Ras Mutation Impairs Epithelial Barrier Function to a Wide Range of Nonelectrolytes

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Although *ras* mutations have been shown to affect epithelial architecture and polarity, their role in altering tight junctions remains unclear. Transfection of a valine-12 mutated *ras* construct into LLC-PK₁ renal epithelia produces leakiness of tight junctions to certain types of solutes. Transepithelial permeability of D-mannitol increases sixfold but transepithelial electrical resistance increases >40%. This indicates decreased paracellular permeability to NaCl but increased permeability to nonelectrolytes. Permeability increases to D-mannitol (M_r 182), polyethylene glycol (M_r 4000), and 10,000- M_r methylated dextran but not to 2,000,000- M_r methylated dextran. This implies a "ceiling" on the size of solutes that can cross a *ras*-mutated epithelial barrier and therefore that the increased permeability is not due to loss of cells or junctions. Although the abundance of claudin-2 declined to undetectable levels in the *ras*-overexpressing cells compared with vector controls, levels of occludin and claudins 1, 4, and 7 increased. The abundance of claudins-3 and -5 remained unchanged. An increase in extracellular signal-regulated kinase-2 phosphorylation suggests that the downstream effects on the tight junction may be due to changes in the mitogen-activated protein kinase signaling pathway. These selective changes in permeability may influence tumorigenesis by the types of solutes now able to cross the epithelial barrier.

INTRODUCTION

Mutations in the three closely related *ras* genes, H-*ras*, K-*ras*, and N-*ras*, are among the most common mutations found in human cancer, reaching 50% in some types of tumors, such as colorectal carcinoma (Forrester *et al.*, 1987; Andreyev *et al.*, 1997, 1998, 2001). Vogelstein and colleagues have shown that mutations in K-*ras* occur early in the development of colon carcinomas (Vogelstein *et al.*, 1988). This dominantly acting mutation in *ras* results in its constitutive activation and the subsequent triggering of its signaling pathway by influencing GTPase activity. It is clear that mutations in *ras* contribute both to the recurrence of the disease and to decreased survival (Andreyev *et al.*, 1998). It is less clear, however, what the consequences of *ras* mutations are during the early stages of tumorigenesis. In this regard, one important issue is the effect of Ras activation on epithelial barrier function, perhaps the most basic of all epithelial functions, and a function shared by all epithelial tissues. Ras can exert fundamental effects on epithelial barrier function through the extracellular signal-regulated kinase–mitogen-activated protein (ERK–MAP) kinase pathway or through direct binding to the tight junction-associated protein, AF-6, which binds to ZO-1, which in turn links the tight junction to the actin cytoskeleton. Activated Ras is known to inhibit interaction between the tight junction-associated proteins, AF-6 and

ZO-1, with resultant perturbation of intercellular junctions (Yamamoto *et al.*, 1997). Modulation of tight junctions by G proteins in general has been extensively covered in a recent review (Hopkins *et al.*, 2000).

The role of Ras in barrier function is just one element of the more general issue of epithelial barrier dysfunction and aberrant regulation of epithelial tight junctions during neoplasia. Tight junction leakiness has been observed in preneoplastic growths as well as actual adenocarcinomas (Soler *et al.*, 1999). Structural alteration of tight junctions in tumors of various tissues has likewise been reported (Martinez-Palomo, 1970; Polak-Charcon *et al.*, 1980; Robenek *et al.*, 1981; Swift *et al.*, 1983; Mullin *et al.*, 1986; Ma *et al.*, 2004). In addition, there has been extensive documentation of the tight junction leakiness induced by tumor-promoting cocarcinogenic chemicals, most notably phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (Mullin and O'Brien, 1986; Mullin *et al.*, 1990). Phorbol esters have caused increased tight junction permeability not only to salts and water (Mullin and O'Brien, 1986) but also to nonelectrolytes ranging in size from molecular weight 180–200,000,000 (Mullin *et al.*, 1997b). The increased permeability also allows growth factors such as epidermal growth factor (EGF) and insulin to traverse phorbol ester-exposed cell layers at dramatically increased rates while retaining biological activity (Mullin and McGinn, 1987; Mullin *et al.*, 1998a).

Work by Chen *et al.* (2000) in Madin-Darby canine kidney (MDCK) epithelia suggests that *ras* transformation reduces the ability of epithelial cells to form a barrier (Chen *et al.*, 2000). Negating the effect of the *ras* transformation by inhibiting mitogen-activated protein kinase kinase (MEK) 1 re-

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sulted in translocation of tight junctional proteins to the cell borders, appearance of tight junctional strands in freeze fracture electron microscopy, and dramatic increases of transepithelial electrical resistance (R_t). However, still earlier studies (Schoenenberger *et al.*, 1991), also conducted on MDCK epithelia, reported that although Ras activation resulted in multilayering of cells with partial loss of cell polarity, there was no change in nonelectrolyte flux across the epithelium, and transepithelial resistance was fourfold higher in the *ras*-transfected cell sheets.

The specific goals of the present study were to 1) verify the effect of a *ras* mutation on tight junction permeability in an epithelial model; 2) characterize the nature of the leak in terms of the types of solutes, regarding both size and charge, able to transit the cell layer at increased rates of flux; and 3) determine changes in abundance and subcellular localization of specific tight junction proteins that occur in association with the changes in permeability.

MATERIALS AND METHODS

Cell Culture

LLC-PK₁ cells, differentiated renal epithelial cells with marker properties of the renal proximal tubule, were a gift of Dr. Robert Hull (Eli Lilly & Co., Indianapolis, IN) (Hull *et al.*, 1976; Mullin *et al.*, 1980) and were used between passages 186 and 202. The procedures for culture of the LLC-PK₁ cell line have been described previously (Mullin and O'Brien, 1986; Mullin *et al.*, 1997b). LLC-PK₁ cells were grown in α -modified MEM (without nucleosides and deoxynucleosides) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and additional 2 mM L-glutamine (Mediatech). Vector control (pBabe) and *ras*-transfected cells were cultured in the above-mentioned medium supplemented with 2 μ g/ml puromycin-HCl to maintain selection pressure on the transfected populations. Routine propagation entailed seeding 1×10^5 cells in a 75-cm² culture flask for 1-wk incubation at 37°C, 5% CO₂. For transepithelial permeability studies involving measurement of electrical resistance or transepithelial flux of radioisotopes, cells were seeded onto permeable filters (1×10^6 cells/4.2-cm² Millicell PCF, Millipore, Billerica, MA). Cells seeded on Millicell PCFs were allowed to form monolayers for 4 d before use in experiments.

