Inactivation of Retinoblastoma Family Proteins by SV40 T Antigen Results in Creation of a Hepatocyte Growth Factor/Scatter Factor Autocrine Loop Associated with an Epithelial-Fibroblastoid Conversion and Invasiveness

Cécile Martel, Francis Harper, Silvia Cereghini, Veerle Noé, Marc Mareel, and Chantal Crémisi

CJJF 94-02, Université René Descartes, 45 Rue des Saints-Pères, 75270 Paris 06, France [C. M., C. C.]; Laboratoire: Organisation Fonctionnelle du Noyau, Centre National de la Recherche Scientifique, UPR 9044, Villejuif, France [F. H.]; Institut National de la Santé et de la Recherche Médicale U423, Hopital Necker Enfants Malades, Paris Cedex 15, France [S. C.]; and Laboratory of Experimental Cancerology, University Hospital, B-9000 Ghent, Belgium [V. N., M. M.]

Abstract

SV40 T antigen (LT) is an oncoprotein that inactivates nuclear regulators such as retinoblastoma (RB) family proteins and p53. We recently reported that in Madin-Darby canine kidney (MDCK) epithelial cells the binding of LT to RB family proteins results in a massive apoptosis and a concomitant down-regulation of c-myc. Here, we show that LT causes loss of epithelial differentiation and induces invasiveness. MDCK cells expressing wild-type LT, but not mutants unable to bind RB, exhibit a fibroblast-like morphology, show a strong down-regulation of the vHNF1 transcription factor and acquire invasive properties. The stable retransformation of MDCK(LT) with a RB and/or c-myc-expressing vector restores the expression of epithelial characteristics. Our data therefore suggest an important role for RB and c-myc in modulating the epithelial phenotype both during normal tissue development and in invasive processes. In addition, when grown in collagen gels, the MDCK(LT) cells form branching tubules, and their conditioned media produce the scattering of monolayer cultured MDCK cells. These last properties are reminiscent of those induced by hepatocyte growth factor/scatter factor (HGF/SF). Moreover, the HGF/SF protein was detected by Western blotting in the MDCK(LT)-conditioned medium. The production of HGF/SF is specifically induced by LT-RB inactivation, because Ras

transformation of MDCK cells fails to induce the production of this factor. These results demonstrate that inactivation of RB family proteins in these cells is at the origin of a HGF/SF autocrine loop.

Introduction

Epithelial cells are at the origin of 90% of all human tumors (1), and they also form the early vertebrate embryo. In the adult, all tissues and cells, including mesenchymal cells, are derived from this embryonic epithelium. Epithelia are characterized by an extraordinary degree of plasticity. Several organs, such as nervous system, kidney, ovary, and prostate, undergo epithelial-mesenchymal conversion during their formation; i.e., the cells no longer express epithelial characteristics, such as cell polarity or cell adhesion molecules, including cadherin, desmoplakin, catenin, and keratin-containing intermediate filaments. Instead, they acquire mesenchymal properties and become elongated, fibroblast-like, and motile (2). Similar processes also occur during tumor progression, i.e., invasion and metastasis of carcinoma cells. The malignant mesenchyme-like cells then break through the basement membrane and invade the underlying mesenchyme (3).

DNA tumor viruses, adenoviruses, herpes viruses, papilloma viruses, Polyoma, and SV40 all show varying degrees of epithelial tropism. For example, papilloma viruses grow exclusively in vivo and ex vivo in keratinocytes. Adenoviruses and SV40 can express some of their genes ex vivo in non-epithelial cells and eventually transform them. SV40 virus is a latent virus in rhesus monkey kidney, and its oncoprotein, LT, has been widely used to transform mainly rodent fibroblasts in vitro. A few primary differentiated cells have been transformed by Polyoma and SV40 to obtain information about the effect of transformation on differentiated functions or to establish permanent lines of the cells themselves. In most cases, the differentiated phenotype was conserved after transformation (4). However, permanent nonpermissive epithelial cell lines have rarely been transformed by SV40. In a recent study, we transformed a nontumorigenic differentiated epithelial cell line of kidney origin with SV40 LT (5). This MDCK cell line was chosen because, in addition to these characteristics, it has a specific origin, it was derived from

1 The abbreviations used are: LT, large T antigen; MDCK, Madin-Darby canine kidney; RB, retinoblastoma; CM, conditioned medium; HGF/SF, hepatocyte growth factor/scatter factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2 To whom requests for reprints should be addressed, at CJF 94-02, 45 Rue des Saints-Pères, 75270 Paris 006, France. Phone: 33 (1) 01 42 86 20 77; Fax: 33 (1) 01 42 86 33 06.

Received 8/8/96; revised 12/2/96; accepted 12/16/96. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
the distal tubule (6), and its epithelial properties have been characterized extensively. MDCK cells, therefore, represent an ideal model system to study the effect of a nuclear oncoprotein on epithelial properties. MDCK cells have been transformed previously by membrane and cytoplasmic oncogenes such as v-src (7, 8), Moloney and Harvey sarcoma virus (9), and K-ras (10), which leads to specific alterations in their intracellular adhesion system.

It is thought that oncoproteins such as LT exert their transforming properties by binding and inactivating nuclear regulators, including RB and p53 (11). RB deletions and mutations have been associated with several human tumors (11). In cell culture, RB acts in some cell types, and especially in tumor cells, as a negative regulator of cell growth (12), but it is also involved in vivo and ex vivo in hematopoietic, nervous, and muscular differentiation processes (13–17). Moreover, it was recently found to act in some cell types such as MDCK as a survival gene (5, 18, 19). For these reasons, we transformed MDCK cells with a mutated LT unable to bind to RB and/or RB-related family proteins.

