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Suppression of tumorigenicity in an aggressive cervical carcinoma induced by protein zero, a nervous system IgCAM

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SUMMARY

In mammals, protein zero (P_0), a neural IgCAM, is expressed solely in the peripheral nervous system where it mediates self-adhesion of Schwann cell membranes as compact myelin is generated. We show that when P_0 is expressed in HeLa, a cervical carcinoma cell line, cells regain adhesion-mediated growth control, including the acquisition of contact inhibition and loss of anchorage-independent growth. Additionally, P_0-expressing HeLa cells lose the ability to invade an artificial matrix, which correlates with decreased secretion of matrix-degrading enzymes. Lastly, and of great interest, unlike the aggressively metastatic cell line from which they were derived, P_0-HeLa cells are neither tumorigenic nor metastatic when injected into athymic nude mice. By all these criteria, P_0 expression appears to efficiently suppress in the long term, the transformed state of this carcinoma cell line. N-cadherin and its intracellular partners plakoglobin, α- and β-catenin were significantly upregulated in the P_0-HeLa cells. It appears therefore that P_0 induces epithelialization and suppression of tumorigenicity in HeLa through the activation of the cadherin/catenin signaling systems. We conclude that the forced expression of bona fide adhesion molecules, such as P_0, may serve as 'upstream' inducers of an essentially dormant but undamaged adhesion program in carcinoma cells that ultimately triggers the re-acquisition of normal epithelial characteristics, thereby suppressing tumorigenicity. Therapeutically, it may be that intercellular adhesion, no matter how it is induced, may serve as a single master event that is able to induce reversion of the carcinomatous state.

Key words: Adhesion, Tumorigenicity, Cadherin, P_0, HeLa

INTRODUCTION

More than 85% of tumors in humans are carcinomas; that is to say they are derived from epithelia. Epithelia have certain fundamental features that distinguish them from other cell types. Typically, epithelial cells assemble tight junctions, adherens junctions and desmosomes at lateral cell borders. They grow in a contact-inhibited manner, and require matrix attachment for cell division. Epithelial cells lack invasive capabilities and so normally express low or null levels of matrixmetalloproteinases (MMPs), enzymes that at high levels in metastatic tumor cells compromise the integrity of the extracellular matrix, thereby facilitating invasion. Carcinomas to one extent or another lose the typical structural and functional characteristics of the epithelia from which they are derived. Cell to cell adhesion is disrupted, and the stereotypic array of junctions becomes disorganized and may disappear altogether (Birchmeier and Behrens, 1994). Additionally, they do not exhibit contact inhibition typical of epithelia and may grow uncontrollably. Carcinomas do not require attachment to the basement membrane for continuous cell proliferation; they are 'anchorage-independent' (Freedman and Shin, 1974). As some carcinoma cells become more aggressive, they secrete MMPs (Khokha and Denhardt, 1989) and, after breaking through the basement membrane, seed tumors at distant sites.

These properties are not exhibited as uniform characteristics of all carcinomas, nor are they necessarily immutable; instead, carcinomas may display a spectrum of these features. Thus, there is a transitional relationship between the intact epithelium and carcinomatous transformation. Many factors, soluble or membrane-associated, can drive differentiation in either direction. For example, P19 mouse embryonal carcinoma cells are pluripotent and can differentiate into neuroectodermal, mesodermal or endodermal cell types depending upon treatment with retinoic acid or activation/inactivation of specific transcription factors (Oulad Abdelghani et al., 1996; Suzuki et al., 1996). There is now substantial evidence that the levels of surface membrane expression of certain adhesion molecules influence the degree of aggressiveness or metastatic potential of a carcinoma. For example, the normal formation and maintenance of an epithelium is dependent upon cell-cell and cell-matrix contacts elicited by members of the classic cadherin and integrin families. These proteins mediate the initial adhesive steps necessary to trigger a cascade of adhesive

and molecular events that ultimately leads to the differentiated phenotype characteristic of the epithelial cell.

