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Protein kinase C-dependent regulation of L-arginine transport activity in Caco-2 intestinal cells

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Abstract

The regulation of plasma membrane L-arginine transport activity was investigated in differentiated and undifferentiated states of the human intestinal cell line, Caco-2. The sodium-independent, leucine-insensitive uptake of L-arginine measured in this study has been assigned by us previously to system y^+ in Caco-2 cells. Treatment of cells with serum-free media containing epidermal growth factor (EGF), transforming growth factor α (TGFα), or the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA), stimulated system y^+ arginine transport activity in Caco-2 cells. Transport upregulation by these growth factors or by TPA was blocked by cycloheximide or the PKC inhibitor chelerythrine. Arginine uptake was diminished during the course of differentiation, attributable to a reduction in the transport system y^+ capacity (V_{max}) with no change in apparent affinity (K_m). TPA stimulated arginine uptake required at least 3 h of continual exposure, and increased the membrane's transport capacity (V_{max}) in both undifferentiated and differentiated cells. TPA elevated the diminished transport V_{max} of differentiated cells TPA to the elevated V_{max} value associated with undifferentiated cells. We conclude that upregulation of arginine transport is part of a pleiotropic response to EGF/TGFα, and that this involves PKC and de novo synthesis of polypeptides associated with system y^+ transport activity.

Keywords: Arginine; Transport; System y^+; Epidermal growth factor; Transforming growth factor α; Phorbol ester; Protein kinase C

1. Introduction

Following the recent discoveries of putative regulatory subunits of nutrient transport systems, interest has surged regarding the cellular control of membrane transporters serving arginine and related metabolic intermediates [1–5]. Although it has been postulated that epithelial cells can modify their ability to transport amino acids [2,6–9], the cellular mechanisms are unknown.

The intestinal epithelium is a rapidly renewing tissue exhibiting high rates of cell proliferation (hyperplasia) and growth (hypertrophy). As proliferating epithelial cells arise from the crypt stem cells, they modify their plasma membrane capacity to transport certain nutrients required for acute cell growth [10]. Arginine and other conditionally essential amino acids are among such nutrients [11,12]. The present study was initiated because little is known regarding the signals involved in the regulation of epithelial membrane amino acid transport [6,13–17]. We previously demonstrated [8] that glutamine transport was upregulated in intestinal epithelial apical membranes isolated from rats injected with epidermal growth factor (EGF).

EGF and its analogue, transforming growth factor α (TGFα), stimulate normal and malignant epithelial cells [18–21]. These growth factors are mucosal paracrines supplied to the rapidly proliferating undifferentiated crypt cells by adjacent paneth cells [22,23]. EGF is also abundant in the intestinal lumen, supplied orally primarily by the submaxillary salivary glands, and intestinally by Brunner's glands and pancreaticobiliary secretions [19,24]. Food sources, especially milk (10–400 ng/ml EGF), supply exogenous EGF which is stable in the presence of gastric acid and digestive enzymes [18,22,25]. EGF and TGFα likely mediate effects through cellular kinases, including the protein kinase C family (PKC) and various oncogene products [19,26].

Regulation mechanisms are completely unknown concerning the transport of arginine and related metabolic intermediates associated with epithelial cell growth and proliferation. In this study we investigated the role of EGF

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and regulators of PKC in modulating arginine transport via system y^+ in Caco-2 cells. System y^+ is a major transporter selective for arginine, lysine, and ornithine uptake in most cell types, and has been kinetically characterized by us in Caco-2 cells [27,29]. Caco-2 is an established cell line derived from a human colon adenocarcinoma, and is widely used as a model to study transport phenomena [30–33]. Newly passaged undifferentiated Caco-2 cells undergo spontaneous enterocytic differentiation after the culture attains confluence [33,34]. A preliminary account of a portion of this work has been presented elsewhere [27,28].

