Dissecting Tumor Cell Invasion: Epithelial Cells Acquire Invasive Properties after the Loss of Uvomorulin-mediated Cell-Cell Adhesion

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Abstract. The generation of invasiveness in transformed cells represents an essential step of tumor progression. We show here, first, that nontransformed Madin-Darby canine kidney (MDCK) epithelial cells acquire invasive properties when intercellular adhesion is specifically inhibited by the addition of antibodies against the cell adhesion molecule uvomorulin; the separated cells then invade collagen gels and embryonal heart tissue. Second, MDCK cells transformed with Harvey and Moloney sarcoma viruses are constitutively invasive, and they were found not to express uvomorulin at their cell surface. These data suggest that the loss of adhesive function of uvomorulin (which is identical to E-cadherin and homologous to L-CAM) is a critical step in the promotion of epithelial cells to a more malignant, i.e., invasive, phenotype. Similar modulation of intercellular adhesion might also occur during invasion of carcinoma cells in vivo.

CARCINOGENESIS is usually a multistage process that involves the activation, mutation, or loss of different genes. In the affected cells these changes result in loss of growth control, invasion of neighboring tissue, attraction of blood vessels (angiogenesis), or metastasis to distant organs (for reviews cf. Nicolson, 1982; Klein and Klein, 1985). Some of these progression steps clearly require the modulation of cellular interactions, and therefore cell adhesion molecules may play a critical role.

In recent years a series of specific cell-cell and cell-substrate adhesion molecules have been identified and extensively characterized (for cell-cell adhesion molecules cf. Rutishauser et al., 1978; Hyafil et al., 1981; Ocklind and Öbrink, 1982; Gallin et al., 1983; Damsky et al., 1983; Shirayoshi et al., 1983; Behrens et al., 1985; for cell-substrate adhesion molecules cf. Hynes, 1987; Wewer et al., 1986). It has been suggested that the modulation of a limited number of these molecules triggers some of the essential morphogenetic events during embryonal development (for reviews see Steinberg, 1970; Edelman, 1984). The evidence for this is based mainly on developmental changes that are observed in the tissue distribution of the different molecules and on the fact that certain steps of differentiation could be manipulated by specific antibodies directed against these components (cf. Rutishauser et al., 1978; Hyafil et al., 1981; Thiery et al., 1982*a*; Fraser et al., 1984). Examples of developmental processes where differential cell adhesion might be important are the various epithelial-mesenchymal transitions. For instance, when neural crest cells leave the neural ectoderm they loose the cell-cell adhesion molecule N-CAM and begin to express mesenchymal characteristics; e.g., they migrate on fibronectin (Thiery et al., 1982*b*). In the embryonal kidney, mesenchyme is induced to differentiate into epithelial tubules which then express the cell adhesion molecule uvomorulin (Ekblom, 1981; Vestweber et al., 1985).

Changes in cellular phenotypes and in the expression of cell adhesion molecules might also occur during invasion and metastasis of tumor cells. Over 90% of the malignant human tumors are carcinomas (Cairns, 1978); transformed epithelial cells grow in epithelial tissues, break through the basement membrane, and invade the underlying mesenchyme. It is clear that the loss of growth control and the phenomenon of invasion are different processes – growth is affected in both benign and malignant tumors, but invasiveness is associated exclusively with malignancy. Thus, a benign tumor may grow expansively and achieve enormous size, but will not invade connective tissue. On the other hand, some malignant tumors are small and yet readily invade mesenchymal structures and widely metastasize throughout the body (cf. Weinstein et al., 1976). It has been observed in dimethylhydrazine-induced rat colonic carcinomas that the invasiveness correlates well with the degree of tumor dedifferentiation (Gabbert, 1985; Gabbert et al., 1985), which results in the dissociation of tubular structures and the appearance of single cells at the invasion front. Thus, one of the prerequisites for the release of carcinoma cells from epithelia might

be a breakdown of intercellular adhesion. It is known that epithelial cells express specific cell adhesion molecules such as L-CAM/uvomorulin or cell-CAM 105 (for reviews cf. Edelman, 1983; Öbrink, 1986). However, the role of these molecules in invasion processes has not been studied in any great detail.

The Madin-Darby canine kidney (MDCK) epithelial cell line represents an ideal model system to study the impact of cell-cell adhesion on various epithelial properties. In tissue culture, MDCK cells form tight epithelial monolayers with all the characteristics of epithelia in vivo; i.e., they are well polarized, form junctional complexes, and transport ions in a vectorial fashion (Rodriguez-Boulan et al., 1983; Behrens et al., 1985; Gumbiner and Simons, 1986). These monolayers can be dissociated by antibodies against the cell adhesion molecule uvomorulin (e.g., by our anti-Arc-1 monoclonal antibody), and the separated cells then assume a fibroblastic morphology (Imhof et al., 1983; Behrens et al., 1985; see also Vestweber and Kemler, 1985; Gumbiner and Simons, 1986). Evidently, a loss of intercellular adhesion is achieved by disturbing one specific cell surface component, uvomorulin (which is identical to E-cadherin), which has first been characterized in mouse tissue and is homologous to L-CAM, Arc-1, and cell-CAM 120/80 in chicken, canine, and human tissue, respectively (Hyafil et al., 1981; Gallin et al., 1983; Damsky et al., 1983; Shirayoshi et al., 1983; Vestweber and Kemler, 1984; Behrens et al., 1985). These adhesion molecules are glycoproteins with molecular weights of 120,000 which have recently been molecularly cloned (Gallin et al., 1987; Nagafuchi et al., 1987; Ringwald et al., 1987) and from which 80-kD tryptic fragments can be released in the presence of Ca^{2+}. Furthermore, uvomorulin has specifically been localized at the junctional complex of the intestinal epithelium; i.e., at regions where cell-cell adhesion molecules are expected to exert their function (Boller et al., 1985; Behrens et al., 1985).

In the present study we show that MDCK epithelial cells invade both collagen gels and embryonic chick heart tissue when they are dissociated by anti-uvomorulin monoclonal antibodies. MDCK cells transformed with Moloney or Harvey sarcoma virus are invasive without antibody treatment and, concomitantly, they lack uvomorulin on their cell surface. These data suggest that transformed and adhesion-deprived epithelial cells may acquire the invasive properties by a common mechanism; i.e., by the loss of uvomorulin-mediated intercellular adhesion.

Materials and Methods

Cell Culture and Antibodies

The nontransformed MDCK cell line used for most of the experiments (here designated MDCK-1) was cultured as described (Imhof et al., 1983; Behrens et al., 1985). Moloney sarcoma virus-transformed MDCK cells (line $DoCl_1$, designated MSV-MDCK) were obtained from American Type Culture Collection (Rockville, MD). MDCK cells transformed with Harvey murine sarcoma virus (line T_4, designated ras-MDCK) and the corresponding nontransformed MDCK cell line (designated MDCK-2) were provided by Dr. M. C. Lin (National Institutes of Health, Bethesda, MD; see Darfler et al., 1986). We also selected additional subclones of Harvey murine sarcoma virus-transformed MDCK cells by limiting dilution of an uncloned cell population that exhibited either epithelial (lines epithelial-1 and -2) or fibroblastic morphologies (lines fibroblastic-1 to -6). The nontransformed MDCK cells used for the invasion assay with chick heart tissue (MDCK-3) were obtained from J. Leighton (Philadelphia, PA). Cells were cultured in DME; MDCK-3 cells in Eagle's medium with Earle's salts and nonessential amino acids (Flow Laboratories, Inc., McLean, VA) containing 10% FCS.