Cadherins are single pass integral membrane surface glycoproteins that mediate Ca^{2+}-dependent cell to cell adhesion in virtually all tissues (Geiger and Ayalon, 1992). Cadherins, and in particular E-cadherin, are believed to be initiators and mediators of cell aggregation, which ultimately leads to the organization of cellular junctions that define the polarized and adhesive phenotype of epithelial cells. E-cadherin-mediated adhesion seems to be the most 'upstream' event that triggers all subsequent pathways (Gumbiner et al., 1988; Mareel et al., 1996, 1995; McNeill et al., 1993). If E-cadherin-mediated adhesion is disrupted, collapse of epithelial junctional organization ensues and cells may display aspects of the transformed phenotype (Amagai et al., 1995; Behrens et al., 1989; Birchmeier and Behrens, 1994; Bracke et al., 1996; Pignatelli and Vessey, 1994; Rodriguez-Boulan and Nelson, 1989; Shiozaki et al., 1996). If E-cadherin expression is engineered in certain carcinomas, tumorigenicity and invasion are decreased (Miyaki et al., 1995; Perl et al., 1998; Vleminckx et al., 1991). These studies show that the acquisition of functional cadherin expression is inversely related to the degree of carcinomatous transformation, and suggest that control over cellular adhesive mechanisms may be one avenue by which tumorigenic potential may be influenced.

The transformed cervical epithelium has been a useful and informative model for studying morphological and behavioral properties of tumor cells. The glandular element of the cervix is normally composed of tall columnar secretory epithelial cells which express and assemble the full complement of epithelial junctions. HeLa is a poorly differentiated cervical carcinoma cell line originally harvested from a highly aggressive adenocarcinoma of the cervix. HeLa cells lack characteristic features of columnar epithelial cells. Although occasional tight junctions and desmosomes may be visualized, the normal repertoire of adhesion associated-proteins is severely reduced or even undetectable (Doyle et al., 1995). Functionally, HeLa is not self-adhesive, lacks contact inhibition, and is highly tumorigenic and invasive (Arai et al., 1976; Vessey et al., 1995).

P_0 is a member of the immunoglobulin gene family and is expressed exclusively in compact myelin synthesized by Schwann cells. The extracellular domain of P_0 is relatively small, containing a single variable-like Ig-fold (Lai et al., 1987; Lemke et al., 1988; Shapiro et al., 1996) that is responsible for membrane juxtaposition of Schwann cell membranes in the extracellular space of the peripheral nervous system. Although P_0 is naturally expressed only in peripheral nerve myelin, it can behave as a vigorous adhesion molecule when artificially expressed in a variety of cell types (D'Urso et al., 1990; Doyle et al., 1995; Filbin et al., 1990; Schneider-Schaulies et al., 1990) and can thus be thought of as a mediator of 'obligatory' membrane adhesion. In HeLa cells, P_0 expression leads to ultrastructural rearrangements and biochemical changes consistent with epithelial junction formation (D'Urso et al., 1990; Doyle et al., 1995).

In this report, we show that constitutive P_0 expression in HeLa cells restores several important aspects of normal epithelial cell physiology including adhesion-mediated growth control, loss of invasiveness and decreased secretion of matrix-degrading enzymes. Of greatest interest, HeLa cells constitutively expressing P_0 do not form tumors in athymic nude mice. These data, and the finding that obligatory P_0-mediated adhesion in HeLa is followed by sustained increases in cadherin and catenin levels, suggest that this IgCAM can elicit an inherent but dormant or 'sluggish' intracellular pathway which, when activated, triggers epithelialization and the suppression of the tumorigenic and transformed properties of this cervical carcinoma cell line.