Ras Transfection

The expression plasmid containing an activated *H-ras* cDNA (valine-12 mutation, *RasV12*) in the pBabe retroviral vector (Morgenstern and Land, 1990) was provided by Dr. Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring, NY). This Ras cDNA construct was sequenced to verify the presence of the codon 12 mutation using primers derived from the cDNA sequence of Ras and primers derived from the pBabe plasmid sequence 5' to the ATG of the Ras cDNA. The retroviral constructs were transfected into the Phoenix packaging cell line (kindly provided by Dr. Gary Nolan, Stanford University, Stanford, CA), which was used for retrovirus production. The LLC-PK₁ cells were subsequently infected with retroviral particles when the cultures were 60–80% confluent and allowed to recover for 24 h after infection before antibiotic selection in media containing puromycin. Cells surviving in the Ras- and pBabe-infected cultures were harvested and used as a pooled culture for experiments to determine tight junction permeability and abundance of Ras, ERK, and tight junction proteins. The Ras cell line was later subjected to single-cell dilution cloning, with various clonal sublines (Ras-A, Ras-B, and so on) selected based upon the increased degree of multilayering that was evident (compared with the Ras parental cell line). The Ras-A subline, which exhibited extensive multilayering, was used in several subsequent studies.

Microscopy

Cells grown on permeable polycarbonate filters as described above were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After postfixation in 1% OsO₄ for 1 h, filters were rinsed, cut into small pieces with a razor blade, dehydrated in a graded ethanol series, and infiltrated and embedded in Spurr's epoxy resin (Electron Microscopy Sciences, Hatfield, PA). Tissue blocks were sectioned with glass knives to produce 1.5- μ m sections (for light microscopy) or with a diamond knife to produce 80-nm sections (for transmission electron microscopy) on a Leica Ultratrac E ultramicrotome (Leica Microsystems, Deerfield, IL). Toluidine blue O (1%) was used to stain sections for light microscopy; ethanolic uranyl acetate and lead citrate were used to stain sections for transmission electron microscopy. An Olympus AX70 (Olympus, Tokyo, Japan) was used for light microscopy, and a JEOL JEM 1010 (JOEL, Tokyo, Japan) was used for transmission electron microscopy. Digital cameras were used to capture images for both.

Electrophysiology and Radiotracer Flux Studies

For measurements of transepithelial voltage at 37°C, 1 M NaCl salt bridges in series with calomel electrodes were used. Voltage was read on a Fluke 8020B multimeter (Fluke, Everett, WA). Cell sheets were refed in fresh culture medium 1–3 h before measuring voltage. To measure transepithelial electrical resistance (R_t), 10 μ A/cm² current pulses (1-s duration) were passed across the cell sheet (in culture medium) using Ag/AgCl electrodes in series with 1 M NaCl salt bridges. The resulting voltage deflection was measured on a Keithley 197A autoranging multimeter (Keithley Instruments, Cleveland, OH) using calomel electrodes in series with 1 M NaCl salt bridges as described above. The resistance was determined using Ohm's law.

For measurement of transepithelial permeability by transepithelial diffusion of D-mannitol or polyethylene glycol (PEG), cell sheets were incubated at 37°C with 0.1 mM (0.1 μ Ci/ml) [¹⁴C]D-mannitol (M_r 182) (PerkinElmer Life and Analytical Sciences, Boston, MA) or [¹⁴C]polyethylene glycol (M_r 4000) (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) in their basal-lateral fluid compartment. Samples of medium from the apical fluid compartment were removed at 30-min intervals for liquid scintillation counting. Samples of basal-lateral medium taken for liquid scintillation counting provided the specific activity measurement (dpm per picomole) necessary to express flux rates as picomoles per minute per square centimeter of cell sheet. The molecular identity of the radioactivity crossing the cell sheets was analyzed by thin layer silica-gel chromatography using 4:1 isopropanol/water and 120:30:50 n-butanol/acetic acid/water solvent systems.

For transepithelial diffusion studies of 10,000 and 2,000,000- M_r ¹⁴C-methylated dextrans (Sigma-Aldrich, St. Louis, MO), a similar procedure to that described above was followed. The concentration of dextran in the culture medium was 0.1 mM (0.2 μ Ci/ml) for the 10,000- M_r species and 3.3 μ M (0.4 μ Ci/ml) for the 2,000,000- M_r species. Because the purchased radiolabeled dextran as supplied was impure with regard to molecular weight, the radioactivity which transited the cell sheet was analyzed by gel filtration chromatography using Sephadex G25 in a 60-cm (length) by 1-cm (diameter) column for the 10,000- M_r dextran, and a Sephacryl 300 column (50 cm [length] by 1.5 cm [diameter]) for the 2,000,000- M_r dextran. This was done for each cell sheet studied. For the G25 column, gravity feed was used. For the Sephacryl 300 column, a pressure head of 4 psi was used to achieve a flow rate of 1 ml/min. That portion of the amount of radioactivity that transited the cell sheet and that was found to correspond to the molecular weight range in question (M_r 10,000 or 2,000,000) was then used to determine the flux rate. The flux rate of, e.g., the 10,000- M_r dextran, is therefore reflective of only 10,000- M_r material.

Cell Fractionation and Western Blot Analysis

Confluent cultures in Falcon 75-cm² culture flasks were washed twice in phosphate-buffered saline (PBS) at 4°C and scraped from culture flasks into 2 ml of buffer A (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, and 2 mM EDTA) and 1:100 dilution of protease inhibitor cocktail III [Calbiochem, San Diego, CA; final concentrations 0.8 μ M aprotinin, 20 μ M leupeptin, 50 μ M bestatin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 10 μ M pepstatin] and phosphatase inhibitor cocktails I and II (Sigma-Aldrich) at 4°C. The cell mixture was sonicated while on ice and then centrifuged at 39,000 rpm for 60 min in a Beckman Ti50 rotor (Beckman Coulter, Fullerton, CA). The supernatant (cytosolic fraction) was transferred to a separate tube, aliquoted, and then frozen at –70°C until ready for analysis. An aliquot of 500 μ l of buffer A with 1% Triton X100 and protease inhibitors at 4°C was added to the pellet from the first centrifugation. This pellet was mechanically disrupted, and the sample was placed on a rocker platform at 4°C for 1 h. After centrifugation at 39,000 rpm at 4°C as described above, the supernatant (membrane-associated fraction) was removed to a separate tube, aliquoted, and then frozen at –70°C until ready for analysis. The pellet was then resuspended in 500 μ l of 4°C lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, and 1:100 dilution of protease and phosphatase inhibitor cocktails I and II as described above for buffer A) and shaken for 1 h at 4°C. After centrifuging 1 h at 4°C, 39,000 rpm in a Beckman Ti50 rotor, this supernatant was also aliquoted and stored frozen at –70°C (cytoskeletal fraction).