The mouse monoclonal antibody anti-Arc-1 (directed against uvomorulin of MDCK cells; cf. Behrens et al., 1985) was purified from ascites fluid on DEAE-Sepharose (Imhof et al., 1983). For preparation of a polyclonal antiserum against uvomorulin, the 80-kD tryptic fragment of uvomorulin was first purified from canine liver: tissue was homogenized in 1 mM $NaHCO_3$, 1.5 mM $CaCl_2$ (10 ml/g liver), centrifuged at 18,000 rpm in a rotor (SS 34; Sorvall Instruments Div., Newton, CT), and the pellet was resuspended in L-CAM assay buffer (Cunningham et al., 1984) at a protein concentration of 7.5 mg/ml. The membranous material was then digested for 40 min at 37°C with 300 μg/ml trypsin (type XI; Sigma Chemical Co., St. Louis, MO), digestion was stopped with a threefold excess of egg white trypsin inhibitor (Sigma Chemical Co.), the suspension centrifuged at 25,000 rpm in a rotor (type 30; Beckman Instruments, Inc., Fullerton, CA) for 60 min, and the supernatant was loaded onto an affinity column prepared by coupling anti-Arc-1 monoclonal antibody to CNBR-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The 80-kD fragment of uvomorulin was eluted with 200 mM diethylamine, pH 11.5, and the solution was neutralized with 1 M Hepes buffer. A rabbit was then immunized with 20 μg protein by lymph node injection (Louvard et al., 1982) and boosted three times by subcutaneous injection. Anti-uvomorulin antibodies were affinity purified on the 80-kD uvomorulin fragment coupled to CNBR-Sepharose. Rat monoclonal antibody DECMA-1 (directed against mouse uvomorulin) was a gift of Dr. R. Kemler (Max-Planck-Institute, Tübingen, FRG; see Vestweber and Kemler, 1985). Anti-FC-1 monoclonal IgG1 (purified on DEAE-Sepharose; Oesch and Birchmeier, 1982) or IgG isolated from rabbit preimmune serum served as control antibodies.

Preparation of Collagen Gels and Invasion Assay

Type I collagen (5 mg/ml, extracted from calf skin with 1% acetic acid; Mauch et al., 1986) was dialyzed against 0.05% acetic acid. 0.1 vol of 10 × DME (containing 22 mg/ml sodium bicarbonate) was then added, and the solution was neutralized. Aliquots of 1.2 ml/well were allowed to gel in 6-well culture dishes (Nunc, Roskilde, Denmark) at 37°C.

For the invasion assays, 4×10^5 MDCK cells (in 1.5 ml DME plus 10% FCS) were plated per well onto the collagen surfaces. After 1 d of culture, new medium containing the antibodies (at a final concentration of 100 μg/ml) was added, and these solutions were replaced every second day. Cells invading the collagen gel were photographed by focusing down into the matrix, and the number of penetrating cells were determined in duplicate by counting fields of 1.4 mm^2 (a total area of 28 mm^2 was scanned to obtain one data point). For transmission electron microscopy, collagen gels were fixed in 2.5% glutaraldehyde, epon embedded, and ultrathin sections were prepared. For scanning electron microscopy, glutaraldehyde-fixed gels were gently disrupted with forceps to reveal cells inside the collagen. The gels were then processed as described (Imhof et al., 1983).

Immunological Methods

Binding assay and immunofluorescence of MDCK cells using the anti-Arc-1 monoclonal antibody were performed essentially as described (Imhof et al., 1983; Behrens et al., 1985). For the binding assay, MDCK cells (3×10^4 cells/well) were cultured overnight on 96-well flexible microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA), fixed with 3% formaldehyde, pH 8.2, and permeabilized with 1% Triton X-100 in PBS. They were then incubated in culture medium for 3 h at room temperature with ^{125}I-labeled anti-Arc-1 antibody (3×10^5 cpm/well, 3×10^7 cpm/ug). For determination of nonspecific binding, the radioactive antibody was competed with 100 μg/well unlabeled antibody. After extensive washing, the wells were cut off and the radioactivity was determined in quadruplicate in a gamma counter. For immunofluorescence, both the normal and the transformed MDCK cells were plated at near confluency to optimize contact formation. After fixation and permeabilization, cells were incubated for 1 h with either 50 μg/ml anti-Arc-1 or 1 μg/ml affinity-purified rabbit anti-uvomorulin antibody, stained by appropriate fluorescein- or rhodamine-coupled second antibodies, and inspected in a fluorescence microscope (Orthoplan; E. Leitz, Wetzlar, FRG).

For immunoprecipitation of uvomorulin, cells in 10-cm tissue culture dishes (Falcon Labware, Oxnard, CA) were labeled for 20 h with 40 μCi/ml of [^{35}S]methionine (Wieland et al., 1986). The cells were then scraped into 1 ml cold L-CAM assay buffer containing 1% Triton X-100, 1 mM PMSF and extracted at 4°C for 1 h. The 100,000-*g* supernatants of the lysates were

precleared with 200 μl of a 10% suspension of *Staphylococcus aureus*, and incubated for 2 h with 100 ng of either affinity-purified anti-uvomorulin antibodies or preimmune IgG. For comparison of the uvomorulin content of normal and transformed MDCK cells, lysates containing equal amounts of TCA-precipitable radioactivity were used. The antigen-antibody complexes were then pelleted with 50 μl *S. aureus* solution, washed with NET buffer containing 1% Triton X-100 (Kessler, 1975), eluted with SDS dissociation buffer, and analyzed by SDS gel electrophoresis and fluorography (Wieland et al., 1986).

For immunodetection of uvomorulin on Western blots, Triton X-100 cell extracts were electrophoresed on 8% SDS-polyacrylamide gels (20 μg of protein/lane), transferred to nitrocellulose filters (Towbin et al., 1979), and the filters blocked for 1 h with 5% low fat milk (Nestlé, Münich, FRG), 0.5% Triton X-100 in PBS. Filters were then incubated overnight with affinity-purfied anti-uvomorulin antibodies (0.2 μg/ml) followed by peroxidase-coupled goat anti-rabbit IgG (Dianova) and stained with 0.5 mg/ml diaminobenzidine and 0.015% H_2O_2 in L-CAM assay buffer.

Assay for Invasion into Embryonic Chick Heart Tissue

MDCK cells were scraped from stock cultures, and clusters of these were confronted with precultured heart fragments of 9-d-old chick embryos on top of semi-solid agar as described earlier (Mareel et al., 1979). After overnight attachment to each other, confronting pairs were transferred into individual 5-ml Erlenmeyer flasks containing 1.5 ml culture medium for further incubation on a Gyrotory shaker at 120 rpm. In some cultures, anti-Arc-1 monoclonal antibody was added to the medium at a concentration of 70 μg/ml. Cultures were fixed after 4, 7, and 14 d and embedded in paraffin for serial sectioning. Consecutive 8-μm-thick sections were stained either with hematoxylin-eosin, an antiserum against chicken heart tissue (Mareel et al., 1981), or an antiserum against MDCK cells. This latter antiserum was prepared as follows: MDCK-3 cells were grown in roller bottles, scraped off with a rubber policeman, homogenized, sonicated, and lyophilized. Two rabbits were immunized four times fortnightly with 3 mg each lyophilized MDCK powder in Freund's complete adjuvant at multiple sites in the neck (subcutaneously) and in the gluteal region (intramuscularly). The antiserum was tested immunocytochemically and found to be sufficiently specific to distinguish between MDCK cells and chick heart fragments on sections of confrontation cultures. Sections were evaluated by two independent observers in accordance with the grading system described (Bracke et al., 1984).

Results

Morphology of MDCK Epithelial Cells after Disturbance of Cell-Cell Adhesion and Malignant Transformation