MATERIALS AND METHODS

Assessment of anchorage-independent growth

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamate (Gibco, Grand Island, NY). Cells permanently expressing both P_0-pECE and pSV2-neo or expressing only pSV2-neo (control lines), were generated in our laboratory as described (D'Urso et al., 1990; Doyle et al., 1995), and maintained in supplemented DMEM containing 400 μg/ml of Geneticin G418 (Gibco). The ability of cell lines to grow and form colonies in the absence of a matrix was measured by a standard agar assay (Hoffman et al., 1993; Rodriguez-Fernandez et al., 1993). Cells were harvested in 0.1% trypsin (Gibco), resuspended, and 10^3 or 10^4 cells were placed in top agar (0.35% Bacto-agar in complete DMEM with G418). This suspension was placed on solidified bottom agar (0.6% Bacto-agar in DMEM). Cells were fed every 2-3 days with top agar containing FCS. Colony formation was assessed and counted (colonies ≥100 μm) after 2 and 4 weeks. Each experiment was done in triplicate. Data was expressed as cloning efficiency (colonies formed/cells plated ×100).

In vitro assessment of invasion

A chemoinvasion assay was performed as described (Albini et al., 1987). Matrigel matrix (50 μg) was coated on 12 mm polycarbonate filter inserts (12 μm pore size) and placed into a 12-well plate creating a modified Boyden chamber apparatus. Conditioned medium, obtained by incubating NIH-3T3 cells in DMEM with added 0.1% bovine serum albumin (BSA) and ascorbic acid (50 μg/ml) for 24 hours, was placed in the bottom chamber to serve as a chemoattractant. Cells were harvested with 0.1% trypsin and then resuspended in DMEM containing 0.1% BSA. Suspensions containing 2.5×10^5 cells were placed in the upper chamber. The plates were incubated for 12 hours at 37°C in 5% CO_2. Cells remaining on the upper side of the filter were removed with a cotton swab. In order to better visualize the cells that had invaded to the bottom side of the filter, fixation with 95% ethanol and staining with Hematoxylin and Eosin were performed. Cells that had invaded the matrix were quantified by counting several fields under the 100× lens of an inverted light microscope (Olympus Corp., Tokyo, Japan).

Secretion of matrixmetalloproteinases

The enzymatic activity and expression of two matrixmetalloproteinases (MMP-2 and MMP-9) were analyzed by gelatin zymography (Heussen and Dowdle, 1980). Conditioned medium was obtained from growing cells and was diluted in 4× sample buffer containing no reducing agents. The cells from which the conditioned medium was harvested were counted with a hemocytometer to adjust for cell number when loading samples. Samples were loaded in a non-reducing 7.5% SDS-Tris protein gel containing 0.2% gelatin. After the gel was resolved, it was incubated in 2.5% Triton X-100 (23°C, 30 minutes) to remove SDS, rinsed extensively with distilled water and placed in 50 mM Tris-HCl, pH 7.7, 5 mm $CaCl_2$ and 0.02% sodium azide (37°C, 40 hours) to allow

enzymatic digestion. Gels were stained with Coomassie Blue and destained in a standard acetic acid/methanol solution.

In vivo assessment of tumor formation

Confluent monolayers of P_0 or control cells grown in 100 mm dishes were pretreated with 5 mm sodium butyrate for 16 hours to upregulate P_0 expression; this treatment does not alter other cellular properties (D'Urso et al., 1990; Doyle et al., 1995). Cells were harvested with 0.1% trypsin and resuspended in DMEM. Athymic nu/nu 5-week-old nude mice were injected intraperitoneally with 10^6 cells; there were 14 mice in each group. The growth rate of these cells was assessed by plating 10^6 cells in 100 mm dishes (without any further butyrate treatment), which were counted after 2 weeks with a hemocytometer.