In a previous study, we reported that LT transformation of MDCK cells induces a massive apoptosis at high cell density (5). Here, we show that the specific inactivation of RB family proteins results in an epithelial-mesenchymal conversion associated with invasiveness. These modifications induce the production of HGF/SF in the CM of MDCK(LT) cells, which causes the scattering of the parental MDCK and the formation of branching tubules when the MDCK(LT) cells are grown in collagen gels. Stable retransformation of MDCK(LT) cells with a RB- or c-myc-expressing vector partially restored cell viability (5), along with reexpression of some epithelial markers and epithelial morphology. Our results suggest that RB and c-myc play a crucial role in regulating the plasticity of the epithelial phenotype in both normal and pathological situations.

Results

LT Expression Causes Conversion to a Fibroblastoid Morphology. We have established previously individual clones of MDCK cells expressing either wild-type SV40 LT or nontransforming mutants unable to bind to RB family proteins, but which still inactivate p53 (5). LT expression dramatically altered the phenotype of MDCK cells (Fig. 1). All MDCK(LT) lines, such as MDCK(1-6) cells, lost the epithelial morphology and became elongated and spindle shaped, like fibroblasts (Fig. 1, b and c). In contrast to parental MDCK cells, which at low density formed small characteristic compact cell islets (Fig. 1a), the MDCK(1-6) cells grew individually, which is typical of mesenchymal cells (Fig. 1b; see also Fig. 3C, cytokeratin). Even at high density, the MDCK(1-6) cells retained their elongated shape (Fig. 1c). Although the medium was changed before taking the picture, round apoptotic cells can be observed (Fig. 1c). Indeed, 40–50% of the cells died by apoptosis at confluence (5). The same altered morphology was observed for all other clones transformed by LT, including MDCK(1-1) cells. In contrast, the MDCK(2a4) and MDCK(2a5) cells transformed by a LT mutant unable to bind to RB were morphologically indistinguishable from the parental MDCK cells (Fig. 1d). Electron micros-copy studies confirmed all of these observations, including the similarity between MDCK cells and MDCK(2a4) cells. MDCK(2a4) cells, like the MDCK cells, retained the apico-basolateral polarized morphology of epithelial cells interacting laterally by desmosomes and tight junctions (compare Fig. 2, a and d). In contrast, the MDCK(1-6) cells totally lost the apico-basolateral polarity. The membranes were completely smooth without microvilli, they were not closely apposed, and they no longer formed desmosomes and tight junctions (Fig. 2, b and c). The cells were particularly elongated, nucleus included. Thus, the specific inactivation of RB family proteins by LT in MDCK epithelial cells resulted in the development of a novel cell type with dramatic morphological changes.

Down-Regulation of Expression of Epithelial-specific Markers in LT-expressing Cells. Western and Northern blotting and immunofluorescence were used to further investigate the expression of epithelial-specific markers involved in cell-cell adhesion and cytoskeletal organization. Since the cell-adhesion molecule E-cadherin plays a crucial role in the establishment of epithelial polarity and mediates strong intercellular adhesion (reviewed in Ref. 20), we determined its expression in the different MDCK clones by Western blotting using the DECMA-1 antibody. Fig. 3 shows a strong down-regulation in the amount of E-cadherin in the two LT-expressing clones MDCK(1-6) and MDCK(1-1), whereas its level in the two LT mutant-expressing lines MDCK(2a4) and MDCK(2a5) was comparable to the parental MDCK cells. This result was confirmed by immunofluorescence (data not shown). Expression of desmosomal proteins such as desmoplakin and desmoglein was investigated by immunofluorescence using a mixture of two monoclonal antibodies.
against these proteins. In parental MDCK cells, the desmosome-specific proteins were typically confined to the plasma membrane in punctuate arrays along lateral cell-cell boundaries (Fig. 3C), whereas in MDCK(1-6) cells there was a disappearance of immunoreactivity from the cell periphery (Fig. 3C). Because cytoskeletal organization plays a major role in the morphology of differentiated epithelial cells, cytokeratin RNA expression and cytokeratin fiber formation were analyzed by Northern blotting and immunofluorescence, respectively. Fig. 3A shows in MDCK(1-6) and MDCK(1-1) cells a strong inhibition of mRNA homologous to murine cytokeratin endoA, in contrast to the MDCK(2a4) and MDCK(2a5) clones transformed by mutated LT. In contrast, mRNA expression of the mesenchymal marker vimentin was specifically induced in LT-expressing MDCK(1-6) and MDCK(1-1) cells (Fig. 3A). In MDCK cells, immunofluorescence with the TROMA-1 antibody stained cytokeratin fibrils extending from the cell surface to the perinuclear region; in some cases, it was possible to distinguish the intermediate filaments containing cytokeratin anchored to the desmosomes. In MDCK(1-6) cells, overall immunostaining was much lower, and the fibrils formed a disorganized cytoplasmic network that had apparently lost its membrane anchorage.

All of these results suggest that RB and/or RB-related protein inactivation by LT specifically induced a mesenchyme-like conversion in immortalized epithelial MDCK cells.

The Mesenchyme-like Conversion Is Correlated with Down-Regulation of the Transcription Factor vHNF1.