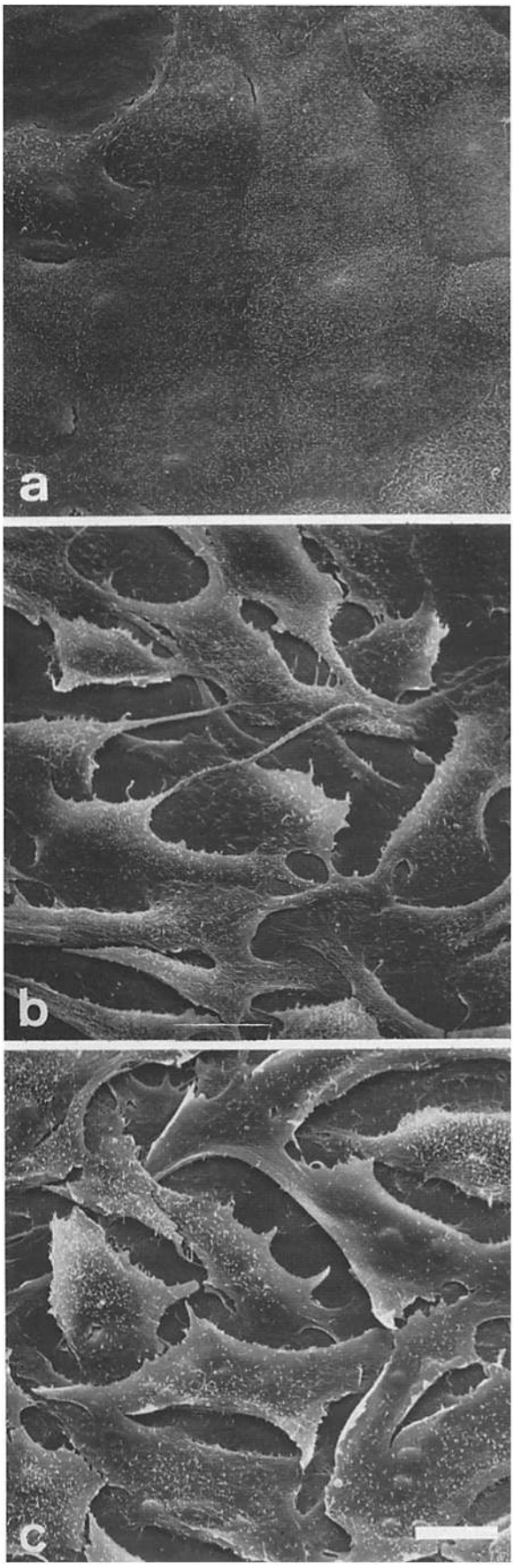
As analyzed by scanning electron microscopy, nontransformed MDCK cells (e.g., line MDCK-1; see Materials and Methods) form closed epithelial monolayers; i.e., the individual cells are arranged in a cobblestone-like manner (Fig. 1 *a*). After treatment of the cells with anti-uvomorulin monoclonal antibodies (e.g., with anti-Arc-1) the monolayer is broken up and the single cells assume a fibroblastic morphology (Fig. 1 *b*; cf. also Imhof et al., 1983, Behrens et al., 1985). MDCK cells transformed with Moloney or Harvey sarcoma virus also exhibit a fibroblastic morphology (Figs. 1 *c* and 2 *b*); these transformed cells do not form close intercellular contacts, have lost their cuboidal shape, and often crawl over one another. We also subjected an uncloned population of Harvey sarcoma virus-transformed MDCK cells to limiting dilution and found that the majority of subclones (87.5%, $n = 48$) showed a fibroblastic phenotype whereas a minority was epithelial (Fig. 2, *a*, *c*, and *d*, shows examples of such epithelial and fibroblastic lines). Apparently, transformation of MDCK cells by sarcoma viruses can interfere with the epithelial phenotype and produce cells resembling those dissociated by anti-uvomorulin antibodies. Similar shape changes have been observed after transformation of MDCK cells by $pp60^{v\text{-}src}$ (Warren and Nelson, 1987).

Nontransformed MDCK Cells Dissociated by Anti-uvomorulin Monoclonal Antibodies Invade Collagen Gels

We then investigated whether the antibody-dissociated MDCK cells acquire further properties of fibroblastic lines; e.g., exhibit an increased cellular motility. Hence, nontransformed MDCK-1 cells were plated on three-dimensional gels of collagen type I, and they were found to grow and form tight monolayers on this matrix. However, when the dissociating antibody anti-Arc-1 (at 100 μg/ml) was added to the preformed monolayer, cells began to invade the underlying collagen within 3–4 d (Fig. 3). Approximately 1% of the cells invaded within this time period when anti-Arc-1 was continuously present (see also legend to Fig. 3), whereas a control antibody of the same isotype at the same concentration was ineffective. In addition, a second anti-uvomorulin monoclonal antibody, DECMA-1, and our polyclonal antibody produced against the 80-kD tryptic fragment of uvomorulin (see below) also induced invasiveness of the cell. The invasive MDCK cells could be visualized in the light microscope by focusing down into the gel matrix (Fig. 4 *a*), and they expressed a spindle-shape morphology. The invasive cells could also be detected in the scanning electron microscope after gentle disruption of the fixed collagen gels (Fig. 4 *c*); they were elongated and tightly surrounded by collagen fibers. Transmission electron microscopy of epon-embedded specimens showed that the invasive cells had lost their polarity; i.e., expressed protrusions all over the cell surface (Fig. 4 *d*). These results show that the disturbance of MDCK epithelial cells through one specific adhesion molecule allows them to escape the monolayer and penetrate underlying collagen substrate.

Transformed MDCK Cells Invade Collagen Gels without Prior Antibody Treatment and Do Not Express Significant Amounts of Uvomorulin

Since the virally transformed MDCK cells closely resemble the antibody-treated cells with respect to their morphology (cf. Fig. 1 and 2), we also examined their invasive potential on collagen gels. Both Moloney and Harvey sarcoma virus-transformed MDCK cells were found to invade even in the absence of anti-Arc-1 antibody, and the addition of antibody did not significantly enhance invasiveness (shown for MSV-MDCK cells in Fig. 5). Fig. 6 summarizes the invasive capacity for collagen of the various nontransformed and transformed MDCK cell lines and also lists the corresponding uvomorulin contents, as quantified with an antibody binding assay on fixed and permeabilized specimens. Thus, all cell lines expressing an epithelial phenotype (MDCK-1 and -2, as well as the sublines epithelial-1 and -2) were found to be noninvasive, whereas all cell lines expressing a fibroblastic phenotype (Harvey and Moloney sarcoma virus-transformed MDCK cells, as well as the subclones fibroblastic-1 to -6) were highly invasive (Fig. 6, *left*). The measurement of uvomorulin in these cell lines revealed an intriguing correlation: the highly invasive (fibroblastic) clones exhibited very low levels of uvomorulin while the noninvasive (epithelial) cell lines expressed high amounts of the molecule (Fig.



6, *right*). These results could be confirmed by immunofluorescence staining with both anti-Arc-1 monoclonal and anti-uvomorulin polyclonal antibodies: uvomorulin was absent from the cell borders of the invasive fibroblastic cells (Fig. 7, *c* and *d*) whereas the noninvasive epithelial cells expressed the characteristic pattern of uvomorulin at the cell–cell contacts (Fig. 7, *a* and *b*).

Immunoprecipitation and Western blotting of uvomorulin in the various MDCK cell lines revealed similar results. Immunoprecipitation from noninvasive MDCK cells resulted in the characteristic pattern of three proteins at apparent molecular masses of 120, 102, and 98 kD, as well as in some 80-kD uvomorulin digestion product (Fig. 8 *a*, lanes *a* and *b*). In contrast, the invasive MDCK cell lines exhibited a reduced amount of all three proteins (Fig. 8 *a*, lanes *c* and *d*). It has been shown by others that the 120-kD component represents full-size uvomorulin, whereas the two lower molecular mass components are most likely coprecipitated (Peyrieras et al., 1985; Vestweber et al., 1987). The same reduction of uvomorulin in the invasive MDCK cells was observed when immunoprecipitation was carried out with an antiserum directed against cell-CAM 120/80, which is the analogue of uvomorulin in human cells (data not shown; cf. Damsky et al., 1983). Furthermore, pulse–chase experiments suggested that the reduced amount of uvomorulin in the invasive MDCK cells is mainly due to a lower rate of synthesis (data not shown). In Western blot experiments (where the 120-kD component is the predominant band) both nontransformed MDCK cell lines and noninvasive epithelial subclones were again high in uvomorulin content whereas the invasive fibroblastic lines were low (Fig. 8, *b* and *c*). Western blot experiments also showed that treatment of nontransformed MDCK cells with anti-Arc-1 antibody did not lead to a significant reduction of the level of uvomorulin (data not shown). Apparently, the disturbance of uvomorulin function is sufficient for invasion to occur in the nontransformed cells, whereas down regulation of the molecule accompanies the invasive phenotype in the transformed cells.