Protein concentrations of each fraction were determined by protein assay as per the manufacturer's instructions (Bio-Rad, Hercules, CA). For gel electrophoresis, frozen aliquots were thawed on ice then combined with an equal volume of 2 $\times$ sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, and 0.001% bromophenol blue), followed by boiling for 3 min, cooling, and addition to gel wells. An aliquot of 15 μ g of protein from each fraction was added to each lane.

PAGE was performed using 4–20% gradient Tris-glycine polyacrylamide gels in a Novex mini cell apparatus (Invitrogen, Carlsbad, CA) at 125 V. Protein transfer to 0.2- μ m polyvinylidene difluoride (PVDF) membranes (Invitrogen) was performed at 30 V using a Novex XCELL II blotting module.

After protein transfer to PVDF membranes, Ponceau-S staining was performed to verify equal protein loading. This use of Ponceau-S has been described previously (Klein *et al.*, 1995; Moore and Viselli, 2000) and actual examples occur throughout the published literature (Schivell *et al.*, 1996; Bakiri *et al.*, 2000). Western blotting was performed under standard conditions using 5% milk in PBS with 0.3% Tween 20 as a blocking agent. The transblot

Figure 1. Ras expression in LLC-PK₁ cells. LLC-PK₁ epithelial cells transfected with either the pBabe vector or that vector containing the activated ras gene were isolated by selection with puromycin and examined for Ras expression using a pan-Ras antibody. Western blot analysis (A) for Ras protein revealed that those LLC-PK₁ cell lines harboring the pBabe ras construct express much higher levels of Ras than do either the parental LLC-PK₁ or LLC-PK₁ cells with the pBabe vector. Ponceau-S staining of the immunoblot confirmed equal protein loading. To determine the uniformity of Ras expression, these same cells were grown on a coverslip and examined by immunofluorescence staining (B) using the same Ras antibody. A side-by-side comparison of DAPI-stained cell nuclei and FITC-labeled cells showed heterogeneous expression of Ras in the cells. Only one of the three island groupings of cells in this micrograph of the Ras-transfected cell line exhibits elevated Ras protein levels. Bar, 200 μ m.

B.

was then incubated with the specific primary antibody at a concentration of 0.3–1 μ g/ml for 1 h at room temperature. A horseradish peroxidase-labeled secondary antibody was then used in conjunction with Western Lightning chemiluminescence reagents (PerkinElmer Life and Analytical Sciences). The labeled immunoblot was placed against reflection autoradiography film (Eastman Kodak, Rochester, NY) and developed in a Kodak M35A X-OMAT processor.

The abundance of Ras was determined using a monoclonal antibody (mAb) that recognized both normal and mutant Ras (BD Biosciences PharMingen, San Diego, CA). All primary antibodies to occludin and various claudins were purchased from Zymed Laboratories (South San Francisco, CA). The occludin and claudin-1, -3, and -7 antibodies were rabbit polyclonals. Anti-claudin-2, -4, and -5 were mouse monoclonals. The abundance of ERK-2 and activated ERK-1/2 was detected using a mAb to diphosphorylated MAP kinase (clone MAPK-YT; Sigma Aldrich) and monoclonal ERK-2 (clone D2; Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence

Transfected LLC-PK₁ cells were grown on glass coverslips or on 0.4- μ m filters to confluence (cells were subconfluent for Ras staining) and stained for proteins of interest by immunofluorescence. Filters were cut in half and stained with or without primary antibody. Initially, the cells were fixed in cold methanol and then stored in PBS until ready for staining. Before incubating with the primary antibody, the cells were subjected to a blocking solution of 10% goat serum in PBS. The specific primary antibodies were diluted in the blocking buffer and incubated overnight at 4°C with the cells on coverslips or the filters. After washing (3 $\times$ 5 min in PBS), the cells were incubated with the appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories; West Grove, PA) for 1 h at room temperature in the dark. After incubation with the secondary antibody and washes, coverslips (or filters) were mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to counterstain DNA and visualize the nuclei of all cells. The primary antibodies used here were the same

as those described above in the Western blot section: anti-Ras (BD Biosciences PharMingen) and anti-claudins-1, -2, and -5 (Zymed Laboratories). In all experiments, secondary antibody alone served as a negative control. Immunostained cells were then visualized by fluorescence microscopy using a Zeiss microscope. Images were captured using a digital camera with the AxioVision software (Carl Zeiss Microimaging, Thornwood, NY).

RESULTS

To begin to dissect the effect of a specific *ras* mutation on tight junction permeability, we transfected the LLC-PK₁ renal epithelial cell line with a construct that contained an activated *ras* mutation (valine-12). As shown in Figure 1A, the *ras*-transfected confluent cell sheets exhibited elevated levels of Ras compared with the untransfected and vector control (pBabe) cultures, in which endogenous Ras expression could be observed. The vector control showed expression level similar to LLC-PK₁. Expression of Ras in the transfected population was heterogeneous, as shown by immunofluorescence in Figure 1B, with expression levels varying from one cell grouping to another. Expression of Ras in the Ras-A clonal subline was more homogeneous (our unpublished data).

We first examined growth and morphology properties of the *ras*-transfected subline. As shown in Figure 2, compared with vector control cells (left), confluent cultures of the Ras transfectant (right) showed smaller domes, indicative of a change in transepithelial transport, barrier function, and/or cell adhesion properties. As shown in Table 1, the Ras trans-

Figure 2. Morphology of confluent cultures of the pBabe vector control and the Ras-transfected LLC-PK₁ cells. Cells were grown to confluence and examined by phase contrast light microscopy. The three-dimensional, fluid-filled domes or hemicysts, which are characteristic of confluent LLC-PK₁ cultures, are indicated by the solid black arrows (left). The Ras subline (right) typically exhibits smaller and more numerous domes (black arrow) at confluence as well as occasional foci of piled up cells (white arrow) compared with pBabe vector control cells (left). Bars, 250 μ m.

fectant shows a statistically greater number of domes, which are 50% smaller. The relationship of domes to fluid transport, and barrier function, properties described in Table 3, has been discussed previously (Leighton et al., 1970; Lever, 1979; Tanner *et al.*, 1983).