Mesenchyme conversion is likely to be accompanied by changes and/or down-regulation of some of the transcription factors participating in the establishment of an epithelial
phenotype. The expression of the vHNF1 homeoprotein is restricted to epithelial tissues and is highly expressed in the kidney (reviewed in Refs. 21 and 22). Since LT expression in MDCK cells resulted in a mesenchymal-like conversion, we wanted to determine whether this change was accompanied by a decrease in the expression of vHNF1. Therefore, we first examined vHNF1 expression at both the mRNA and protein levels by Northern and Western blot analysis. Monkey kidney CV1 cells, as well as COS-1 cells, the LT-transformed derivative, were also included in these analyses. Fig. 4, A and B, shows that the levels of both vHNF1 mRNA and protein were similarly decreased in COS-1 compared with CV1, and in both MDCK(1-6) and MDCK(1-1) LT-expressing cells compared with MDCK. It is interesting that no decrease was observed in MDCK(2a4) and MDCK(2a5) cells expressing the mutated version of LT. We have also observed a similar decrease in the vHNF1-active DNA-binding protein by performing gel retardation experiments with a double-stranded oligonucleotide encompassing the HNF1 binding site present in the albumin promoter and specific anti-vHNF1 antibodies (Fig. 4C). Addition of vHNF1 antibody resulted in the complete displacement of the retarded complex and subsequent formation of the indicated supershifts (Fig. 4C). Addition of preimmune serum did not affect the mobility of the vHNF1 complex (not shown). Thus, the changes induced by LT transformation also involved down-regulation of the vHNF1 transcription factor specifically expressed in epithelial cells. Since N-myc is also involved in the mesenchyme conversion (23), its expression was examined. However, very low levels of N-myc mRNA were detected in MDCK and in MDCK(LT) cells (data not shown).

Invasiveness of MDCK(LT) Cells in Vitro. As mentioned previously, mesenchyme conversion occurs during both development (2) and tumor invasion (3). Because the LT oncogene was at the origin of this mesenchyme-like conversion, it was important to determine the potential invasive behavior of these cells. This was investigated by: (a) invasion in collagen gels; (b) invasion of embryonic chick heart fragments; and (c) protease secretion. Fig. 5, A and B, shows that MDCK(1-6) cells exhibited a remarkable induction of inva-
Down-regulation of vHNF1 in wild-type LT-expressing cell lines. A, Northern analysis. Cytoplasmic RNA was extracted from cycling CV1 cells (CV1), from COS cells, CV1 transformed by LT (COS), from MDCK cells (MDCK), MDCK(LT) cell clones (1-6 and 1-1), and from MDCK cells transformed by a LT mutant unable to bind RB (clone 2a4), then analyzed by Northern blot and probed with vHNF1 and rat GAPDH. B, Western blot analysis. Total protein samples were extracted from the same cells as in A and in addition from a second MDCK clone transformed by mutated LT (2a5), fractionated on 7.5% SDS polyacrylamide gels, and analyzed by Western blotting using vHNF1 antibody. C, decrease in vHNF1 binding activity in MDCK(LT) cells. Nuclear extracts were prepared from the indicated cell lines. Gel electrophoretic mobility shift assays were carried out using as probe double-stranded PER56 oligonucleotide encompassing the HNF1 binding site of the rat albumin promoter (22).

The activity of proteinase secreted from MDCK-LT-expressing cells was analyzed using gelatin zymography of the culture medium of subconfluent cells. Electrophoresis of the culture medium from MDCK(1-6) cells through polyacrylamide gels containing SDS and type A gelatin detected a major band at M, 62,000, which was absent from the culture medium from parental MDCK cells (Fig. 5C). These data show that the LT-induced mesenchyme-like conversion is accompanied by the acquisition of highly invasive properties.

MDCK(LT) Cells Constitutively Produce the HGF/SF Factor, Which Induces Morphogenesis and Scattering Activities. When grown within three-dimensional collagen gels, in contrast to parental MDCK cells, which form cysts (Ref. 24 and Fig. 6g), the MDCK(1-6) cells formed tubular-like structures. Within the collagen gels, the MDCK(1-6) cells very rapidly became elongated and oriented toward one another, forming long, thin structures that gave rise after 4–5 days to tubular-like structures (Fig. 6, a–f). Thin sections of branching structures formed by MDCK(1-6) into collagen and further electron microscopic observations confirmed the tubular nature of these structures and the presence of an extended lumen as observed by phase-contrast microscopy. Moreover, these sections revealed that the cells lining these tubules recovered polarity with some apical microvilli and junctional complexes (Fig. 7). Apoptotic cells were sometimes present inside the lumen. The analysis of MDCK cyst sections revealed a round, central lumen surrounded by cells with a more pronounced polarity and numerous apical microvilli and junctional complexes (data not shown). Since invasive behavior can be induced in MDCK cells by other oncogenes, we compared the growth within collagen of cells, such as the fibroblastic MDCK-ras-f cells (9). Under the same experimental conditions, these cells never formed such tubular structures, or even cysts, after prolonged growth in collagen. They grew isolated with a characteristic morphology: an extremely long, thin and sharp cytoplasmic tail on one end, and at the other a small swelling enclosing the nucleus, giving them a comet-like appearance (Fig. 6, h and i). Thus, this tubular structure was specifically generated by RB inactivation. The MDCK(2a4) cells transformed by mutated LT did not form either structure (data not shown).