MDCK Cells Deprived of Uvomorulin Function Invade into Heart Fragments

We also examined whether antibody-dissociated and transformed MDCK cells are invasive for embryonal heart tissue. This assay has been developed to discriminate between benign (noninvasive) and malignant (invasive) tumor cells in vitro (Mareel, 1983). Nontransformed MDCK cells (line MDCK-3) were confronted with fragments of embryonic

Figure 1. Scanning electron microscopy of MDCK epithelial cells: change in cell morphology upon anti-uvomorulin antibody treatment and upon malignant transformation. (*a*) Nontransformed MDCK-1 cells, (*b*) MDCK-1 cells treated with anti-Arc-1 antibody at 100 μg/ml for 3 h, and (*c*) Moloney sarcoma virus-transformed MDCK cells (MSV-MDCK). Note that antibody-treated and transformed cells exhibit a similar, i.e., fibroblastic, morphology. Specimens were prepared for scanning electron microscopy as described (Imhof et al., 1983). Bar, 10 μm.

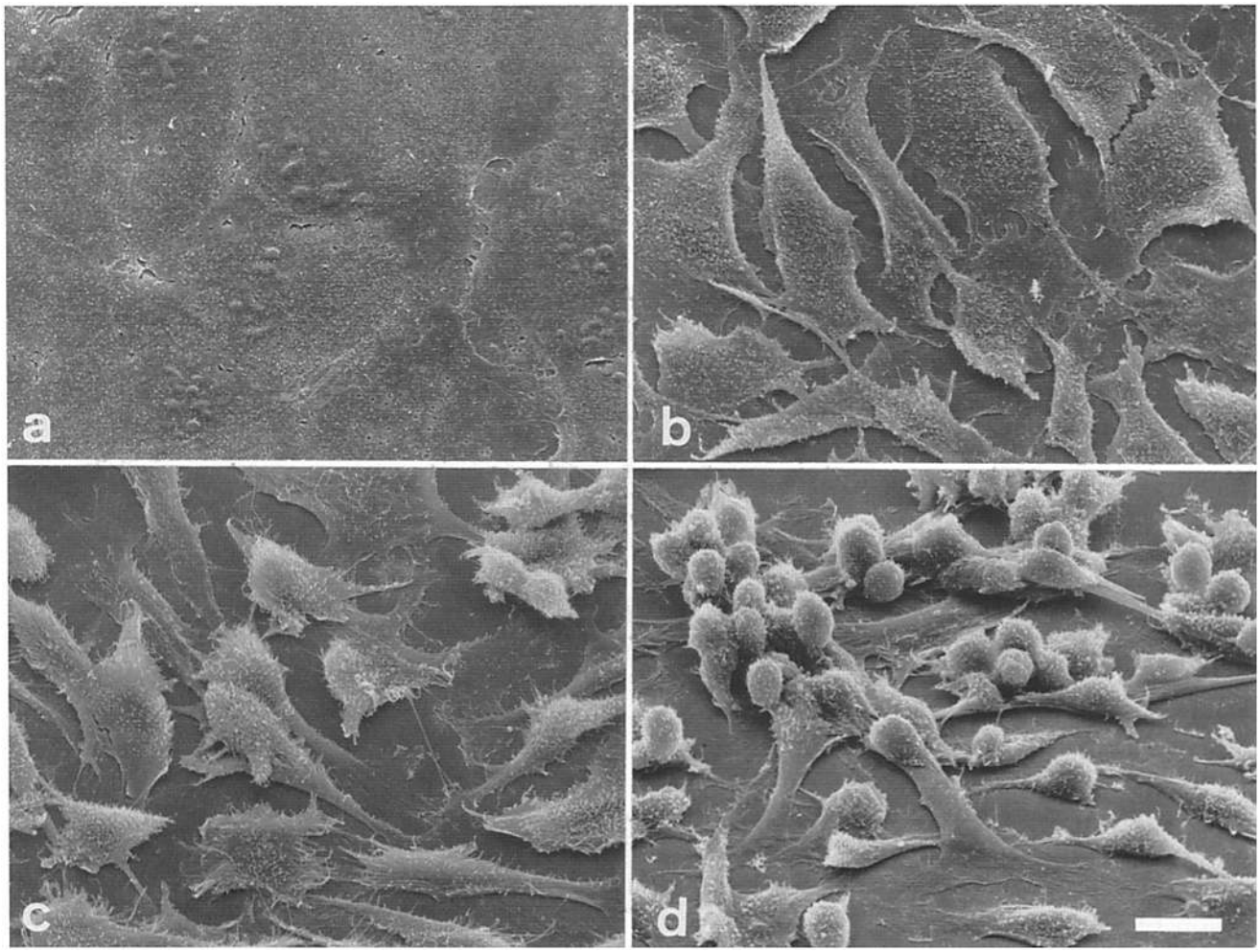


Figure 2. Scanning electron microscopy of subclones of Harvey sarcoma virus–transformed MDCK cells. Selected examples of fibroblastic and epithelial sublines of Harvey sarcoma virus–transformed MDCK cells are shown (see also Materials and Methods). (*a*) Clone epithelial-2; (*b*) clone T4, ras-MDCK; (*c*) clone fibroblastic-2; and (*d*) clone fibroblastic-4. Note that the line epithelial-2 has a morphology similar to nontransformed MDCK cells (see Fig. 1 *a*), whereas the morphology of lines T4 and fibroblastic-2 is similar to antibody-treated MDCK cells (see Fig. 1 *b*). The line fibroblastic-4 also contains some rounded-up cells. Bar, 10 μm.

chick heart in suspension culture (see Materials and Methods), and during 4–14 d of incubation the cells formed closed epithelial monolayers around the heart tissue (Fig. 9, *a–c*). Confirming previous results (Schroyens et al., 1984), no invasion was seen, as demonstrated by specific immunocytochemical staining of both heart tissue and MDCK cells (Fig. 9, *b* and *c*). However, when confrontation was carried out in the presence of 70 μg/ml anti–Arc-1 monoclonal antibody for 4 ($n = 3$) and 7 d ($n = 2$) all cultures contained groups of MDCK cells within the heart fragments (Fig. 9, *d–i*, *arrowheads*). The invasive MDCK cells could unequivocally be identified by specific immunocytochemical staining with the anti-MDCK antiserum (Fig. 9, *f* and *i*) and by complementary staining for the chick heart tissue (Fig. 9, *e* and *h*).

Selected MDCK cell lines with both epithelial and fibroblastic phenotypes were also tested for invasion of the chick heart fragments in the absence of anti-uvomorulin antibodies. It was found that both nontransformed MDCK cells and the epithelial subline-1 (which expressed a high uvomorulin content; see Figs. 6–8) were noninvasive and exhibited epithelial characteristics on the heart surface (Table I). In contrast, the fibroblastic Harvey sarcoma virus–transformed MDCK cells and the lines fibroblastic-1 and -3 (which expressed virtually no uvomorulin; cf. Figs. 6–8) progressively invaded the heart tissue.

Discussion

In the present study we describe two aspects of epithelial cell invasion which seem to be interrelated. First, nontransformed MDCK epithelial cells perturbed in their cell–cell contacts by the action of anti-uvomorulin antibodies become invasive for collagen gels and embryonic heart tissue. Second, MDCK cells transformed with Moloney or Harvey sarcoma virus are constitutively cell–cell contact deficient and invasive in both assays, and they express virtually no uvomorulin on the cell surface. The data suggest that cell–cell adhesion involving L-CAM/uvomorulin stabilizes the epithelial phenotype and counteracts invasive properties of the cells. A loss of uvomorulin-based intercellular adhesion

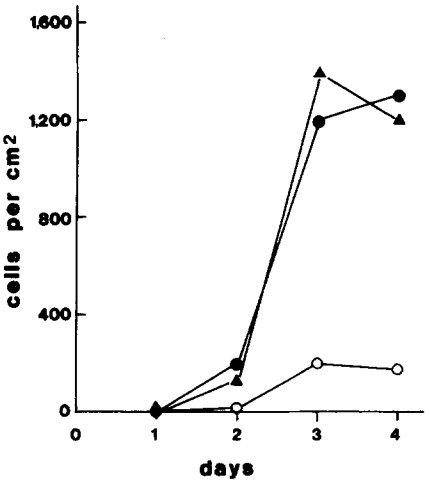


Figure 3. Invasiveness of nontransformed MDCK epithelial cells after dissociation by anti-uvomorulin monoclonal antibodies. MDCK-1 epithelial cells were cultured on gels of collagen type I and at day 0, the anti-uvomorulin monoclonal antibodies anti-Arc-1 (●) and DECMA-1 (▲) or control IgG1 (○) were added (at 100 μg/ml). Cells invading the collagen gels were quantified in the light microscope (see Materials and Methods and Fig. 4, *a* and *b*). It was found that ~1% of the population invaded the gel matrix within 3 d (i.e., 1.4×10^3 invading cells were counted per cm^2, compared to ~2×10^5 cells/cm^2 present on top of the collagen gel).

might also occur when carcinoma cells invade into neighboring tissues in vivo.