Animals were perfused with 4% paraformaldehyde (PFA) at 2, 4 and 8 weeks. Representative tissues (lung, liver, spleen, brain and any grossly apparent tumors) were removed and placed in 10% sucrose in 4% PFA for 1.5 hours at 4°C, and then in 20% sucrose in 4% PFA overnight at 4°C. Tissue was embedded in Tissue-TEK OCT compound (Miles Inc.) on dry ice and was cut on a cryostat into 10 μm sections, and stored at –80°C until use. Standard Hematoxylin and Eosin staining was performed on sections to visualize the parenchyma and overall architecture of the selected tissues. Immunohistochemical analysis was also performed on frozen tissue sections. Sections were hydrated in PBS, then placed in blocking solution (5% normal goat serum in PBS) and incubated for 2 hours with a monoclonal mouse cytokeratin 18 antibody (ICN Immunochemicals, Costa Mesa, CA) or affinity-purified polyclonal rabbit anti-P_0 serum (D'Urso et al., 1990). After several washes, the samples were incubated with a fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Coverslips were placed on slides with mounting solution (50 mM Tris, pH 8.6, 2.5% DABCO [1,4-diazabicyclo(2.2.2) octane]; Sigma Chem. Co.). A Leica TCS 4D confocal laser scanning microscope (CLSM) was used to assess immunofluorescence of tissue sections.

Western blotting

Cell fractions enriched for cytoskeletal elements and membranes (Doyle et al., 1995) were separated on a 7.5% polyacrylamide gels and transferred to nitrocellulose paper which was blocked with 5% nonfat milk, incubated with primary antibody, washed, and incubated with peroxidase-conjugated secondary antibodies (Sigma Chem. Co.). Blots were developed by chemiluminescence (Amersham Corp.). A standard BCA protein assay (PIERCE) was performed to standardize the amounts of protein loaded.

RESULTS

Components of the cadherin/catenin signaling pathway are upregulated in P_0-HeLa cells

Previously, we showed that when HeLa, a cervical carcinoma cell line largely devoid of epithelial junctions, was forced to express P_0, strong cell-cell adhesion and close membrane apposition was induced. Junctional structures were found to assemble intermittently at lateral cell borders of expressing cells and concomitantly, certain proteins known to be associated with these structures were upregulated (Doyle et al., 1995).

Electron microscopy revealed that in long term (1-2 week) cultures of confluent monolayers of P_0-HeLa cells, multiple epithelial junctions form along the lateral cell borders, including cadherin-based adherens junctions (Fig. 1A). This is in contrast to control cells which exhibit membrane interdigitations with no direct membrane contact (Fig. 1A). Additionally, P_0