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, sodium bicarbonate, penicillin, streptomycin, non-essential amino acids, trypsin, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Hepes, (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), tris(hydroxymethyl)aminomethane were of the highest grade from Sigma, St. Louis, MO. The 0.2 μM filters used to sterilize media were from Millipore, Bedford, MA. L-[^{3}H]Arginine was obtained from Amersham, Arlington Heights, IL. Liquiscint scintillation fluid was from National Diagnostics, Atlanta, GA. The protein assay reagent was from Bio-Rad Lab, Richmond, CA. The established human intestinal epithelial cell line Caco-2 was initially obtained as passage #16 from American Type Culture Collection, Rockville, MD. The cells were cultured in 6-well tissue culture dishes (Falcon type 3046), or 100 mm tissue culture dishes (Falcon type 3003). EGF and TGFα were human recombinant peptides obtained from Promega, Madison, WI, and were prepared from concentrated stocks in 0.1 M acetic acid. Chelerythrine chloride was obtained from LC Services Corporation, Woburn, MA. All other reagents were obtained from Sigma.

2.2. Caco-2 cell cultures

Caco-2 stocks (passages #19–#40) stored in liquid nitrogen were harvested from 100 mm Falcon tissue culture dishes containing 15 ml of DMEM, 4.5 g/l glucose, 0.584 g/l glutamine, 10% fetal bovine serum, 3.7% sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids [6,34]. Cells were grown in a humidified incubator at 37°C in 10% CO_2/90% O_2. The day of seeding was designated as day 0.

Caco-2 cells were subcultured by washing with 37°C isotonic calcium-free saline solution containing 0.05% trypsin and 0.02% EDTA, and flooding the dish with 10 ml of the same solution for 5 min. The cell/trypsin mixture was dispersed with a narrow tip glass pipette, then added to DMEM containing 10% FBS. The dispersed cells were sedimented in a sterile conical centrifuge tube at $1000 \times g$ for 5 min, and the supernatant was removed. Next, growth medium was added to re-suspend the cells (using a narrow tip glass pipette) until cells were separated. Cell clumps were allowed to settle for a few min at $1 \times g$, and only the top layer of medium containing single cells was used for sub-culturing, as confirmed by phase contrast microscope examination. Cells were seeded at a cell density of $4.5 \cdot 10^6$ cells per 100 mm dish for future sub-culturing, or seeded in the 6-well cluster Falcon tissue culture dishes at a density of $3.86 \cdot 10^5$ cells per 35 mm well for transport experiments. All cell culture solutions were filter-sterilized (0.2 μm membrane filter) or/and autoclaved. The growth medium was changed daily, and cultures were inspected daily using a phase contrast microscope.

2.3. Arginine uptake measurements

Uptake of arginine was measured in cells two days post-seeding (undifferentiated) through 14 days post-seeding (differentiated). Cultures attained confluence at about day 6. Studies designed to compare transport in cells two days post-seeding and 9 days post-seeding, were conducted using cells started from the same seeding parent cells. System y^+ transporter activity was measured at room temperature (23°C ± 1.0°C). System y^+ is defined as sodium-independent, 10 mM leucine-insensitive uptake of L-[^{3}H]arginine, as described in Caco-2 by us [2,29]. Following pre-treatment of cells with various agents (described below), the medium was aspirated, and cells were rinsed three times with uptake buffer (23°C) containing 137 mM choline chloride, 10 mM Hepes/Tris buffer (pH 7.4), 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.5 mM $CaCl_2$. The uptake was initiated by adding 1 ml uptake buffer containing L-[^{3}H]arginine (2 μCi/ml, 0.5 μM to 1 mM unlabeled arginine) and 10 mM L-leucine. Culture dishes were continuously shaken by an orbital shaker (1 Hz) during the uptake period. Uptake was arrested by aspirating the uptake buffer, and washing three times with ice-cold buffer lacking substrate. Radioactivity of isotope extracted from the cells with 1 ml 1 M NaOH was neutralized with acetic acid, then assayed by liquid scintillation spectrometry. Protein in the NaOH extract was measured using the Bio-Rad protein assay. Initial rates of system y^+ transport activity were determined during the linear uptake period (2 min), with zero time points serving as blanks [29]. Uptake rates are expressed as moles arginine per minute per mg cell protein.