It has previously been shown that MDCK epithelial cells, although immortalized in tissue culture, are nonmalignant and only grow poorly in adult nude mice (McRoberts et al., 1981). It is therefore quite remarkable that treatment of these cells with anti-uvomorulin antibodies makes them invasive in the heart invasion assay. This assay generally differentiates between benign and malignant cells in vitro (Mareel, 1983); i.e., carcinoma cells, transformed fibroblasts, or melanoma cells all invade heart tissue, whereas nontransformed epithelial cells or primary fibroblasts are noninvasive (Schroyens et al., 1984; Van Roy et al., 1986). The collagen assay also measures invasiveness in vitro (Schor, 1980); it is not as elaborate as the heart assay, and the invasive cells can easily be quantified in the course of the process and their morphology closely examined. However, this assay seems to be somewhat less restrictive since, for instance, fibroblasts invade independently of whether they are transformed or not. Nevertheless, nontransformed MDCK epithelial cells were noninvasive in the collagen assay, and anti-uvomorulin antibody treatment resulted in invasion. The question arises how specific interference with cell adhesion might transfer nontransformed epithelial cells from a noninvasive to an invasive state. We could hypothesize that the separated cells, having lost their lateral bonds, might now turn their "interests" toward the lower substrate; e.g., might produce powerful matrix-digesting proteases. Although the detailed mechanism of this transition remains to be explored further, it is tempting to speculate that the loss of uvomorulin-mediated intercellular adhesion promotes MDCK epithelial cells toward a more malignant state.

In both invasion assays, we have specifically abolished uvomorulin function by the antibody treatment, and this must be the primary cause for the release of the cells from the monolayer. We have also observed that virally transformed MDCK cells are invasive by themselves and do not express uvomorulin on their cell surface. Since these transformed cells resemble the antibody-treated cells with respect to their morphology, it is tempting to speculate that again the absence of functional uvomorulin contributes to their invasiveness. We are aware of the fact, however, that the antibody dissociation experiments are much stronger in revealing a direct interrelationship between the loss of uvomorulin function and the invasiveness of the cells. In the virally transformed cells the loss of adhesion is certainly only one of the many changes that result upon transformation (see also below). It is interesting in this context that mesenchymal cells, which are invasive for collagen even in their nontransformed state, do not possess L-CAM/uvomorulin-type adhesion molecules (Edelman, 1983; Vestweber and Kemler, 1984; Takeichi et al., 1985). Furthermore fibroblasts transfected with cloned mouse E-cadherin cDNA (E-cadherin is identical to uvomorulin) now express the epithelial adhesion molecule on the cell surface and this stimulates intercellular contact formation (Nagafuchi et al., 1987; see also Mege et al., 1988). Using the same cloned cDNA (plasmid pSTEM1, kindly provided by Dr. A. Nagafuchi, Kyoto University, Kyoto, Japan, to Dr. M. Mareel) in cotransfection with a cosmid encoding resistance to G418 (pHSG272), clones of MDCK fibroblastic-3 cells that express murine E-cadherin have been obtained. Such clones exhibit an epitheloid morphology and do not invade into the chick heart in vitro (Mareel, M. M., and F. M. Van Roy, unpublished observations). This experiment demonstrates the possibility to correct the invasive phenotype by transfection with E-cadherin cDNA.

In the collagen invasion assay only a fraction of the antibody-dissociated MDCK cells were found to be invasive; i.e., 1% of the cells penetrated in 4 d (see legend to Fig. 3). The Moloney and Harvey sarcoma virus-transformed MDCK cells invaded to a similar degree, as did nontransformed MDCK cells dissociated by scatter factor (data not shown; cf. also Stoker et al., 1987). A study of the invasiveness of various fibroblastic cell lines for collagen resulted in comparable values (for baby hamster kidney and human skin fibroblasts no invasion was observed in 4 d, and 2.5 and 5% invading cells, respectively, were seen in 8 d), and only melanoma cells were more highly invasive (Schor, 1980; Schor et al., 1982). Several reasons are possible for the somewhat low yield of invasive cells in the collagen assay; e.g., the composition of the matrix might not be optimal and/or the cells might only be invasive at a small time period of the cell cycle. Inclusion of fibronectin or laminin into the collagen gels (e.g., at 50 μg/ml) did not significantly alter the invasiveness of the dissociated MDCK cells (data not shown). In the heart assay, no quantitation of the invasive cells after antibody dissociation was performed. However, invasion was significant (Fig. 9) but lower than in the case of the transformed MDCK cells, where sometimes more than half of the heart tissue was replaced (Table I). It is clear that transformation induces phenotypic changes in addition to cell dissociation, and this might enhance the invasive potential of the cells in a pleiotropic manner; e.g., by induction of a more com-