Also evidenced in the Ras transfectant were occasional multilayered foci of cells (Figure 2, right, white arrow). In thick section microscopy, this sporadic tendency by the Ras transfectant to multilayer is even better illustrated, and contrasts with the monolayer phenotype of LLC-PK₁ and pBabe

(Figure 3). Perhaps in keeping with this tendency to multilayer, the Ras subline manifested a slightly higher confluent cell density than LLC-PK₁ or pBabe (Table 2), although growth curves have shown similar rates of cell division (our unpublished data).

In spite of the property of multilayering, the normal morphological polarity of the LLC-PK₁ cells (apical microvilli, apically situated desmosomes, and tight junctions) is still in evidence in electron micrographs of the Ras-transfected culture (Figure 4, bottom right). The only cells whose morphological polarity would be in question would be those in the sublayers of the multilayered foci (Figure 4, bottom left). Polarity and junctions are, however, clearly evident in the uppermost layer of the multilayered foci.

This morphological polarity of all three cell lines is reflected in a similar electrical polarity (Table 3), with all three cell lines manifesting a luminal negative potential difference, indicative of the apical- to basal-lateral Na⁺ transport demonstrated by LLC-PK₁ cells (Misfeldt and Sanders, 1981). After observing decreased size of domes in the Ras subline, it was unexpected to find that R_t of *ras*-transfected cell sheets was significantly (40%) higher than R_t of LLC-PK₁ and pBabe cell sheets (Figure 5, top, and Table 3). As mentioned above, transmission electron microscopy did, however, show that tight junctions existed between all cell pairs of *ras*-transfected cells, even those over multilayered sites (Figure 4). A contribution to this increased R_t by the sporadic multilayering of cells is a possibility, but it should be noted

Table 1. Altered dome formation as a result of Ras mutation

	No. of domes/field	Area (%) of largest dome/field	Area (%) of all domes/field
LLC-PK ₁	11.2 $\pm$ 2.1	4.3 $\pm$ 0.6	16.0 $\pm$ 1.9
pBabe	20.0 $\pm$ 1.4 ^a	4.7 $\pm$ 0.5 ^b	19.6 $\pm$ 1.8 ^b
Ras	34.3 $\pm$ 3.4 ^a	2.3 $\pm$ 0.3 ^c	17.2 $\pm$ 1.7 ^b

Data represent the mean $\pm$ SE for n = 6 separate 40 $\times$ fields. Areas per field represent the percentage of area occupied by the largest dome—or all domes—divided by the total area of the field.

^a $p < 0.01$ relative to LLC-PK₁ or each other.

^b Not significant vs. LLC-PK₁.

^c $p < 0.02$ vs. LLC-PK₁ or pBabe.

Figure 3. Thick section light micrograph of toluidine blue-stained confluent cultures of LLC-PK₁ and Ras-transfected cell sheets. Control cell sheets, both LLC-PK₁ (top) and pBabe vector controls (indistinguishable from LLC-PK₁), manifest single-cell layer-thick sheets of cells, whereas the Ras-transfected cell sheets (bottom) exhibit frequent multilayered foci.

that multilayering per se does not coincide with increased R_t . In fact, it has been associated with decreased R_t (Mullin *et al.*, 1992, 1996). The clonal (and more homogeneously multilayered) Ras-A subline showed an even greater R_t and lower short circuit current (I_{sc}) than that exhibited by the Ras line (Table 3).

This increased R_t of the Ras transfectant is in stark contrast to a dramatically increased transepithelial flux of D-mannitol across the cell sheets, an almost sevenfold increase (Figure 5, bottom). Mannitol is a nonelectrolyte, and R_t is a measurement (in this cell line) of paracellular permeability to Na⁺ and Cl⁻. Therefore, it is possible that the paracellular "pore" induced by the Ras overexpression is specific for uncharged solutes. This unusual permeability phenotype seems to be specific for the Ras transfectant because a parallel study with Akt (protein kinase B)-transfected LLC-PK₁ cells did not show this peculiarity (our unpublished data). Akt overexpression was observed to increase transepithelial mannitol flux but transepithelial electrical resistance was unchanged, indicating increased paracellular permeability to nonelectrolytes but unaltered permeability to small electrolytes (our unpublished data).

Table 2. Effect of *Ras* mutation on confluent cell density

	No. of cells $\times 10^3/\text{cm}^2$
LLC-PK ₁	165.3 $\pm$ 8.0
LLC-PK ₁ -pBabe	152.0 $\pm$ 6.7 ^a
LLC-PK ₁ -Ras	184.0 $\pm$ 6.7 ^b

Cells were trypsinized after reaching confluence and counted by hemacytometer at 100 $\times$ magnification. Data shown represent the mean $\pm$ SE of $n = 5$ cell counts.

^a Not significant vs. LLC-PK₁.

^b $p < 0.01$ vs. LLC-PK₁ or LLC-PK₁-pBabe (Student's *t* test, equal variances).

Our main focus in this study was to describe one particular aspect of the nature of the paracellular leak in the Ras-transfected cell sheets. Specifically, we sought to determine how large a solute could now pass across these cell sheets, and whether there was a "ceiling" to where a certain solute was too large to pass. The transepithelial flux of [¹⁴C]D-mannitol (M_r 182), [¹⁴C]polyethylene glycol (M_r 4000), ¹⁴C-methylated dextran (M_r 10,000), and ¹⁴C-methylated dextran (M_r 2,000,000), nonelectrolytes of increasing size, were then compared for each of the three cell lines. Each solute was chosen for its negligible affinity for intracellular uptake and consequent possible transcellular transit. The paracellular pathway was being evaluated in each case. The studies were performed as described in *Materials and Methods* and include the analyses of the molecular nature of the radioactivity coming across the cell sheet. These analyses are especially necessary for the dextran permeability studies because of our observation that both radiolabeled dextrans were heterogeneous as commercially supplied with regard to molecular weight. For the 10,000- M_r ¹⁴C-dextran, only 95% was found to be 10,000- M_r material, the remaining radioactivity being smaller fragment molecules. For the 2,000,000- M_r dextran, an average of only 25% was found to be in fact M_r 2,000,000. These determinations were made by passing the radiolabeled compounds through a Sephadex G25 or Sephacryl 300 column as described in *Materials and Methods*. All radioactivity coming across the cell sheets was similarly analyzed. The flux of total radioactivity was then corrected by this percentage, to reflect the flux of material of the actual molecular weight in question. The results of the gel filtration analyses were confirmed by centrifugation of the radiolabeled dextran material through Centricon filter units with molecular weight cutoffs of 5000 and 100,000.