It is interesting that the CM of MDCK(1-6) cells induced the scattering effect of MDCK cells (Fig. 8Ab), as did the CM of the MRC5 mesenchymal cells containing HGF/SF (Ref. 25; Fig. 8Ac). This scattering effect of MRC5 CM was previously shown to be mediated by the tyrosine c-met receptor (26). To determine whether the scattering effect of MDCK(1-6) CM on MDCK was also mediated by a tyrosine kinase, a 50-μM concentration of the tyrosine kinase inhibitors genistein and/or tyrphostin was added to MDCK(1-6) CM. Fig. 8Ad shows that tyrphostin completely inhibited the scattering effect; the same inhibition was observed with genistein (data not shown). Similar results were obtained with HGF/SF-containing medium produced by MRC5 cells (data not shown).

Since all of these properties are reminiscent of those of HGF/SF, we performed immunoblotting using a polyclonal
human antibody (a kind gift of Dr. Nakamura) to test the expression of the HGF/SF protein in the MDCK(1-6) CM, either after concentration through a Centricon microconcentrator (Amicon, Danvers, MA) or binding on heparin-Sepharose, because it was shown previously that this factor has a high affinity for heparin (27). Fig. 8B shows that, in both cases, the antibodies against human HGF/SF clearly recognized in the MDCK(1-6) CM the three characteristic protein species of HGF/SF: the M, 92,000 uncleaved form, which can be converted into the M, 62,000 and M, 34,000/32,000 subunits (27). Some protein contaminants were also recognized by the human antibody in the MDCK(LT) CM (Fig. 8B, Lane 2). However, the large majority of them disappeared after further purification on heparin-Sepharose (Fig. 8B, Lane 3, and Ref. 27). The size of the first subunit was found to be slightly higher in the MDCK(1-6) CM than in the MRC5 CM and the recombinant human protein, possibly reflecting either a posttranslational modification (27) or a species difference; the canine HGF/SF protein might be slightly different from the human protein. These results unambiguously identified HGF/SF in the MDCK(LT) CM. However, these antibodies could not neutralize the scattering effect of the MDCK(1-6) CM, whereas this effect was abolished when we used MRC5 CM (data not shown), probably reflecting a lack of antibody specificity for neutralizing a biological activity in canine cells (data not shown). Thus, LT transformation of MDCK cells induces the constitutive expression of HGF/SF, which can exert morphogenesis and scattering activities depending on the experimental conditions.

Fig. 5. Invasiveness of MDCK(1-6). A, invasion of MDCK(1-6), MCF-7/AZ, and DHD-FIB into collagen type I. Invasion index, number of cells invaded into the collagen gel:total number of cells (on top of the gel + invaded) counted in 15 microscopic fields after 24 h (□) and 48 h (□); bars represent mean ± SD. Invasion of MDCK(1-6) and DHD-FIB cells is significantly higher (P < 0.005) than invasion of MCF-7/AZ cells. Statistical analysis was based on Student’s t test. B, invasion of chick heart tissue by MDCK(1-6) cells. Histological sections from 4-day-old confronting cultures between MDCK(1-6) and chick heart fragments. a was stained with H&E, and b was stained with an immunohistochemical technique to reveal chick heart antigens. Bar, 50 μm. C, gelatin zymography of culture medium of MDCK cells transformed by LT: MDCK, parental MDCK; 1-6, clone MDCK(1-6).
Partial Reexpression of Epithelial Characteristics in MDCK(LT) Cells by RB and c-myc Gene Transfer. Our results suggested that LT-mediated mesenchyme-like conversion was due in part to RB inactivation, because the use of SV40 LT mutants unable to bind RB or a RB-related protein failed to convert MDCK cells. To further establish this fact, we analyzed previously isolated MDCK(1-6) clones retransformed with a human RB-expressing vector (5). We had observed a correlation between RB inactivation, loss of the epithelial phenotype, and strong down-regulation of c-myc expression (5). To investigate whether this correlation was fortuitous or linked to the mesenchyme-like conversion in these cells, we analyzed MDCK(1-6) cells retransformed with a human c-myc expression vector (5). In all of the selected clones, the expression of LT was examined (data not shown). Cell morphology, cell polarity, and intercellular contacts were analyzed by phase and electron microscopy studies. The reexpression of some epithelial-specific markers was inves-
Fig. 8. A, scattering effect of MDCK(1-6) CM on parental MDCK cells cultured in standard medium. a, MDCK cells cultured in standard medium; b, MDCK cells cultured in MDCK(1-6) CM; c, MDCK cells cultured in HGF/SF-containing MRC5 CM; d, MDCK cells cultured in MDCK(1-6) CM with 50 μM tyrphostin. Same effect was observed with 50 μM genistein. All photographs were taken 24 h after plating. Bar, 60 μm. B, detection of the HGF/SF protein in MDCK(1-6) conditioned medium by Western blotting using a rabbit antiserum against human recombinant HGF/SF. Lane 1, human recombinant HGF/SF protein. Lane 2, MDCK(1-6) CM concentrated in a Centricron 10 microcentrifuge. Lane 3, proteins from MDCK(1-6) CM eluted from heparin-Sepharose. Lane 4, proteins from MRC5 CM eluted from heparin-Sepharose. The uncleaved form M, 92,000 and the two M, 62,000 and 34,000/32,000 subunits of HGF/SF are indicated by black arrows. The corresponding M, 62,000 subunit from MDCK(1-6) CM is slightly higher and is indicated by the dotted arrow.