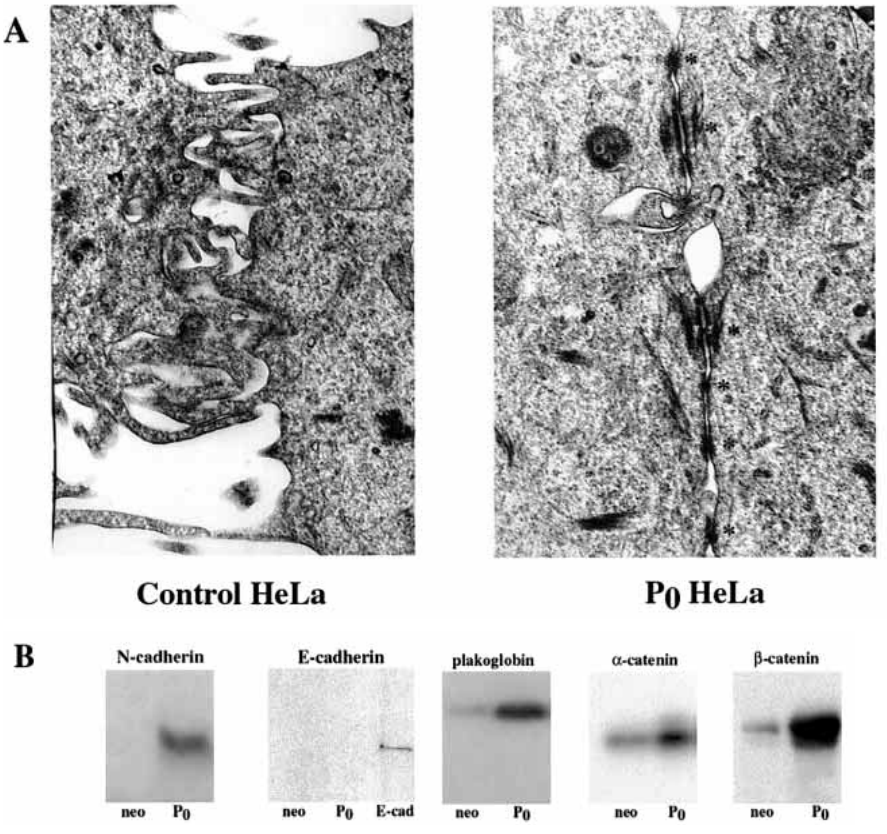

Fig. 1. Components of the cadherin/catenin signaling pathway were upregulated in P_0-HeLa cells. Electron microscopy demonstrated that long term cultures of P_0-HeLa cells assembled multiple rows of intercellular junctions (left panel; asterisks), whereas control HeLa (neo cells) only display membrane interdigitations (A). Western blot analysis of cytoskeletal-enriched cell lysates (B) demonstrate that control pSV2-neo cells express low amounts of N-cadherin and the catenins. P_0-HeLa cells upregulated and maintained the synthesis of N-cadherin, plakoglobin, α-catenin and β-catenin; there is no increase in E-cadherin synthesis. neo, control HeLa cells; P_0, P_0 transfected HeLa; E-cad, E-cadherin expressing HeLa.

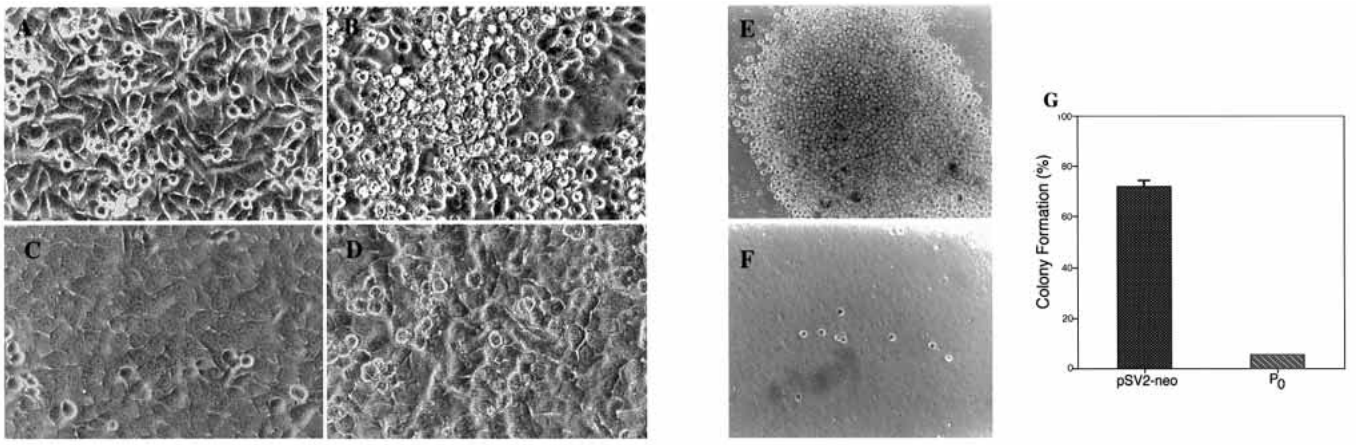

Fig. 2. Anchorage-dependent and -independent growth parameters. Phase contrast images show that pSV2-neo cells continue to divide and pile up on one another at confluency (A) and post-confluency (B). In contrast, P_0 expressors maintain their distinctive morphology even several days post-confluency (C,D), suggesting that P_0 cells acquire contact inhibition. Additionally, these cells were grown in soft agar to assess anchorage-independent growth. PSV2-neo formed large colonies with a cloning efficiency of 72% (E,G), whereas P_0 cells were unable to proliferate in the absence of a matrix and had a cloning efficiency of 5% (F,G). This indicates that P_0-HeLa could not grow in an anchorage-independent manner.