2.4. Treatments

To treat cells with various agents (EGF/TGFα, cycloheximide, chelerythrine, phorbol esters), growth medium

was first replaced with serum-free media (i.e., DMEM containing non-essential amino acids, penicillin and streptomycin, but lacking fetal bovine serum) for 2 h at 37°C in the humidified incubator. The cells were then exposed to each agent at various times and concentrations described below. Pretreatment buffers were removed and replenished every 6 h. Caco-2 cells remained healthy (viability > 99% by dye exclusion) during at least 24 h of exposure to serum-free media. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-dibutyrate were prepared from DMSO stocks, giving < 0.5% DMSO in final media exposed to cells. This concentration of DMSO did not influence uptake.

2.5. Statistical analysis

All experiments were conducted at least in triplicate (including the zero-time blanks), and all experiments were confirmed using at least two independently passaged generations of cells. Experimental means are reported ± S.E. Comparisons of means were made by ANOVA with pairwise multiple comparisons by the Newman-Keuls method at $P < 0.05$. Transport kinetic parameters were obtained by fitting data to the Michaelis-Menten equation by nonlinear regression analysis [6,7] using the Enzfitter computer program (Biosoft, Cambridge, UK).

3. Results

3.1. Effect of EGF and TGFα on system y^+ activity

Caco-2 cells two days post-seeding were exposed to EGF (100 ng/ml), TGFα (20 ng/ml), glucagon (2 μg/ml), or insulin (1 μg/ml) in serum-free medium for 12 h continuously. The data of Fig. 1 demonstrated that TGFα and EGF each significantly stimulated system y^+ activity compared to controls ($P < 0.05$). Uptake of arginine in cells treated with either insulin or glucagon were not significantly different from controls ($P > 0.05$; data not shown).

3.2. Effect of chelerythrine and cycloheximide on growth factor stimulation

Inclusion of 20 μM cycloheximide or 6.6 μM chelerythrine [35,36] in the serum-free treatment media containing TGFα or EGF blocked the stimulatory effect of the growth factors, as demonstrated in Fig. 1. In an independent set of experiments, we confirmed previous observations in Caco-2 cells that [^{3}H]thymidine incorporation and protein synthesis were each stimulated by EGF, and were inhibited by cycloheximide [31,32]. In cycloheximide-treated cultures, the accumulated moles of arginine, protein content and the absolute numbers of cells in the dishes were each reduced by 40% compared to control cultures.

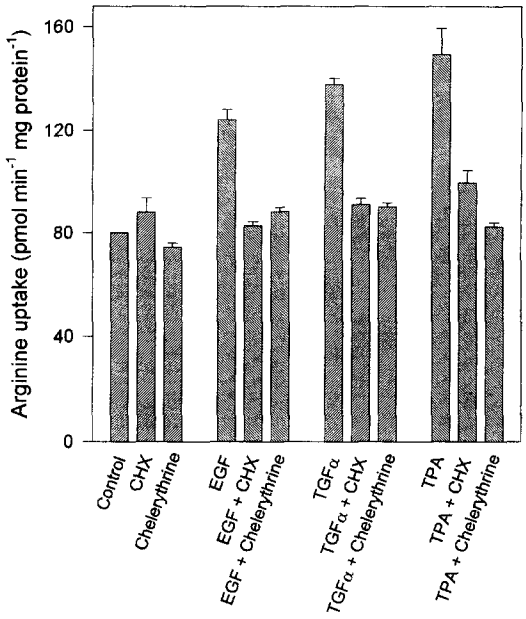

Fig. 1. System y^+ uptake of 5 μM L-arginine in Caco-2 cultures (2 days post-seeding). Prior to measuring transport activity, cells were exposed continually for 12 h to DMEM media lacking fetal bovine serum, containing 100 ng/ml EGF, 20 ng/ml TGFα, 100 nM TPA, 20 μM cycloheximide, and/or 6.6 μM chelerythrine chloride. Each point represents mean ± S.E of 3–7 determinations.

3.3. Effect of phorbol ester, cycloheximide or chelerythrine on system y^+ activity

Caco-2 cells were incubated in serum-free medium with 100 nM TPA with and without 20 μM cycloheximide, and/or 6.6 μM chelerythrine chloride during a 12 h period prior to measuring arginine uptake. The data of Fig. 1 indicated that system y^+ transport activity was stimulated by TPA, and that simultaneous incubation with chelerythrine or cycloheximide blocked the TPA stimulation. Chelerythrine by itself did not influence arginine transport.

