.

The mean number of BEC (in millions) obtained was 2.42 (4.08)—specifically 3.18 (4.5) for samples from resective surgery and 0.15 (2.2) from bronchial biopsies. The mean expansion obtained, expressed as a percentage of the primary cultures, was 300% (143%)—specifically 343% for bronchial biopsies and 200% for surgical resections. Overall mean cell viability was 91.9%