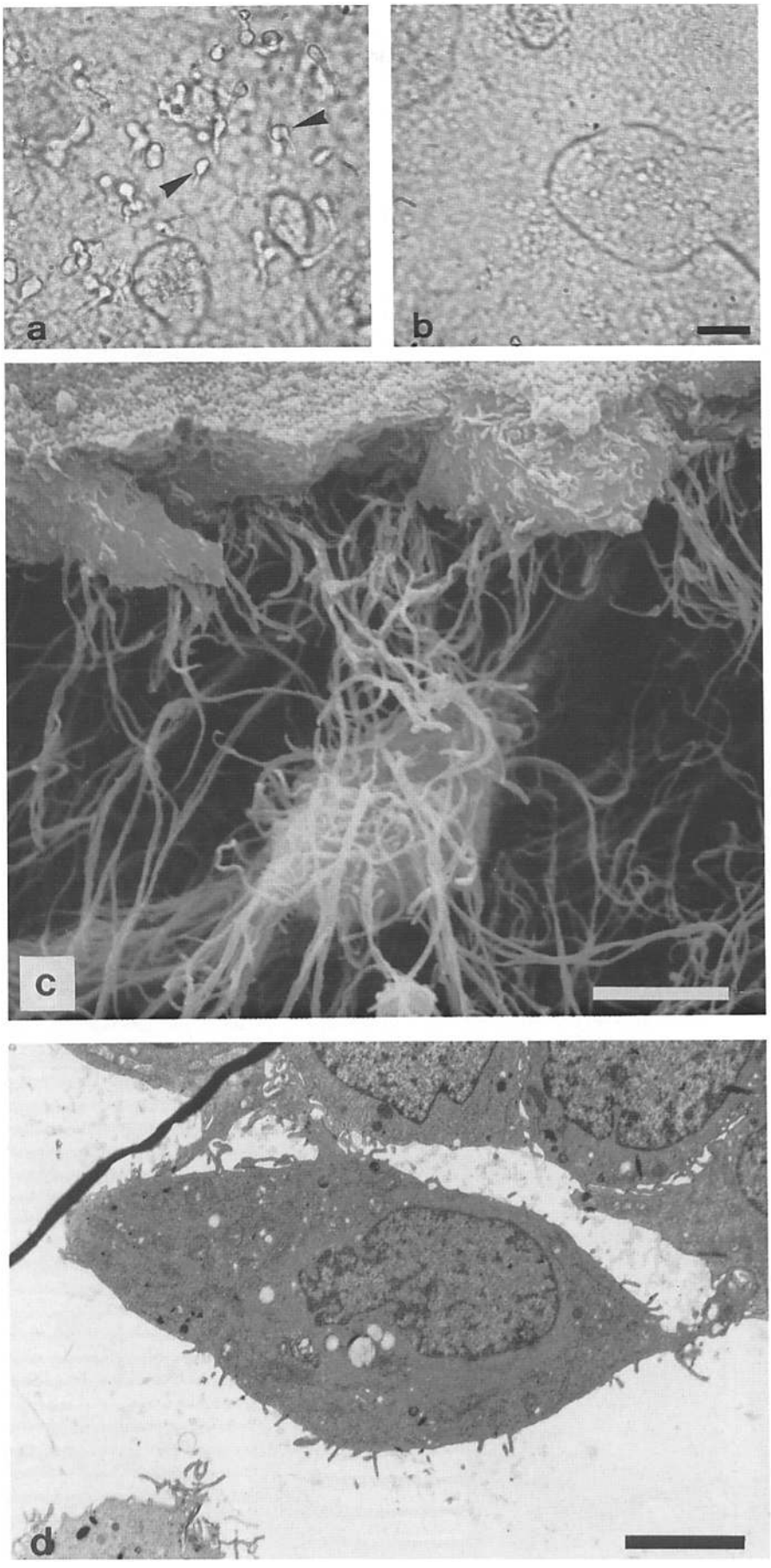


Figure 4. Microscopic observation of the penetrating MDCK cells. (*a* and *b*) Overview by light microscopy. (*a*) MDCK-1 cells penetrating the collagen gel after dissociation by the anti-Arc-1 antibody; (b) MDCK cells treated with the control IgG1 at the same focus. Note that the invading MDCK cells (*a*) exhibit a fibroblastic morphology (in focus, see *arrowheads*) and that the MDCK monolayers on the collagen surface are slightly out of focus. Bar, 40 μm. (*c* and *d*) Scanning (*c*) and transmission (*d*) electron microscopy. The invasive MDCK cells were visualized by scanning electron microscopy after gentle disruption of the collagen gels and by transmission electron microscopy after ultrathin sectioning of epon-embedded specimens. In both cases, an invasive cell is shown below the MDCK monolayer. Note the collagen fibers surrounding the invasive cell in (*c*). Bars, 4 μm.

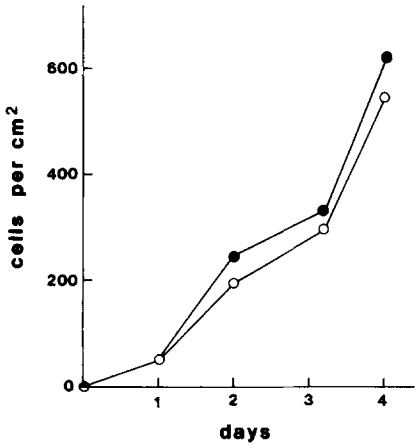


Figure 5. Invasiveness of Moloney sarcoma virus-transformed MDCK cells on collagen gels. MSV-transformed MDCK cells were cultured on gels of collagen type I and the invasive cells were quantified daily as in Fig. 3. Penetration of cells in the presence of 100 μg/ml anti-Arc-1 antibody (●) and in the presence of the control IgG1 (○) is shown.

plex spectrum of matrix-digesting proteases. Or, the heart tissue might release factors which specifically promote the invasion of the transformed cells, but to a lesser degree those of the antibody-treated cells.

In this and the preceding studies (Imhof et al., 1983; Behrens et al., 1985) we have provided strong evidence that cell–cell adhesion involving functional uvomorulin plays a crucial role in maintaining the epithelial phenotype. When this adhesion molecule is disturbed or absent, the cells lose epithelial characteristics and acquire new properties: i.e., they show a spindle-shaped morphology, lack a pronounced apical–basolateral polarity, and exhibit invasiveness for collagen and heart tissue. These are all qualities that have generally been assigned to mesenchymal and/or malignant cells. The results from our studies in vitro may therefore also be significant for the understanding of epithelial–mesenchymal transitions as they occur during embryonal development (cf. Hay, 1984). For instance, epithelial tubules of the kidney are derived from mesenchymal precursor cells (Ekblom, 1981), and uvomorulin expression was found to be turned on during this transition (Vestweber et al., 1985). However, the administration of anti-uvomorulin antibodies did not inhibit tubule formation. Apparently, up regulation and down regulation of uvomorulin is not the only factor to shift the equilibrium between the two states in this particular system. It is therefore important to examine what other factors (e.g., other cell adhesion molecules, hormones, or components of the extracellular matrix) also modulate these complex transitions in vivo (cf. Sariola et al., 1988; Klein et al., 1988).

There are important pathological conditions where epithelial cells invade mesenchymal structures. During invasion of carcinomas, transformed epithelial cells escape the established adhesion barriers within epithelia and penetrate the underlying tissues. The data of our study in vitro give some hints that this could be accomplished by the disturbance of specific cell–cell adhesion molecules. Moreover, the extent to which carcinoma cells are able to repress the function of their cell adhesion molecules could be a decisive factor for the degree of their invasiveness. For instance, in the MDCK system described, two types of *ras*-expressing cell lines were identified: (*a*) cells retaining their epithelial characteristics and maintaining a high uvomorulin content (the lines epithelial-1 and -2); these are noninvasive although they express the *ras* oncogene (as shown by immunofluorescence) and produce tumors with tubular differentiation in nude mice; and (*b*) cells assuming fibroblastic properties (lines fibroblastic-1 to -6); these are highly invasive in vitro and they form fully undifferentiated tumors in nude mice (Mareel,

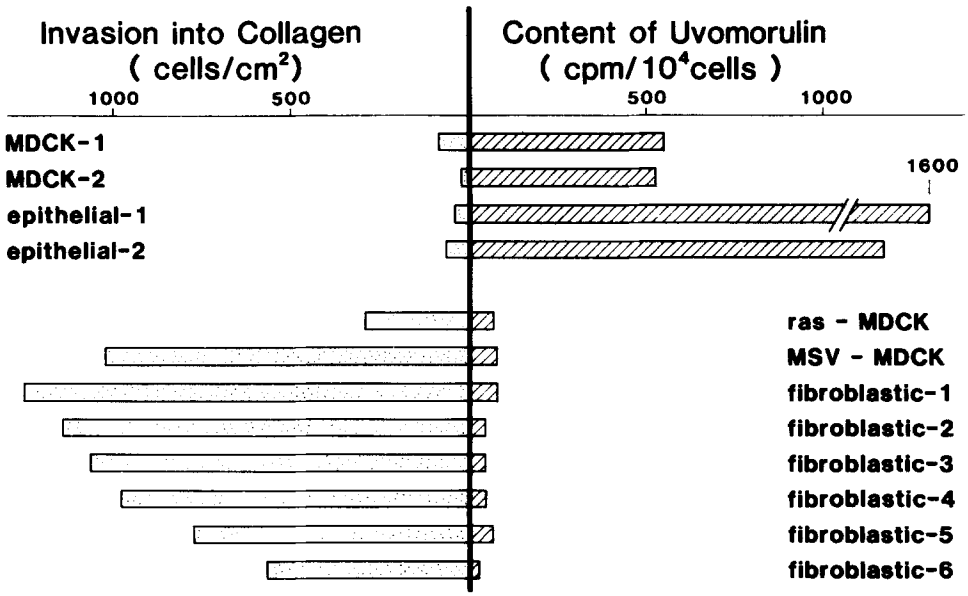


Figure 6. Invasiveness on collagen gels and corresponding uvomorulin content of the various MDCK cell lines. The invasiveness was determined as in Fig. 3 after 3 d of culture on collagen gels. The expression of uvomorulin in tissue culture was quantified with a cell binding assay using ^{125}I-labeled anti-Arc-1 antibody (see Materials and Methods). Note that the cell lines with an epithelial phenotype (the nontransformed lines MDCK-1 and -2 and the *ras*-transformed clones epithelial-1 and -2) are noninvasive and express a high amount of uvomorulin, whereas the fibroblastic lines (ras-MDCK, MSV-MDCK, and the *ras*-transformed clones fibroblastic-1 to -6) are highly invasive and do not express uvomorulin.