expressors exhibited greatly increased levels of N-cadherin and its associated proteins, α- and β-catenin (Fig. 1B). The catenins are downstream mediators of cadherin adhesion and are necessary for classic cadherins to function appropriately in epithelia. Another intracellular cadherin binding partner is plakoglobin (M_r 86,000), and its expression is severely reduced in HeLa cells as well as in other carcinoma cell lines (Fig. 1B; Lewis et al., 1997; Simcha et al., 1996). The synthesis of plakoglobin was also increased in P_0-HeLa (Fig. 1B).

In epithelia, E-cadherin is usually the classic cadherin that is responsible for adherens junction formation and structure. P_0 expression in HeLa, however, was not found to trigger E-cadherin synthesis (Fig. 1B). Rather, the upregulation of N-cadherin and the associated catenins, α-catenin, β-catenin and plakoglobin in P_0-HeLa occurred independently of E-cadherin. This suggests that perhaps P_0 adhesion may activate N-cadherin/catenin signaling pathways in this cervical carcinoma, which in turn may change its physiological or tumorigenic behavior to be more epithelial-like in an analogous fashion, as has been demonstrated in multiple carcinoma types with E-cadherin (Miyaki et al., 1995; Perl et al., 1998; Vleminckx et al., 1991).

P_0 expression changed anchorage-dependent and -independent growth parameters

Normal epithelia grow in a controlled and contact-inhibited manner. Carcinoma cells, including HeLa cells, have lost contact inhibition and, upon confluency, pile up and continue to divide due to the loss of density-limited growth (Fig. 2B). Since P_0 expression restores cell to cell adhesion in HeLa cells, we considered the possibility that their growth properties might be altered.

Once P_0 expressors reach confluency, they become reversibly contact inhibited, stop dividing, and maintain their polygonal and flattened morphology (Fig. 2C,D). Cell to cell boundaries become indistinct due to close apposition of membranes (approx. 5 nm) mediated by P_0 adhesion (Doyle et al., 1995; Shapiro et al., 1996). In contrast, control cells continue to divide at confluency, forcing them to round up and pile on top of one another, and eventually to lose attachment to the plate surface (Fig. 2A,B). This data demonstrates that the anchorage-dependent growth pattern of P_0 expressors is similar to that of normal epithelial cells rather than to parental or control lines.

Additionally, epithelial cells require attachment to a basement membrane or matrix when undergoing cell division. Transformed cell lines, including HeLa, have a decreased requirement for cell-substrate attachment for continual proliferation which may lead to tumor formation. Anchorage-independent growth can be evaluated in vitro by determining if a particular line can form colonies when grown in suspension or in a semisolid medium such as soft agar. HeLa cells and the pSV2-neo transfected control, like most tumor cell lines, divide and form large colonies when grown in agar with a cloning efficiency of approximately 72% (Fig. 2E) (Celis et al., 1978). In contrast, virtually none of the P_0 expressors form colonies when grown under these conditions and have a much lower cloning efficiency (5%), similar to normal epithelial cell lines (Fig. 2F). The majority of the P_0 expressors remain as single cells.

P_0 expressors secrete low levels of matrixmetalloproteinases and were not able to invade an artificial basement membrane

Another property that contributes to the aggressiveness of a carcinoma is their ability to invade. One mechanism that enables carcinomas to invade basement membranes is the abnormally high synthesis and secretion of matrixmetalloproteinases (MMPs), which degrade the basement membrane and the surrounding matrix. Epithelial cells do not express and secrete these enzymes and are therefore less invasive.