Discussion
Conversions between epithelial cells and mesenchymal cells occur in normal conditions during development (2) and in pathological conditions during tumor invasion (3). The molecular understanding of the regulatory mechanisms involved in these interconversions is an important goal of cellular biology. Our results indicate that two nuclear regulators, RB and/or RB-related proteins and c-myc, play a crucial role in the maintenance of the epithelial phenotype. The use of LT mutants allowed us to show that specific RB inactivation by LT transforms nontumorigenic epithelial MDCK cells into highly invasive fibroblastoid cells with the creation of a HGF/SF autocrine loop. These changes were concomitant with a massive apoptosis at high cell density and a strong down-regulation of c-myc (5). Moreover, the restoration of RB expression in these MDCK(LT) cells by RB gene transfer again induced a strong morphological change, but in this case toward an epithelial phenotype with reexpression of some epithelial-specific markers. These changes were now concomitant with an increase in cell viability and high c-myc expression (5). C-myc gene transfer induced similar modifications, although to a lesser extent than RB, suggesting that the RB effects we observed might be mediated at least partly by c-myc up-regulation, because, in addition, all RB clones reexpressed high levels of c-myc (this study and Ref. 5).

RB Inactivation Induces Dedifferentiation of Epithelial Cells. Together with our previous study (5), our results show a correlation between the maintenance of the epithelial state and cell viability. Loss of the former may result in loss of the latter. RB family proteins and, to a lesser extent, c-myc appear to be at the crossroads between these two related processes in MDCK epithelial cells. Our results and interpretation are consistent with other recent investigations performed in vivo and ex vivo. In RB knockout mice, aberrant differentiation of erythrocytes and neurons occurred with apoptotic death (13-16). Specific inactivation of RB by the...
Fig. 9. Electron microscopy analysis of MDCK(1-6) cells re-transformed by a RB- or c-myc-expressing vector. Tangential sections: a, parental MDCK(1-6) cells; b-e, MDCK(1-6) cells re-transformed by a RB-expressing vector, clone 1-6(RB22). Perpendicular sections: e, 1-6(RB22); f, MDCK(1-6) cells re-transformed by a c-myc-expressing vector, clone 1-6(M23). Parental cells (a) display a smooth cell surface. No desmosomes or tight junctions were observed between neighboring cells. In contrast, 1-6(RB22) cells in b-e displayed microvilli (stars), desmosomes (arrows), and desmosome-like structures (arrowheads), some of them being developed over a long membrane surface (c). Similar cell organization was observed in 1-6(M23) cells (f), which also exhibited microvilli (star) and desmosomes (arrowhead). Bar, 1 μm.

Viral oncoprotein E7 targeted in the retinas of transgenic mice prevented terminal differentiation, and cells instead underwent apoptosis (28, 29). Similar processes occur in mouse embryonic carcinoma cells, in which the absence of functional RB during differentiation into neuroectoderm results in apoptosis (30). Thus, in all of these cases, similar to our findings, the absence of a functional RB and/or RB-related protein interfered with a differentiation program and resulted in apoptosis.

However, the role of RB in epithelial differentiation had not been firmly established previously, although it was predictable by a recent study showing the highly specific expression pattern of RB protein during embryogenesis (31). In several tissues, RB protein is largely confined to differentiated epithelial cells. In the squamous epithelia, skin, and buccal epithelium, RB is detected in the more highly differentiated layer. A similar differentiation-dependent pattern of RB expression is found in the mucous membranes of the digestive tract, where RB is expressed exclusively in the differentiated enterocytes. In addition, RB is highly expressed in kidney-collecting tubules. Thus, our conclusions are consistent with all of these examples illustrating high RB expression in differentiated epithelia. Moreover, we found that an epithelial-specific marker, the cytokeratin endoA promoter, was tran-
mice died early from hematopoietic and neuronal problems before other defects could be detected elsewhere (13-15).

**Participation of c-myc in the Epithelial Phenotype.** We showed here that c-myc also participated in the maintenance of the epithelial phenotype. C-myc is thought mainly to participate almost exclusively in cell proliferation, and it is believed that the activation and/or deregulation of this function is at the origin of its role in tumorigenesis. However, several studies have suggested that c-myc might have another, antagonistic function. C-myc expression was shown to be highly and constitutively expressed in several quiescent epithelial cell lines (32, 33) and remained high during keratinocyte differentiation (32), and was induced during differentiation of PC12 (34) and lens cells (35). In addition, the cytokeratin genes endoA and endoB (36), together with the plasminogen activator inhibitor 1 (37), which prevents the degradation of extracellular matrix components and thus preserves the interactions with epithelial cells, were found to be activated by a wild-type c-myc construct. The correlation between c-myc and the epithelial phenotype was strengthened by the results obtained with MDCK-ras-f and MDCK-ras-e cells (9). We found that c-myc expression remained high in noninvasive, differentiated epithelial cells and was low in dedifferentiated invasive fibroblast-like cells. Moreover, the synthesis of c-Myc1, the larger Myc protein, was shown to be increased at high cell density (38) and to inhibit cell growth (39). Therefore, it was suggested that the two Myc proteins would have opposing functions (39). In addition, Myc 1 was demonstrated to activate transcription through a CAAT/enhancer-binding protein sequence, suggesting that the transcription of genes containing this sequence and the differentiation processes where they are involved may be positively regulated by c-myc (39). Our findings are also supported by an earlier report showing that a wild-type c-myc was able to reverse the neu-induced transformed phenotype (40).