Although the radiolabeled D-mannitol and polyethylene glycol were received as pure compounds of a specific molecular weight (M_r 182 and 4000, respectively), we likewise checked the chemical nature of the radioactivity crossing the cell sheets for [¹⁴C]mannitol and [¹⁴C]polyethylene glycol fluxes by TLC. In the [¹⁴C]mannitol flux studies, all radio-

Figure 4. Transmission electron micrograph cross-sections of LLC-PK₁ and Ras-transfected cells from postconfluent cultures seeded on permeable filters. Cells were seeded onto Millicell PCF filters and after 4 d fixed and processed as described in *Materials and Methods*. A pair of LLC-PK₁ cells on a permeable filter (top left) exhibiting evidence of morphological polarity such as microvilli on their apical membrane facing the overlaying culture medium (10,000 $\times$). At higher magnification (36,000 $\times$) one can see the apically situated tight junction (arrow) sitting atop a desmosomal junction (top right). The ras-transfected subline (bottom) typically exhibits piled up foci. The uppermost layer of these foci has cells showing typical morphological polarity, a feature not seen in the under layers. The uppermost cell layers (bottom right) also manifest apically situated tight junctions (arrow) in this higher magnification view of the framed area of the lower left micrograph.

activity crossing all cell sheets was, in fact, mannitol. In the [¹⁴C]polyethylene glycol flux studies, only 50% of the radioactivity crossing pBabe cell sheets was actually polyethylene glycol, whereas for the (leakier) Ras cell sheets, 97% of the radioactivity coming across was true polyethylene glycol.

Table 4 shows transepithelial flux results from the untransfected and pBabe and Ras-transfected cell lines comparing 10,000- and 2,000,000- M_r dextrans with mannitol (M_r 182) and PEG (M_r 4000). Compared with the untransfected LLC-PK1 cell sheets, a near sevenfold increase was observed

in the transepithelial flux of D-mannitol across the Ras-transfected cell sheets, with no significant difference between the flux across LLC-PK₁ versus pBabe. The flux for [¹⁴C]mannitol across Ras and Ras-A cell sheets was not significantly different (our unpublished data). The flux of [¹⁴C]polyethylene glycol was also increased by sevenfold across Ras cell sheets compared with the pBabe cell sheets. The 10,000- M_r methylated dextran showed the greatest relative increase of all, with the flux across *ras*-transfected cell sheets being more than ninefold higher than across parental and vector control cell sheets. This indicates that solutes at least as large as M_r 10,000 are able to pass through the tight junctions of *ras*-transfected cells, depending upon the exact conformation and net charge of the solutes.

It needs to be emphasized that "paracellular" transit can be through tight junction strands and lateral intercellular space or through gaping 500- μ m holes in the epithelium. Kinetically these routes would seem very similar except for their size exclusion limit.

With the observation that solutes as large as M_r 10,000 can diffuse paracellularly across the *ras*-transfected cell sheets, the possibility of actual "holes" in the cell sheet needed to be considered. By "holes" we refer to the space that would be created if one or more cells detached from the cell sheet and left actual gaps in the barrier, which would be on the order of many micrometers in width. We saw no indication of this in the microscopy that we performed, but the observation of decreased cell substratum and cell-cell adhesion in the *ras*-transfected cells made this a distinct possibility. Simply not finding such a hole (a negative result) in a limited number of visually observed microscope sections would not constitute

Table 3. Effect of *Ras* transfection on transepithelial electrical parameters

	Potential difference	Resistance	Short circuit current
pBabe	1.2 $\pm$ 0.1	161 $\pm$ 6	7.3 $\pm$ 0.3
Ras	0.5 $\pm$ 0.1 ^a	229 $\pm$ 19 ^c	2.1 $\pm$ 0.1 ^a
Ras-A	0.3 $\pm$ 0.1 ^{a,b}	342 $\pm$ 32 ^{c,d}	1.0 $\pm$ 0.1 ^{a,e}

Data represent the mean $\pm$ SE of $n = 7$ cell sheets. Potential difference is given in millivolts, resistance is given in ohms $\times$ square centimeter, and short circuit current is given in microamps per square centimeter. All potential differences were lumen negative.

^a $p < 0.001$ relative to pBabe.

^b $p < 0.02$ relative to Ras.

^c $p < 0.01$ relative to pBabe.

^d $p < 0.01$ relative to Ras.

^e $p < 0.002$ relative to Ras.

Figure 5. Ras-transfected LLC-PK₁ cell sheets exhibit greater transepithelial [¹⁴C]mannitol flux but also greater transepithelial electrical resistance than either control cell sheet. Transepithelial electrical measurements and radiotracer flux studies were performed as described in *Materials and Methods*. Transepithelial mannitol flux was increased almost sevenfold relative to the controls (bottom). However, electrical resistance was also increased by ~40% (top). Data shown represent the mean $\pm$ SE of 13 cell sheets. The two controls were not significantly different for either parameter. Ras had a significantly greater transepithelial mannitol flux ($p < 0.001$), and a significantly greater resistance ($p < 0.03$) than either control.

persuasive evidence. We therefore evaluated the transepithelial flux of a 2,000,000- M_r [¹⁴C]-methylated dextran. As shown in Table 4, there was no relative increase in the amount of 2,000,000- M_r radiolabeled material crossing the *ras*-transfected cell sheet compared with the parental and vector controls. In fact passage of the 2,000,000- M_r material was almost negligible across all three cell sheets. The very low amount of material that did cross all three cell sheets (in roughly equal amounts) could be the result of limited damage of cell sheets in handling and/or a small but finite amount of nonspecific transcytosis. In any event, the *ras*-transfected cell sheets were no more permeable to material than the two controls. In separate experiments we likewise observed that there was the same order of negligible 2,000,000- M_r [¹⁴C]-dextran flux across the clonal Ras-A cell sheets. However, as a positive control, we observed that exposure of pBabe cell sheets to the phorbol ester TPA for 3 h at 37°C, resulted in a huge 1000-fold increase in actual 2,000,000- M_r [¹⁴C]-dextran material coming across the cell sheets (our unpublished data).