3.4. Time-course of phorbol ester stimulation of arginine transport

Caco-2 cells were continually incubated with 100 nM TPA in serum-free medium for various times ranging from 0–24 h prior to measuring arginine uptake. The data of Fig. 2 shows that system y^+ activity was stimulated following a 3 h exposure to 10 nM TPA, and that continual exposure to TPA stimulated transport activity throughout a 24 h exposure period. The inset shows that TPA exposures up to 60 min did not influence arginine transport. Similar results were obtained using 500 nM TPA or phorbol 12,13-dibutyrate (data not shown). When cells were exposed to a 2 min pulse of TPA followed by a 12 h incubation in the same serum-free medium lacking TPA, the uptake rates subsequently measure at 12 h were not significantly different from the control rates (data not shown).

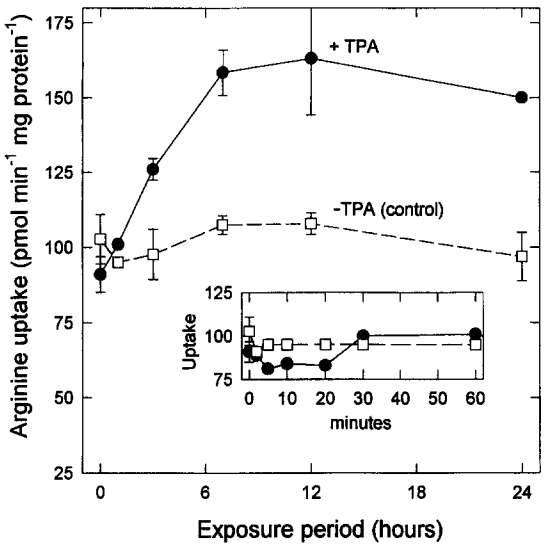

Fig. 2. Time-course of phorbol ester (TPA) stimulation of system y^+ activity. Caco-2 cells (2 days post-seeding) were continuously incubated for various times in serum-free media containing 100 nM TPA (●) or lacking TPA (□) prior to assaying 5 μM arginine uptake via system y^+. The inset shows exposure periods during the 60 min. Each point represents mean ± S.E of three determinations. TPA treated and control cells were from the same seeding generation.

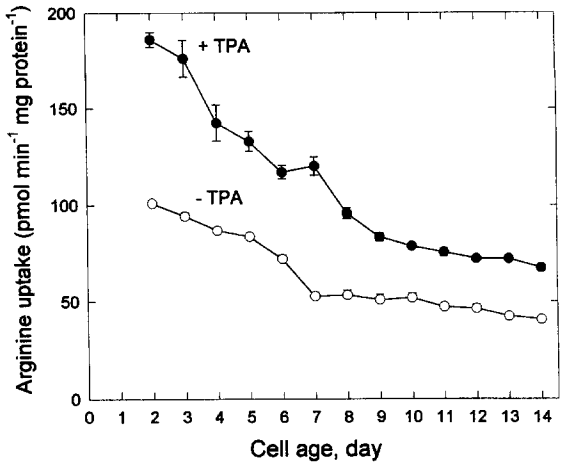

Fig. 3. Differentiation (cell age)-dependent phorbol ester stimulation of system y^+ activity. Cells were exposed to serum-free media containing 100 nM TPA (●) or lacking TPA (□) for a 24 h period prior to assaying 5 μM arginine uptake via system y^+. The confluent monolayer was attained on day 6. All cells were from the same passaged generation in this example; data were confirmed with three individually passaged cultures. Each point represents mean ± S.E of three determinations.

3.5. *Differentiation (cell age)-dependent phorbol ester stimulation of system y^+ activity*

We have shown elsewhere [27,29] that Caco-2 system y^+ activity decreases with progressing cell age post-passaging, with greater rates of uptake in undifferentiated cells (2 days post-seeding) dropping off and leveling after the cells formed a confluent monolayer and become differentiated (> day 6 post-seeding). The data of Fig. 3 confirm this observation and further demonstrated that treatment with 100 nM TPA for 24 h in serum-free medium stimulated system y^+ activity regardless of cell differentiation status (cell age post-passaging).