It is interesting that the 12S E1A viral adenovirus oncogene was also shown to reverse the transformed phenotype of human tumors and to induce the expression of epithelial characteristics in these cells (41, 42). In addition, it can maintain the epithelial phenotype of primary epithelial kidney cells and rescue them from apoptosis (43), like c-myc. These results are particularly important because they contribute additional data to the functional similarities between E1A and c-myc. E1A, like c-myc, codes for two proteins of 289 and 243 amino acids displaying overlapping and antagonistic functions (44).

**Induction of HGF/SF Expression and Activity.** In addition to being highly invasive in collagen gels and chick heart, the MDCK(1-6) cells formed tubular structures when grown within three-dimensional collagen gels. To date, only one factor has been shown to induce such morphological changes in MDCK cells in these experimental conditions, the HGF/SF factor (24, 45). HGF/SF is a pleiotropic factor secreted by mesenchymal cells and known to act essentially in a paracrine fashion, because in addition to branching morphogenesis, it induces epithelial cell movement (25), proliferation (46), and also increases invasiveness (27, 47). HGF/SF causes MDCK cells to dissociate from one another

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*Fig. 10.* A and B, epithelial-specific marker expression in 1-6(RB) and 1-6(c-myc) clones. A, Northern analysis. Cytoplasmic RNA was extracted from MDCK cells (MDCK), MDCK(1-6) cells (1-6), 1-6 cells transformed by the hygromycin vector (Hg), 1-6 cells transformed by a RB-expressing vector, clone 1-6(RB22) (RB22), clone 1-6(RB25) (RB25), clone 1-6(RB27) (RB27), and 1-6 cells transformed by a c-myc-expressing vector, clone 1-6(M23) (M23), clone 1-6(M32) (M32), then analyzed by Northern blot and hybridized with endoA and GAPDH probes. B, Western analysis. Total proteins were extracted from the same cells as in A, fractionated on 5% SDS gel polyacrylamide gels, and analyzed using the anti-E cadherin antibody DECMA-1. C and D, comparative c-myc expression in MDCK-ras-e and MDCK-ras-f. C, Northern analysis. Cytoplasmic RNA was extracted from MDCK-ras-e (M-ras-e) and MDCK-ras-f (M-ras-f), analyzed by Northern blot, and hybridized with c-myc and GAPDH probes. D, Western analysis. Total protein samples were extracted from the same cells as in C, fractionated on 5% SDS polyacrylamide gels, and analyzed using the anti-E cadherin antibody DECMA-1.

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*E. Batsché and C. Crémi s, unpublished data.*
under standard cell culture (25) conditions. Consistent with this property, we found that the CM of MDCK(1-6) cells induced the scattering of MDCK cells in vitro. All of the HGF/SF effects are mediated by a specific membrane receptor, the tyrosine kinase encoded by the c-met protooncogene usually expressed by epithelial cells (26, 48). We report here that the scattering effect of MDCK(1-6) CM is inhibited by two tyrosine kinase inhibitors (genistein and tyrphostin). Finally, we confirmed by Western blotting that the HGF/SF is expressed in the MDCK(LT) CM and specifically retained on heparine-Sepharose as described previously (27). All of these results demonstrate that LT expression in MDCK cells has created an autocrine loop of HGF/SF production. It is important to note that this activity was specific to MDCK(ras-f) cells and was absent in the invasive MDCK(ras-f) cells (9).

Our cell system remarkably illustrates the pleiotropic and somewhat antagonistic effect of the HGF/SF factor. On plastic substrates, i.e., in a two-dimensional culture, the expression of HGF/SF induced a mesenchyme-like conversion associated with dispersion and invasiveness, whereas in a three-dimensional culture when the cells were embedded into collagen gels, tubular structures reflecting its morphogenesis activity were observed (24). The MDCK(LT) cells recovered polarity and junctional complexes, which were previously lost, thus highlighting the extraordinary plasticity of epithelial-derived cells. By following cell movement and tubule formation under the microscope each day, we observed that the cells first started coming together and orienting toward one another and then, after growth for an additional few days, they compacted to form the wall of the tubule structure, eventually giving rise to cell polarity with induction of junctional complexes and lumen, reminiscent of what is observed during compaction of the morula in early development. Work is in progress to firmly establish this fact and to determine whether it is a multistep process and the precise kinetics of the molecular events.

Specificity of LT Effects. As mentioned in the "Introduction," several oncogenes, such as v-src (7, 8), H-ras (9), K-ras (10), and c-fos (49), have been used to transform MDCK and mammary epithelial cells. In all cases, they induced a mesenchyme-like conversion, but with a much less pronounced effect. LT expression in MDCK cells, i.e., in our case inactivation of the RB family proteins, induced a much broader pleiotropic effect creating a completely novel cell type. The morphological changes were more pronounced, and apico-basolateral polarity and microvilli were completely lost. Although the MDCK(1-6) cells were invasive like the MDCK(ras-f) cells (9), they additionally acquired a HGF/SF activity. It is interesting that our results show that the mesenchyme-like conversion is not limited to morphological changes and invasiveness, but also down-regulated the expression of the vHNF1 transcription factor, which is normally expressed exclusively in epithelial tissue and specifically highly in kidney (21). In addition, as mentioned above, the expression of nuclear regulators such as c-myc and c-fos, which is, respectively, usually very low or absent in mesenchymal cells, was repressed in MDCK(LT) clones (5). In an early study involving mammary epithelial cells (49), the mesenchyme-like conversion was linked to c-fos overexpression. The difference with our results might be explained by the fact that these mammary cells expressed very low levels of c-fos (49) and presumably c-myc, although c-myc expression was not explored, whereas our MDCK cells express a high level of both c-fos and c-myc (33). Thus, the inactivation of RB family proteins by the wild-type LT appears to have a very broad pleiotropic and specific effect on the loss of an epithelial-specific program.