With the prospect that *ras* transfection was in fact generating a molecular "pore" to allow the passage of small- and moderate-sized nonelectrolytes through the tight junctional barrier, we examined Ras-related changes in the abundance of several tight junctional proteins. We focused on transmembrane barrier proteins (occludin and various claudins) rather than intracellular tight junctional associated proteins (e.g., ZO-1 and cingulin). Cells were fractionated into membrane-bound (triton soluble) and cytoskeletal (triton-insoluble) pools because certain claudins were barely detectable in whole cell lysates (our unpublished data). Figure 6 shows Western blot analyses comparing occludin and six claudins in cytosolic (S) as well as membrane-bound (M) and cytoskeletal (C) fractions. Tight junctional protein abundance patterns fell into four categories. Occludin, claudin-1, and claudin-4 showed increased levels in the membrane-associated (Triton-soluble) cell fraction in the Ras transfectant in two separate cell passages of each cell line. For this group, only occludin was detectable in the cytosolic fraction, with claudins-1 and -4 being undetectable. The second category includes only claudin-2, whose abundance was greatly decreased in the M and C fractions in the Ras transfectant relative to the parental (L) and vector (P) controls. The third category includes claudins-3 and -5, whose abundance did not noticeably change in the Ras transfectant. In the fourth category, claudin-7 was unique in several respects. It was the only claudin that was readily detectable in the S fraction and may have manifested phosphoprotein bands, which

Table 4. Comparisons of the effect of *Ras* mutation on the transepithelial flux of solutes of increasing molecular size

	J_{mannitol}	J_{PEG}	J_{10k}	J_{2M}
LLC-PK ₁	80.6 $\pm$ 5.6 (1.0) ^a	0.25 $\pm$ 0.04 (1.0) ^a	0.17 $\pm$ 0.02 (1.0) ^a	4.11 $\pm$ 2.53 (1.0) ^a
pBabe	72.2 ^b $\pm$ 3.5 (0.90) ^a	0.20 ^b $\pm$ 0.02 (0.80) ^a	0.16 ^b $\pm$ 0.03 (0.94) ^a	3.99 ^b $\pm$ 1.44 (0.97) ^a
Ras	551.5 ^c $\pm$ 104.8 (6.88) ^a	1.47 ^c $\pm$ 0.21 (5.88) ^a	1.56 ^d $\pm$ 0.38 (9.17) ^a	3.74 ^b $\pm$ 1.23 (0.91) ^a

Transepithelial flux (J) determinations of [¹⁴C]D-mannitol (M_r 182), [¹⁴C]polyethylene glycol (M_r 4000), [¹⁴C]methyl dextran (M_r 10,000), and [¹⁴C]methyl dextran (M_r 2,000,000) were performed as described in *Materials and Methods*. Data shown represent the mean $\pm$ SE for six cell sheets unless noted. All data are in picomoles per minute per square centimeter except for the 2,000,000- M_r dextran, which is in picomoles per minute per square centimeter $\times 10^{-4}$.

^a Relative fold-increase/decrease compared with LLC-PK₁ flux rate.

^b Not Significant vs. LLC-PK₁.

^c $p < 0.001$ vs. LLC-PK₁ or pBabe.

^d $p < 0.005$ vs. LLC-PK₁ or pBabe.

Figure 6. Western blot analysis of integral tight junction proteins in three distinct subcellular fractions of the LLC-PK₁ (L), pBabe-LLC-PK₁ (P), and Ras-LLC-PK₁ (R) cell lines. Cytosolic (S), Triton-soluble (M), and Triton-insoluble (C) fractions were isolated as described in *Materials and Methods* and analyzed by Western immunoblot analysis for abundance of tight junction proteins as indicated. These immunoblots are representative of two separate analyses, on two distinct passage levels of cells with 15 μ g of protein added per lane. Ponceau-S staining of immunoblots verified equivalent protein loading in various lanes. The occludin bands migrated between 65 and 70 kDa. All claudin bands migrated between 20 and 30 kDa.

were distinctly different in the M and C fractions. The abundance of the lower band was significantly greater than the upper band in the M fractions, but much less of the protein was detected in the C fractions. However in both cases, the Ras transfectant (R) showed more claudin-7 protein than the LLC-PK₁ (L) or pBabe (P) controls.

Concerning these relative changes in expression of the various claudins as a result of Ras mutation, one may need to consider that the Ras subline contains a mixed population of cells, one of which may not possess actual tight junctions. As a result of the Ras mutation, multilayering of cells in distinct foci became evident across the Ras-transfected cell sheet (Figures 2–4). As was true for multilayered foci in LLC-PK₁ cell sheets exposed chronically to phorbol esters, the cells of the underlayers do not uniformly manifest morphological polarity nor does one observe tight junctional electron density in these cells. It is therefore possible that the decrease in claudin-2 seen in the Western immunoblot of Figure 6 is in part due to this mixed cell population contributing to the total protein loaded onto the gel. However, it should be mentioned that one does not see a decrease in other claudins in the Ras subline and in fact occludin and claudins-1 and -4 show increased levels in the Ras-transfected mixed population.

To verify the uniformity of the changes in claudin expression as a result of Ras overexpression in the LLC-PK₁ cells,

we examined the cells by immunofluorescence for claudin-1, -2, and -5. Transfected cells were grown to confluence on membrane filters to establish tight junctions without dome formation and then stained and visualized by immunofluorescence microscopy (Figure 7). A comparison between the vector and ras-transfected cells shows that claudin-1 clearly increases and claudin-2 declines as predicted by the Western blot data (Figure 6). We also show claudin-5, which does not change appreciably in its expression pattern, regardless of the status of Ras abundance in the epithelial cells.

Because Ras overexpression seems to alter the expression of specific tight junction claudin molecules, which in turn has a drastic effect on the permeability through those tight junctions, we asked whether this might be mediated through changes in the ERK–MAP kinase pathway, a downstream mediator of activated Ras. Cultures of the transfected cells were grown to confluence, growth arrested by serum deprivation, and stimulated for 1 h with 10% serum. Western blot analysis of whole cell lysates for ERK-2 and phospho-ERK-1/2 is shown in Figure 8. Our results show a dramatic increase in phospho-ERK-2 and a moderate increase in ERK-2 protein in the Ras-transfectant compared with the vector and parental controls. These results suggest that the striking physiological changes that we observed in the junctional permeability and select tight junction proteins as a result of Ras overexpression in the epithelial cells, may be initially triggered by changes in the MAP kinase pathway. Our future efforts will focus on this pathway as a potential mechanism for our observations.