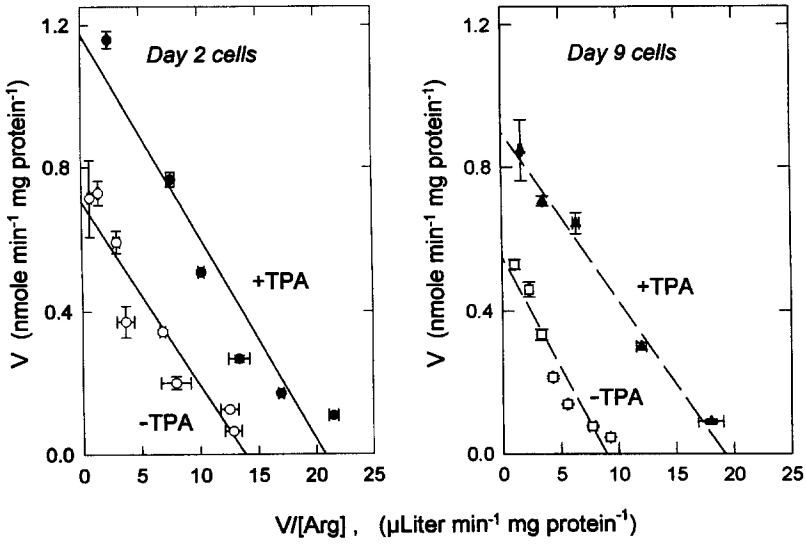

Fig. 4. Eadie-Hofstee plot (v vs. v/[Arg]) of system y^+ arginine transport kinetics measured in undifferentiated (2 days post-seeding) and confluent, differentiated (9 days post-seeding) cells. Cells were exposed to serum-free media containing or lacking TPA for 24 h prior to measuring system y^+ arginine initial uptake rates at various arginine concentrations. Data were obtained for this figure using cells from the same seeding generation, and the data were confirmed in cells from a different passage number. Points represent means ± S.E of three determinations. Kinetic constants were obtained by non-linear regression analysis, as described in the text.

3.6. The effect of phorbol ester on system y^+ transport kinetics

Caco-2 cells were pre-treated with 500 nM TPA in serum-free medium for 24 h prior to measuring system y^+ transport kinetics over the arginine concentration range of 0.5 μM–1.0 mM. As shown in the Eadie-Hofstee kinetic plot of Fig. 4, for cells grown in serum-free media, the system y^+ capacity was greater in the undifferentiated state (cells 2 days post-seeding; $V_{max} = 777 \pm 14$ pmol min^{-1} mg $protein^{-1}$, $K_m = 44 \pm \mu M$ arginine) compared to the confluent and differentiated state (9 days post-seeding; $V_{max} = 542 \pm 32$ pmol min^{-1} mg $protein^{-1}$, $K_m = 55 \pm 5$ μM). Furthermore, the data of Fig. 4 indicated that inclusion of TPA in serum-free media stimulated the system y^+ V_{max} in day 2 cells ($V_{max} = 1174 \pm 64$ pmol min^{-1} mg $protein^{-1}$, $K_m = 56 \pm 5$ μM), and in day 9 cells ($V_{max} = 877 \pm 22$ pmol min^{-1} mg $protein^{-1}$, $K_m = 43 \pm 2$ μM).

4. Discussion

EGF, TGFα, and phorbol ester can each stimulate epithelial cell growth and division [19,20]. Experiments with intact intestine and cultured epithelial cells suggest that EGF and TGFα influence the synthesis and expression of selected cell products, rather than simply non-specifically stimulating protein synthesis [32]. Our results with undifferentiated and differentiated Caco-2 cells (Figs. 1–4) indicated that upregulation of system y^+ transporter activity is part of a pleiotropic response to EGF/TGFα.