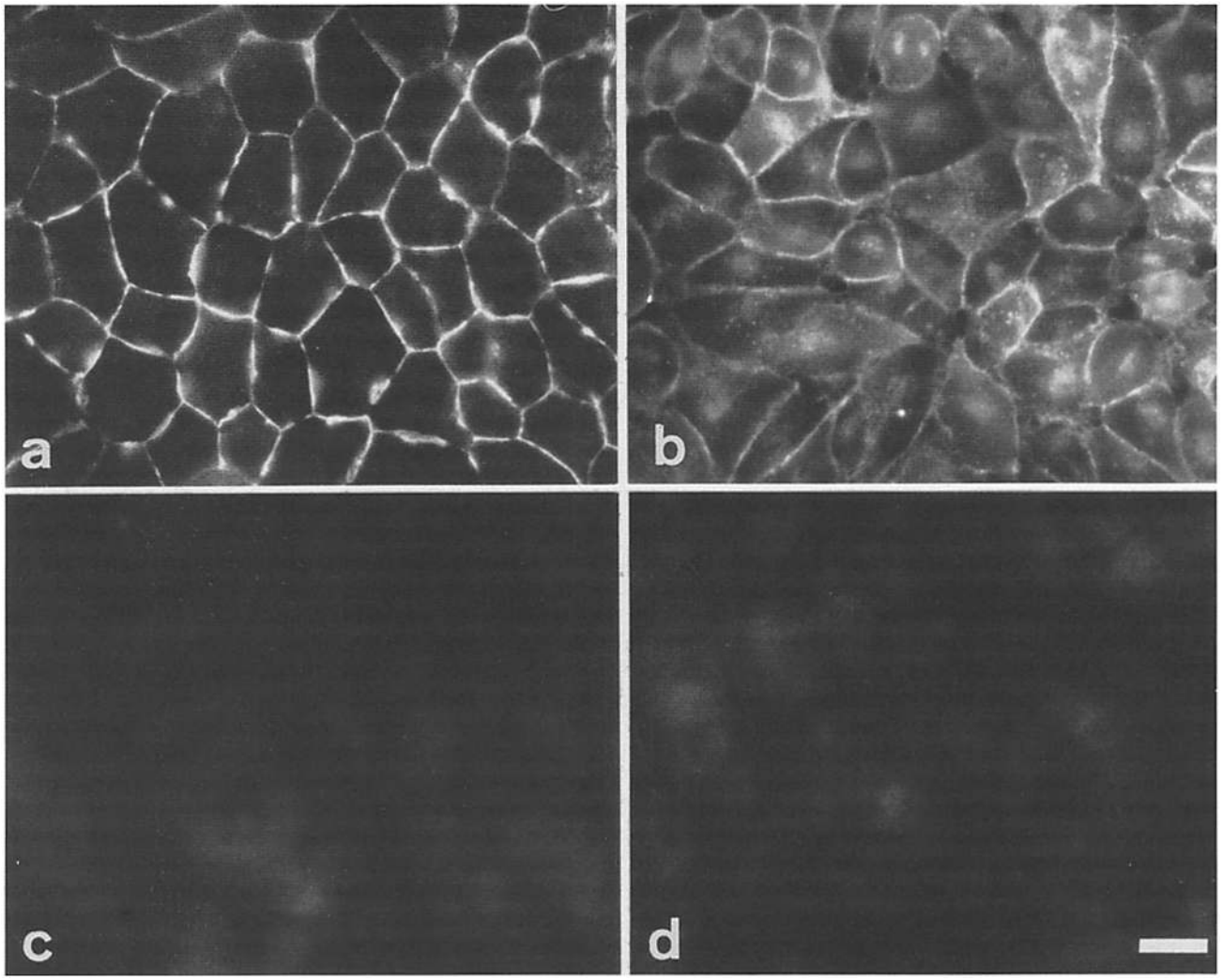


Figure 7. Immunofluorescence of uvomorulin in noninvasive and invasive MDCK cell lines. (*a*) Nontransformed MDCK-1 cells, (*b*) clone epithelial-2, (*c*) MSV-transformed MDCK cells, and (*d*) Harvey sarcoma virus–transformed MDCK cells. The immunofluorescence was performed on fixed and permeabilized cells using monoclonal anti-Arc-1 (*a* and *c*) and affinity-purified polyclonal anti-uvomorulin antibodies (*b* and *d*). Bar, 20 μm.

M. M., F. M. Van Roy, J. Behrens, and W. Birchmeier, manuscript in preparation). Apparently, these two variants seem to represent two stages of tumor progression of epithelial cells; i.e., a noninvasive (benign) and an invasive (malignant) stage. To prove whether similar stages are in fact relevant in vivo, one should examine cell adhesion molecules of carcinomas in the course of the invasion process. The available data provide us only with a static picture of the in vivo situation. For instance, junctional deficiencies are quite common in human tumors, and this is particularly true for carcinomas (Weinstein et al., 1976). Furthermore, in an experimental system of rat colon carcinomas, high invasiveness has been correlated with the loss of tumor differentiation; i.e., the disappearance of tubular structures and the presence of incompletely developed junctional complexes (Gabbert, 1985; Gabbert et al., 1985). However, the amount of cell adhesion molecules of the L-CAM/uvomorulin-type have not yet been measured in situ. On the other hand, the uvomorulin homologue cell-CAM 120/80 has been found in certain human carcinoma cell lines, and it seems to exert adhesive function in vitro (Damsky et al., 1983). We could hypothesize that during tumor progression in this latter case, carcinoma cells underwent only a temporal breakdown of the uvomorulin adhesion system. However, a permanent down regulation was observed for the cell adhesion molecule cell-CAM 105 in certain hepatocellular carcinomas (Hixson et al., 1985).

The recent research on cellular oncogenes has mainly been directed toward understanding growth control of mesenchymal cells, whereas epithelial cells were somewhat neglected. We have here observed that transformation of MDCK epithelial cells with Moloney and Harvey sarcoma viruses (which introduce the *mos* and *ras* oncogenes, respectively) leads to specific alteration of the intercellular adhesion system. Similarly, expression of $pp60^{v\text{-}src}$ in MDCK cells resulted in transformed cells which exhibit reduced