Relation with Kidney Development. Our experimental cellular system seems to mimic some steps occurring during kidney development. Renal morphogenesis is characterized by two phenotypic conversions: (a) an epithelial-to-mesenchymal transition leading to the generation of mesenchymal nephron progenitors, followed by (b) a mesenchymal-to-epithelial transition leading to the generation of diverse nephron epithelial cell types (50, 51). Moreover, a large amount of normal cell death was shown to occur during these processes (52, 53). Metanephric mesenchyme is programmed for apoptosis, and conversion of mesenchyme to epithelium seems to require rescue from apoptosis and induction of differentiation (52). Our results are fully consistent with these reports, since ~50% of the mesenchyme-like MDCK(LT) cells died by apoptosis at confluence, and restoration of the epithelial phenotype was accompanied by a concomitant restoration of cell viability (5).

RB, c-myc, Epithelial Differentiation, and Tumor Progression. C-myc deregulation is associated with tumorigenesis (54), and c-myc is often overexpressed in differentiated epithelial tumors. We showed previously that overexpression of c-myc in human colon carcinoma cells is correlated with increased cytokeratin expression (55). Moreover, we and others have shown that c-myc in certain cellular contexts can act as a survival gene (5, 56). This property would explain why epithelial tumor cells continue to proliferate. They would have a selective advantage over other cells because they would be prevented from dying. If this c-myc function is lost through RB inactivation, for example, the result would be a loss of the epithelial phenotype and acquisition of invasive properties. We suggest that one of the important functions of RB and c-myc, whose inactivation might play an important role in tumor progression, is their role in maintaining the differentiated epithelial state.

Materials and Methods

Cells

MDCK (canine kidney), CV-1 (monkey kidney), and COS-1 (monkey kidney cells transformed by SV40 DNA) were obtained from the American Type Culture Collection (Rockville, MD). MCF-7/AZ, used as negative control in the collagen type I invasion assay, are human mammary carcinoma cells; DHD-FDB rat colon myofibroblasts (57) were used as a positive control. MRC5 (human lung fibroblasts) and the cell lines mentioned above were grown in DMEM (4.5 g glucose/liter) containing 10% FCS, except for MDCK, for which the medium contained 5% FCS. MDCK(LT) (1-1 and 1-6) are clonal derivatives of MDCK cotransformed either with a plasmid containing the SV40 early region (pSV T + I) and a neomycin resistance marker plasmid, or, for MDCK (2a4 and 2a5) clones, with nontransforming LT mutants unable to bind to the RB protein (pAT-D2H and pK1), as described previously (5). Immunocytochemical staining revealed that 96% of these cells were uniformly and strongly positive for LT. Only clones transformed with pAT-D2H were used in this study, but similar results
were obtained with pK1-MDCK-transformed cells. The clone MDCK(1-6) was chosen for further transformations either with a hygromycin resistance marker plasmid alone or in combination with a human RB-expressing vector, or a human c-myc CDNA-expressing vector. Hygromycin-resistant colonies (250 μg/ml) were isolated and tested for ectopic protein expression by Western blotting (5). All cells and serum were Mycoplasma free.

**RNA Isolation and Transfer**

Cytoplasmic RNA was isolated and then subjected to electrophoresis in 1.1% agarose gels as described (33) and transferred onto Hybond N* filters (Amersham) in 20 mM NaOH, then left for 5 h at room temperature. Two controls were routinely performed to verify equal loading of the RNA. Before loading, a 2-μg sample of the 40 μg of treated RNA was run on a separate gel, which was then stained with ethidium bromide (not shown). The transfers were also hybridized with a GAPDH probe as an internal control.

**Preparation of Hybridization-labeled Probes**

C-myc exon-3 is highly conserved and hybridizes with all species (54). Purified human exon-3 c-myc C片段 was labeled by the megaprime DNA labeling kit (Amersham) and hybridized overnight at 60°C in Church buffer. A 2.3-kb EcoRI fragment of the murine endoα pseudogene α and a 1-kb EcoRI fragment of human vimentin cDNA were also used. The pseudogene was preferred to avoid repetitive sequences present in the intron of the endoα gene. The endoα gene is homologous to the K8 human cytokeratin. Purified Pst fragment of rat GAPDH cDNA was used as control.

**Western Blot Analysis**

Exponentially growing or quiescent cells (10–20 × 10⁶) were harvested and lysed in the following buffer: 10 mM Tris-HCl (pH 6.8); 150 mM NaCl; 2 mM MgCl₂; 1 mM EDTA; 1% SDS; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride; and 10 μg/ml leupeptin, aprothinin, and pepstatin A. The mixture was boiled for 5 min, sonicated for 30 sec, and clarified by centrifugation at 10,000 × g for 10 min at 4°C. Protein concentration was determined by the Bradford method, after which an equal volume of 2× Laemmli sample buffer was added. After denaturation, proteins were fractionated on SDS-polyacrylamide gels, and Western blot analysis was performed by standard procedures and with the enhanced chemiluminescence Western blotting detection system (Amersham) using the monoclonal anti-E-cadherin DECMA-1 antibody (gift of R. Kemler). vHNF1-specific antisera was raised against residues 293-424 of mouse vHNF1 and fused to glutathione transferase in the pGEX3 bacterial expression vector (58).