DISCUSSION

Schoenenberger *et al.* (1991) and Chen *et al.* (2000) disagreed on the existence of a tight junction leak as a result of Ras transformation. The latter study reported leakiness, whereas the former did not, even though both reported evidence of cellular morphology and polarity changes as a result of the transformation. The finding that the downstream effector Raf-1 can also induce tight junction leakiness seems to support the contention by Chen *et al.* (2000) that an activated Ras would in fact result in paracellular leak (Chen *et al.*, 2000; Li and Mersny, 2000). However, although Chen *et al.* (2000) and Li and Mersny (2000) reported Ras- and Raf-1-associated tight junction leak, it was not clear that the leak was on a molecular level, rather than a random loss of cells resulting in actual holes in the cell sheet. Either diffusion through tight junctions or leak across multicellular holes in a cell sheet will both permit transepithelial flux of mannitol, PEG, or inulin at increased rates with similar first order kinetics. Because both studies reported effects of Ras on cell-cell adhesion, such a “macroscopic” leak would be possible. It is an important distinction because it determines what aspects of barrier function would be retained by a *ras*-transformed cell layer, and the types of solutes able to cross the barrier at accelerated rates. It must be emphasized that demonstration of “leaky” tight junctions in neoplastic or preneoplastic models is only a preliminary finding to the more biomedically significant question: to what types of solutes have those tight junctions now become leaky? Identification of the nature of the solutes that are able to cross an epithelial monolayer that harbors Ras mutations is critical to the understanding of neoplastic progression in tissues that acquire Ras mutations.

Our current study produced one key finding with potentially very significant implications for novel insights into neoplastic progression: the *ras*-transfection resulted in tight junction leakiness extending to solutes at least as large as M_r

Figure 7. Claudin expression in ras-transfected LLC-PK₁ cells by immunofluorescence analysis. LLC-PK₁ cells transfected with either empty vector (pBabe) or that vector containing Ras were grown to confluence on 0.4- μ m filters and stained by immunofluorescence to detect claudin (Cld)-1, -2, or -5 using a FITC-labeled secondary antibody. Images of the parallel DAPI-stained nuclei are shown in the insets. Ras overexpression causes elevated levels of claudin-1 but depressed levels of claudin-2. Claudin-5 remains unchanged. Bar, 50 μ m.

10,000. This is surprising and suggests a fundamental restructuring of tight junction proteins. It is more surprising given the fact that this increased permeability to moderately

large (but uncharged) molecules (on the order of M_r 10,000) occurs concomitantly with an apparent *decrease* in the permeability of small charged solutes such as Na^+ and Cl^- (as

Figure 8. Increased levels of phosphorylated ERK-2 as a result of Ras transfection. Cultures of LLC-PK₁, pBabe, Ras, and Ras-A were grown to confluence in 75-cm² culture flasks and harvested for total cell lysates as described in text. Immunoblots were probed with monoclonal anti-MAP kinase, activated (clone MAPK-Y T) capable of detecting phospho-ERK 1/2, and then stripped and re-probed with monoclonal anti-ERK-2 (clone

D2). In two separate cell passages (two independently derived lysates and immunoblots), dramatic increases in phospho-ERK-2 were observed in the Ras-transfected cultures. A moderate increase in total ERK-2 protein was also observed.

evidenced by the increased transepithelial resistance). This somewhat counterintuitive situation of increased permeability to nonelectrolytes in tandem with increased transepithelial resistance has been observed previously when the tight junction protein occludin was overexpressed in MDCK renal epithelia (Balda *et al.*, 1996; McCarthy *et al.*, 1996).

This scenario is plausible if the transfection resulted in the generation of a finite and relatively small number of pores in a barrier, which would normally be impermeable to larger molecular weight solutes. The relative large increase in the flux of mannitol, polyethylene glycol and 10,000-M_r dextran is the result of essentially going from very low rates of flux to measurable rates of flux after *ras* transfection. The resistance data suggest that these newly generated pores either do not permit the passage of Na⁺ or Cl⁻ or the number of these pores is so small that they do not significantly affect the total paracellular flux of Na⁺ or Cl⁻, which have measurable permeability across all tight junctions of even control cell sheets.

The simultaneous induction in the Ras transfectant of 1) increased transepithelial resistance (decreased paracellular conductance), 2) increased paracellular permeability to a range of nonelectrolytes (e.g., mannitol), and 3) dramatic loss of claudin-2 expression is noteworthy. There is ample evidence in the literature that claudin-2 seems to confer a cation-permeable "pore" to the paracellular pathway of certain epithelia (Enck *et al.*, 2001; Amasheh *et al.*, 2002). This seems specifically true for LLC-PK₁ epithelia (Van Itallie *et al.*, 2003). It is therefore reasonable that down-regulation of claudin-2, as we observe in this study of *ras*-transfection, results in increased transepithelial electrical resistance. Elevation of transepithelial electrical resistance caused by exposure to EGF was likewise observed to coincide with decreased expression of claudin-2 (Singh and Harris, 2004).

Although *ras* transfection allowed seven-, three- and nine-fold increases in the flux of probes mannitol, polyethylene glycol, and 10,000-M_r dextran, respectively, *ras* transfection did *not* affect the transepithelial flux of a 2,000,000-M_r dextran. This finding indicates 1) the Ras activation did not result in cellular or multicellular "holes" in the epithelial layer; 2) that there is a ceiling on the size of the "pore" generated by the *ras* mutation, and consequently on the size of a molecule which can traverse it; and 3) it is unlikely that increased nonspecific transcytosis accounts for the increased observed flux rates across the *ras*-transfected cell sheets. Our current studies are focused on identifying the maximum size (and charge characteristics) of a molecule that can traverse the junctions of *ras*-transfected cell sheets. Gel filtration column chromatography profiles of ¹⁴C-dextran fragments which crossed cell sheets suggest that the "cutoff" for permeability across *ras*-transfected LLC-PK₁ cell sheets is higher than 10,000 M_r (our unpublished data).