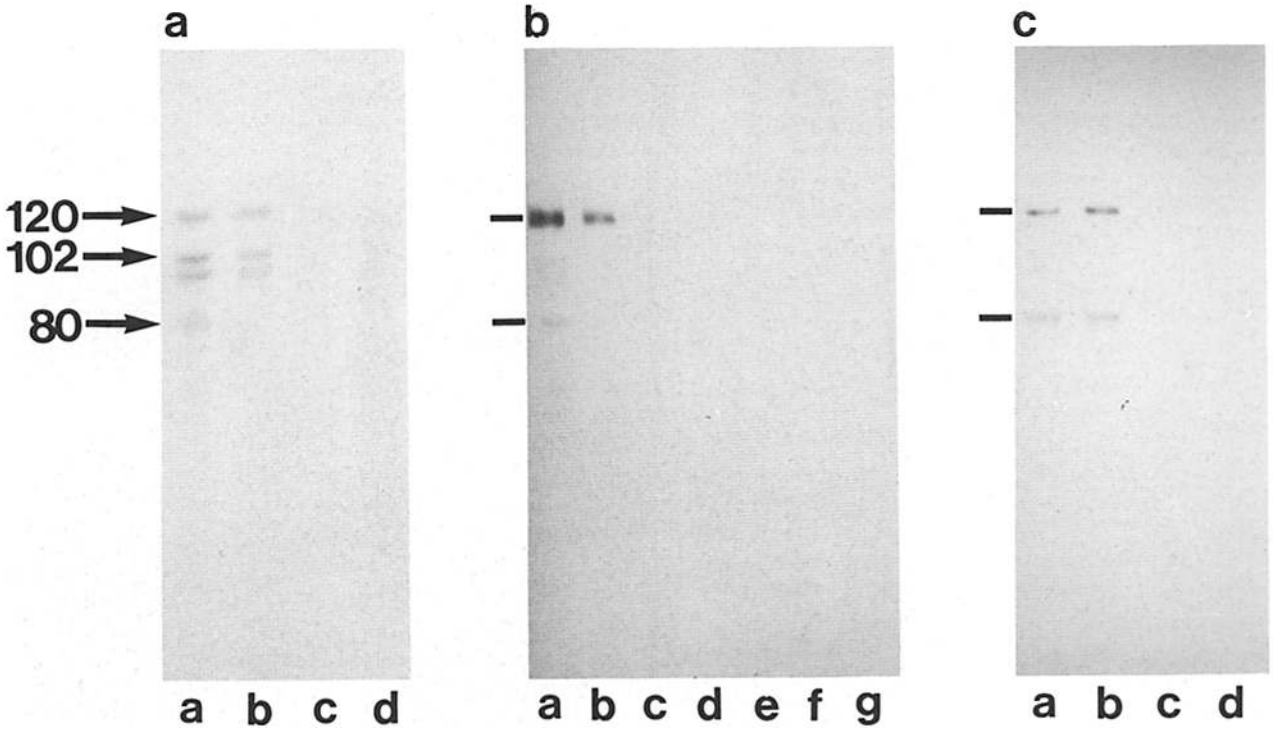


Figure 8. Biochemical comparison of uvomorulin expression in normal and transformed MDCK cells. (*a*) Immunoprecipitation of uvomorulin from [^{35}S]methionine-labeled nontransformed (lane *a*, MDCK-1; lane *b*, MDCK-2) and transformed MDCK cells (lane *c*, MSV-MDCK; lane *d*, ras-MDCK). The cell lines are designated as in Fig. 6 (see also text). Uvomorulin (at 120 kD), the associated components (e.g., at 102 kD), and the 80-kD uvomorulin digestion fragment are indicated with arrows. (*b*) Western blot analysis of uvomorulin in cell extracts of nontransformed (lane *a*, MDCK-1; lane *b*, MDCK-2) and transformed MDCK cells (lane *c*, fibroblastic-4; lane *d*, fibroblastic-5; lane *e*, fibroblastic-6; lane *f*, fibroblastic-1; and lane *g*, fibroblastic-2). Only the 120-kD component of uvomorulin and some 80-kD digestion product are detectable and are indicated with bars. (*c*) Western blot analysis of uvomorulin in cell extracts of (lane *a*) epithelial-1, (lane *b*) epithelial-2, (lane *c*) MSV-MDCK, and (lane *d*) ras-MDCK cells.

cell–cell contacts (Warren and Nelson, 1987). Garcia et al. (1986) have obtained an immortalized cell line after injecting SV-40 DNA into primary rabbit epithelial cells, and these retained some of the original differentiation markers (e.g., tight junctions). However, when SV-40–DNA plus activated *ras* DNA was injected, fully transformed cells were produced, which then became elongated, multilayered, and lost their tight junctions. Furthermore, Rijsewijk et al. (1987) transfected mammary epithelial cells with the *int*-1 oncogene and obtained fibroblastic cells which were tumorigenic (cf. also Brown et al., 1987). No analysis of the relevant cell–cell adhesion systems was performed in these two studies. It will therefore be crucial to examine whether the above mentioned and other oncogenes in fact transmit their transforming message to L-CAM/uvomorulin–type adhesion systems, and on which level they may block the expression of these key molecules.

Table I. Histological Evaluation of Confronting Cultures between Embryonic Chick Heart Fragments and Transformed or Nontransformed MDCK Cell Lines

	‡Grading after		
Cell type*	3 d	7 d	14 d
MDCK-2	I or II	I	I or II
Epithelial-1	I or II	II	ND
ras-MDCK	III	IV	IV
Fibroblastic-1	III	IV	IV
Fibroblastic-3	III or IV	IV	IV§

The grading was performed according to Bracke et al. (1984). Grade I, confronting cells surrounded the heart fragment with a layer of fibroblastic cells separating them from the core of cardiac muscle; grade II, confronting cells surrounded the heart fragment and were apposed to the cardiac muscle; grade III, confronting cells occupied and/or replaced less than half of the heart tissue; and grade IV, confronting cells occupied and/or replaced more than half of the heart tissue. Grades I and II are considered as no invasion, grade III and IV as invasion.

* The cell lines are designated as in Fig. 6.

‡ Three confronting cultures were examined to obtain one data point.

§ Central necrosis due to excessive proliferation of the MDCK cells was observed in this particular case.

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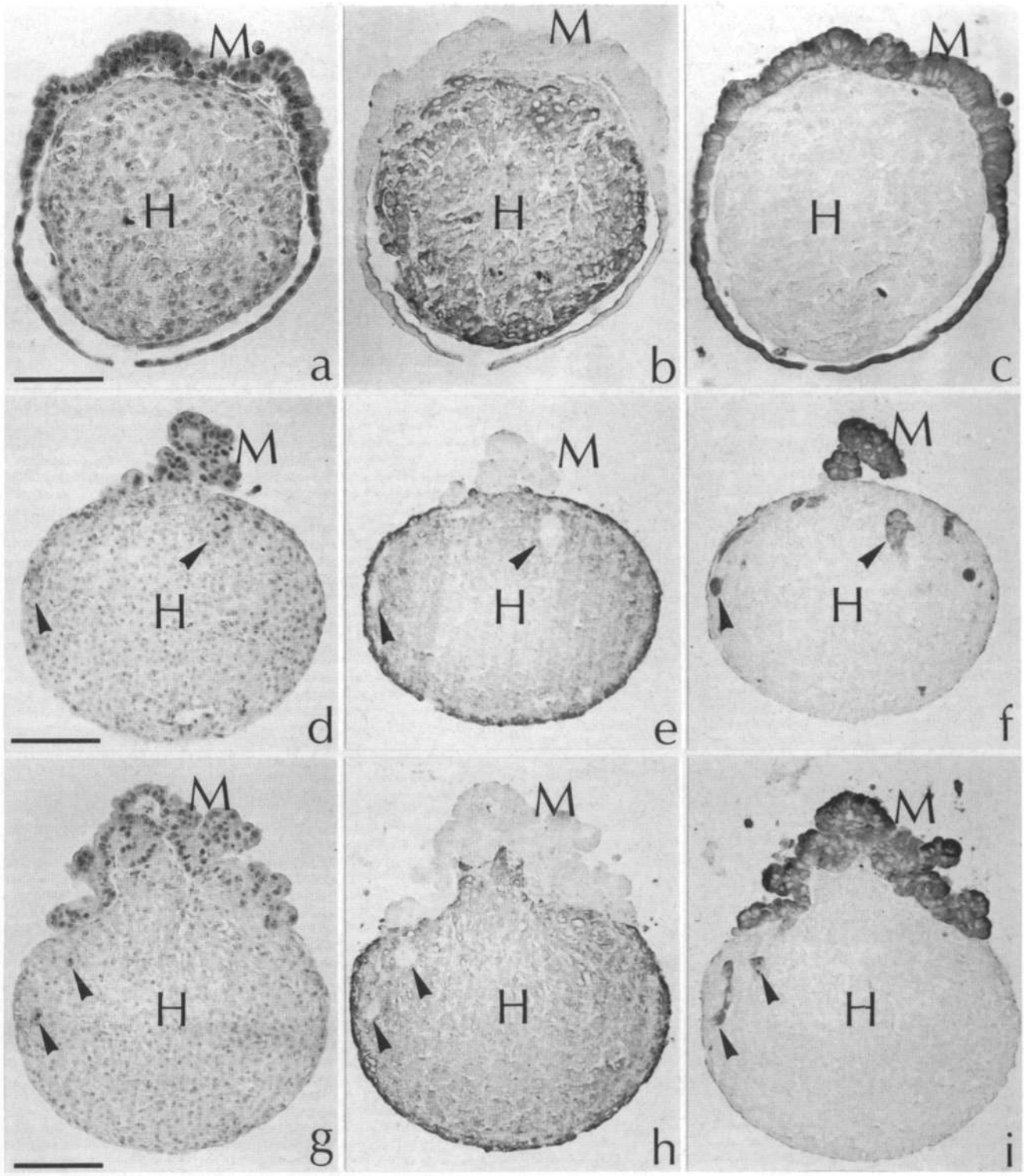

Figure 9. Invasion of chick heart tissue after treatment of MDCK cells with anti-uvomorulin antibody. Sections of 4-d-old confrontation cultures between nontransformed MDCK cells and embryonic chick heart tissue are shown. The cultures were either incubated in the absence (*a–c*) or in the presence of 70 μg/ml anti-Arc-1 monoclonal antibody (*d–i*). Consecutive sections were stained either with hematoxyline-eosine (*left*), anti-chick heart antiserum (*middle*), or anti-MDCK cell antiserum (*right*). *M*, the MDCK cells; *H*, heart tissue. Arrowheads in *d–i* mark invasive MDCK cells after anti-Arc-1 treatment. Bars, 100 μm.

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