**Nuclear Extracts and Gel Mobility Shift Assays**

Nuclear extracts from cell lines were prepared as described (59). Twelve μg of nuclear proteins were used in the DNA binding assays. Gel mobility shift assays were as described (22). Where indicated, vHNF1-specific polyclonal antibody diluted 1 in 10 in PBS + 1 mg/ml BSA was preincubated with nuclear extracts at room temperature for 10 min before adding the labeled probe. Double-stranded oligonucleotide encompassing the HNF1 binding site of the rat albumin promoter (PE56; Ref. 22) was 32P-labeled with T4 polynucleotide kinase.

**Immunofluorescence**

Cells were grown on coverslips. Fixation was with acetone:methanol 1:1 for 10 min at room temperature and 5 min at –20°C with cold methanol, followed by 5 min with acetone for desmosomal proteins. Anti-keratin antibody was monoclonal TROMA-I to murine endoα keratin homologous to the human K8 (gift of P. Brület), and antidesmosomal proteins were a mixture of monoclonal antibodies to desmoglein and desmoplakin I and II (Boehringer Mannheim).

**Assays for Invasion In Vitro**

- **Chick Heart Invasion Assay.** Cells in suspension were placed in contact with precultured embryonic chick heart fragments on top of a semisolid agar. After 24 h, the cocultures were transferred into 5-mI Erlenmeyer flasks with 1.5 ml of culture medium and incubated for 4 days at 37°C in 10% CO₂. After fixation and embedding, invasion was scored on serial histological sections as described previously (see 8).

**Collagen Type I Invasion Assay.** Cells were seeded on top of collagen type I gels, and invasion was scored on cultures after 24 and 48 h of incubation using a microscope with a computer-controlled stage (60). The score was expressed as an invasion index (number of cells inside the gel over total number of cells).

**Morphogenetic Activity**

The tubulogenic activity was assayed as described (24). MDCK and MDCK(1-6) cells (5 × 10⁵ cells/ml) were suspended in 1.2-ml collagen gels in 6-well plates, left for ~10 min at 37°C, then 1.5 ml of complete medium was added, and plates were photographed every day for 1 week.

**Gelatin Zymography**

Gelatinase activities secreted from MDCK and MDCK(1-6) cells were tested by zymography (61). Subconfluent cells grown in 60-mm dishes were washed with PBS and maintained in 5 ml of serum-free medium overnight. Samples (0.2 μg) of CM were electrophoresed at 4°C in a 10% SDS-polyacrylamide gel containing 1 mg/ml type A gelatin (Sigma). After washing in 10 mM Tris-HCl (pH 8.0) containing 2.5% Triton X-100 and conditioning in 10 mM Tris-HCl (pH 8.0), the gel was incubated overnight in 50 mM Tris-HCl (pH 8.0) containing 0.5 mM CaCl₂ and 1 μM ZnCl₂ at 37°C. Gelatinase activity was visualized by staining the gel in 1% Coomassie blue in 5% acetic acid and 10% methanol and destained to visualize unstained areas corresponding to zones of proteolysis.

**Preparation of CM, Scatter Activity Assay, and Binding to Heparin-Sepharose**

MRC5 and MDCK(1-6) cells were grown to subconfluence or confluence, at which time fresh depleted or complete medium was added. After a further incubation of 1–3 days, the CM was collected, centrifuged, and used immediately or stored at –20°C. MDCK cells were seeded in 24-well plates and incubated in 1 ml of complete medium (control) or in undiluted MDCK(1-6) CM or MRC5 CM diluted 1:4. In all cases, the CM was supplemented to a final serum concentration of 5%. When genistein or tyrothrin (50 μM) was used, it was added at plating. The scattering effect on MDCK cells was monitored by light microscopy after overnight incubation. For purification of HGF/SF, MRC5 and MDCK(1-6) CM were incubated at 4°C for 2 h with heparin-Sepharose beads equilibrated in 100 μM phosphate buffer (pH 7.0) and 0.5 mM NaCl. The heparin-Sepharose was then recovered by centrifugation and washed with the same buffer. A total of 50 μl of 2× Laemmli sample buffer were added and boiled for 10 min. After centrifugation, aliquots were fractionated on 7.5% SDS-polyacrylamide gels and analyzed by Western blotting using rabbit polyclonal antisera raised against human recombinant HGF/SF (a gift from Dr. T. Nakamura).

**Electron Microscopy**

Cell monolayers were fixed at 4°C with 1% glutaraldehyde in 0.1 M Sörensen phosphate buffer (pH 7.3). The monolayers were washed in phosphate buffer and post-fixed in 2% osmium tetroxide in 0.05 M Sörensen phosphate buffer. After 5 min of washing in water, cell monolayers were dehydrated in increasing concentrations of ethanol and embedded in situ in Epon 812. Cells were sectioned either parallel (tangential sections) or perpendicular (perpendicular sections) to the monolayer surface. Sections were then stained with uranyl acetate and lead in citrate before examination with a Philips 400 transmission electron microscope at 80 kV.

**Acknowledgments**

We thank Dr. T. Nakamura and Dr. R. Kemler for the generous gift of HGF/SF and E-cadherin protein and antibodies, Dr. J. Jouanneau for advice in collagen and heparin experiments and useful suggestions, Dr. E. Puvion for helpful discussions and V. de Mazon for help in zymography. We are very grateful to Drs. J. Jouanneau, P. Duprey, and G. S. Butler-Browne for critical reading of this manuscript.
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