EGF and TGFα elicit their functions by binding to Caco-2 EGF receptors [13,23], instigating a cascade of intracellular phophorylation events. One of the metabolic consequences of EGF/TGFα binding to receptors is the elevation of 1,2-diacylglycerol (DAG), which can subsequently activate protein kinases such as PKC. Phorbol ester tumor promoters such as TPA can substitute for DAG, and directly activate intracellular PKC [19,26]. Fig. 1 indicates that EGF, TGFα, or phorbol ester individually stimulated Caco-2 system y^+ activity, and that each of their effects was blocked by chelerythrine. Chelerythrine specifically inhibits the catalytic domain of PKC (IC_{50} = 0.66 μM for PKC; IC_{50} = 170 μM for protein kinase A; and $IC_{50} > 100$ μM for all other protein kinases) [35,36]. In the light of the present results (Figs. 1–4), therefore, upregulation of Caco-2 system y^+ is likely mediated through protein kinase C.

The arginine transport activity stimulatory effects of EGF, TFGα, or TPA were also blocked following exposure to cycloheximide over several hours. TPA stimulation was observed only after 3 h of continual exposure (Fig. 2). Therefore, it is unlikely that growth factor/phorbol ester stimulation of transport involved rapid (min) post-translational modifications such as phosphorylation of existing transporter protein. This finding is consistent with the lack of possible phosphorylation sites on putative y^+ proteins [37]. It is likely that stimulation involved de novo protein synthesis, as discussed below.

The upregulation of system y^+ aids in delivering several nutrients that are associated with the rapid growth of undifferentiated epithelial cells. System y^+ transporter is responsible for uptake of lysine and ornithine, and the conditionally essential amino acid arginine, in either the intact intestinal epithelium or Caco-2 cells [2,3,16,29]. Our results (Figs. 3 and 4) indicated that TPA stimulated uptake by increasing the membrane's capacity (V_{max}) to transport arginine in both differentiated and undifferentiated cells. In Fig. 4, it is notable that in the day 9 cells, the depressed arginine transport V_{max} normally associated with cells in the differentiated state [29] was restored by TPA to the elevated V_{max} normally associated with undifferentiated cells. In a manner opposite to arginine transport in the present study, glucose or dipeptide secondary active transporter capacities are suppressed in undifferentiated Caco-2 cells, and are increased when the cells differentiate [30,31]. Therefore, the differentiation-associated change in arginine transport (Figs. 3 and 4) is not simply a non-specific membrane phenomenon.

The plasma membrane 'transport system' is defined as a functional, physiological entity composed of a transporter (carrier) polypeptide alone or in combination with regulatory proteins [4]. The observed upregulation of arginine uptake (Figs. 1–4) likely involved de novo synthesis of polypeptides associated with transport system y^+ activity, because stimulation was cycloheximide-sensitive, was time-dependent over the course of hours following an initial lag period, and involved an increase in membrane uptake capacity (V_{max}). Possible candidate polypeptides include y^+ carriers, putative regulatory subunits, and/or unknown transcription factors.

The present study was focused on the regulation of arginine transport activity via system y^+, which is operationally defined as sodium-independent, leucine-insensitive, NEM-sensitive uptake of L-arginine [1,2]. Recent evidence suggests that cationic amino acid functional transporters are comprised of at least a carrier polypeptide responsible for y^+ activity (or variants such as $b^{0,+}$, $B^{0,+}$, y^+L, b_1^+) and perhaps putative regulatory subunit polypeptides [1–5]. In our attempts to identify the carrier species responsible for Caco-2 system y^+ activity, we have demonstrated the presence of CAT-1 7.9 kb mRNA in Caco-2 cells, and we did not detect transcripts of the related CAT isoforms [1] using reverse transcriptase in conjunction with the polymerase chain reaction [29]. We found [29] that steady-state levels of CAT-1 mRNA were uncorrelated with y^+ activity, remaining relatively constant over the course of differentiation, despite the differentiation-associated dramatic changes in system y^+ activity. It remains to be investigated whether peptide growth factors and protein kinase C play a role in possible transla-

tional/transcriptional control of arginine transport via control of regulatory polypeptide subunits in Caco-2 intestinal epithelial cells.

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