This pattern of increased transepithelial permeability brought about by Ras activation is in partial contrast to the

pattern of increased permeability resulting from exposure of these same LLC-PK₁ cell sheets to phorbol esters (Mullin *et al.*, 1997a). Exposure to phorbol esters was different from Ras activation in two aspects: 1) mannitol and 10,000-M_r dextran flux increased in both situations, whereas transepithelial electrical resistance decreased with phorbol esters but increased with Ras; and 2) flux of a 2,000,000-M_r dextran was increased with phorbol ester exposure but not with Ras activation. Ability of the electron-dense dye ruthenium red but inability of the electron-dense dye cationic ferritin to cross these phorbol ester-treated cell sheets argued against holes in the epithelium (Mullin *et al.*, 1997a). This suggests that tight junction permeability can be "increased" to different degrees and to different solutes and is not a simple "open versus closed" entity. This reflects perhaps the large number of homotypic and heterotypic interactions that can be obtained from the (at least) six different claudins that we show to be present in these cells (Figure 6). This difference between Ras activation and phorbol ester effects on tight junction permeability also suggests that the underlying signal transduction events in play here are more involved than simply activation of the Raf-MEK-ERK pathway common to both effectors. Interestingly, the Ras-MEK1 pathway was recently shown to not affect claudin-1 expression in mammary epithelia, further suggesting that the observed effects of activated Ras on tight junction permeability are being mediated by effectors/pathways other than just MEK-ERK (Macek *et al.*, 2003). Still, the MEK-ERK pathway plays a major role because PD98059 can block phorbol ester effects on LLC-PK₁ tight junction permeability (Mullin, unpublished observations).

We observed greater apparent activation (phosphorylation) of ERK-2 compared with ERK-1 as a result of the Ras mutation in the LLC-PK₁ kidney epithelial cells (Figure 8). Most studies dealing with activation of the Raf-MEK-ERK pathway, however, show relatively equal activation of both ERK-1 and ERK-2. However, one study focusing on Raf-MEK-ERK has shown dramatically greater phosphorylation of ERK-2, specifically after retinoic acid activation of Raf-MEK-ERK signaling in myeloid cell cultures (Miranda *et al.*, 2002). Another study that actually dealt with the valine-12 Ras mutation showed that ERK-1 was preferentially phosphorylated as a result of the Ras mutation, and the phosphorylation state of ERK-2 was relatively unchanged in human kidney 293T cells (Recio *et al.*, 2000). We would hypothesize that these differences in reported results regarding ERK isoform phosphorylation arise in part from the different cell models used in each of these studies, because the regulation of these two different kinases are likely to show significant cell type specificity.

The fact that the transepithelial flux of a 10,000-M_r dextran increases almost 10-fold after *ras* transfection suggests that peptides and moderately sized proteins might be able to transit an epithelium or a focus of cells in an epithelium

Figure 9. Diagram of potential implications of chronically increased tight junctional leak to small growth factors. Top, condition of epithelial wounding where EGF from luminal fluid flows down its concentration gradient into the interstitial (stromal) fluid compartment. Entrance into this compartment gives luminal EGF access to EGF receptors on basal-lateral surfaces of epithelial cells, in turn engendering epithelial cell motility and proliferation changes that can accelerate reformation of the epithelial barrier. In addition to epithelial receptors, EGF can also access receptors on stromal fibroblasts thus eliciting increased VEGF synthesis and secretion which in turn can bind to endothelial receptors thereby promoting angiogenesis. However this second effect would be tempered by back leakage of interstitial fluid constituents such as VEGF into the luminal compartment, thereby reducing their concentration in the stromal fluid. In preneoplastic foci (bottom), EGF leaks across distorted cell junctions and can produce similar effects on epithelial cells; however, this effect does not self-limit as in wound closure. It continues indefinitely and centers its promoting action on initiated, transformed epithelia. It also would have similar action on stromal fibroblasts, namely, eliciting VEGF secretion into what will become the future tumor microenvironment. However, unlike the situation in wounding, a “roof”, namely, the epithelium (no matter how leaky), exists across and over the stroma, which may reflect back diffusing VEGF (substantially larger than EGF) and keep its concentration in the stroma higher than in the stroma below an epithelial abrasion or ulceration (reprinted with permission of Science STKE).

carrying such a mutation. EGF, whose molecular weight is 6100, would fall within this category (Figure 9). EGF exists at extremely high levels in fluids in luminal compartments of certain epithelial tissues such as the upper gastrointestinal tract and the lower urinary tract, even though its receptors are absent from or at least at sharply reduced density in the apical membranes of epithelia (Scheving *et al.*, 1989; Bishop and Wen, 1994). The high luminal EGF concentration makes for a steep concentration gradient from the lumen into the stroma (Gregory and Wilshire, 1975; Schaudies *et al.*, 1989; Jorgensen *et al.*, 1990; Nexo *et al.*, 1992). This very high luminal EGF concentration may routinely serve in rapid repair of acute damage to a barrier as ligand (streaming across a wound in the epithelium) now interacts with basal-lateral epithelial receptors and receptors on stromal cells (Riegler *et al.*, 1996). However, the existence of a focus of epithelial cells with *chronically* leaky tight junctions in the upper gastrointestinal tract or the lower urinary tract epithelial barrier would allow for long-term penetration of EGF into the stroma at these sites with cell kinetic effects on the epithelia as well as stromal fibroblasts. This hypothesized phenomenon has been demonstrated using cell coculture models treated with activators of protein kinase C (Mullin and McGinn, 1987; Mullin *et al.*, 1998a).

The ability of tight junctions to increase their permeability to some molecules but not others is very important biomedically. If, for example, EGF binding to stromal fibroblasts sharply up-regulates the synthesis and secretion of vascular endothelial growth factor (VEGF) by these same fibroblasts, this 34,000- M_r protein may be "trapped" within the stromal compartment and therefore rise to higher levels than if it could leak across the epithelial cell layer. This could be important in the magnitude of the angiogenic response triggered in the stromal compartment and has been described recently (Mullin, 2004). This model is depicted in Figure 9. The overall phenomenon of selectively altered permeability increases by tight junctions has been recently considered in claudin-5 knockout mice.

In addition to protein growth factors leaking through such barrier defects, other molecules may cross the barrier and thereby serve a diagnostic/prognostic purpose. Entry of prostate-specific antigen (PSA) (a protease in prostatic luminal fluid) into the bloodstream may be indicative of this phenomenon. Normally vectorially secreted by polarized prostatic epithelia into the lumen of the prostate, the appearance of PSA in the blood likely reflects breakdown of the prostatic epithelial barrier in neoplasia and/or loss of polarity of prostate epithelia.

In summary, our results suggest that part of the neoplastic process involves the localized breakdown of barrier function at the site of the transforming epithelia to molecules at least as large as 10 kDa. This can have diagnostic/prognostic potential for alerting the patient and physician to the onset of neoplasia or other diseases. It also may serve a causal, promotional function in the progression of neoplasia by allowing growth stimulatory proteins access to compartments and receptor sites from which they are normally sequestered.

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