An in vitro chemoinvasion assay (Albini et al., 1987) was used to compare and quantify the invasiveness of the P_0 expressors and the control cells. This assay measures the number of cells that are able to invade an artificial matrix (Matrigel) that is composed of laminin, type IV collagen, heparin sulfate and other substances found in the basement membrane. We find that after 12 hours, 293 control cells on

average invade the matrix whereas only about 4.5 P_0 cells can invade (Fig. 3A-E).

We compared the enzyme activity and expression of two type IV collagenases (MMP-2 and MMP-9) in the P_0 expressors and other carcinoma cell lines by gelatin zymography. Samples of conditioned medium are run on non-reducing gels containing gelatin, which serves as a substrate for the MMPs. The resolved gel is placed in buffer to allow development of enzyme activity, and then stained. The clear bands reveal the extent to which the gelatin has been proteolyzed (Heussen and Dowdle, 1980). Two highly invasive breast carcinoma cell lines MDA-MB-231 and 453 secrete high levels of MMP-9 or MMP-2 (Fig. 4, lanes A,B). Control pSV2-neo HeLa cells (+ or – butyrate, Fig. 4, lanes C and D, respectively) secrete both MMP-9 and MMP-2. P_0 expressing cells have lost the ability to elaborate these enzymes (Fig. 4, lanes E,F).

These results show that the P_0 expressors cannot invade the artificial basement membrane to the same degree as the controls due to their decreased secretion of matrix-degrading collagenases. This raised the possibility that these cells may be in fact less tumorigenic and invasive in vivo than parental or control cell lines.

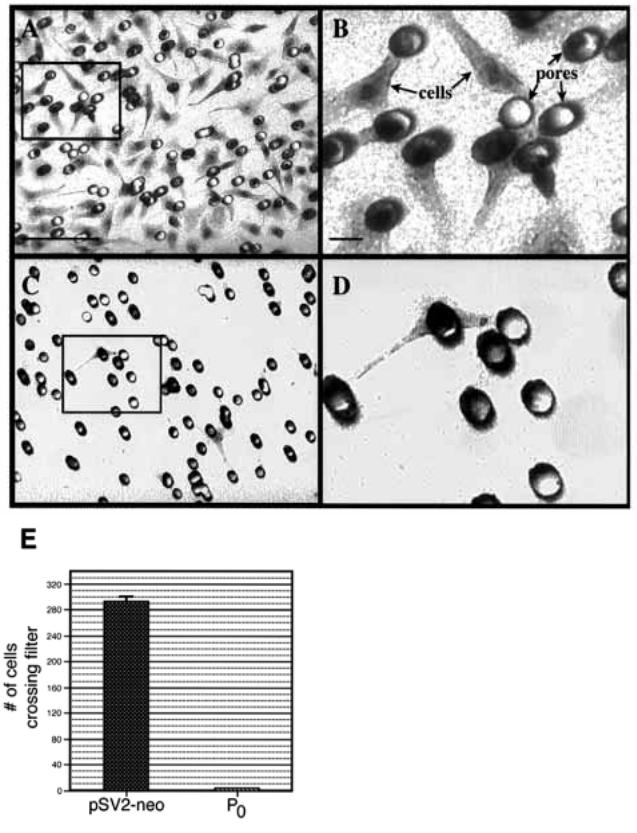

Fig. 3. Comparison of invasion through a basement membrane. The invasiveness of the P_0 and control cells was determined by comparing their ability to cross an artificial basement membrane (Matrigel-coated filters). On average, 296 (per 100× microscopic field) of the control cells (A,B,E) were able to invade through the matrix whereas only 4.5 (per 100× microscopic field) of the P_0 cells could (C,D,E). Qualitatively (A-D) and quantitatively (E), it appears that the P_0 expressors are less invasive that control cells. B and D are high magnifications of the boxed areas in A and C. Bars, 10 μm.

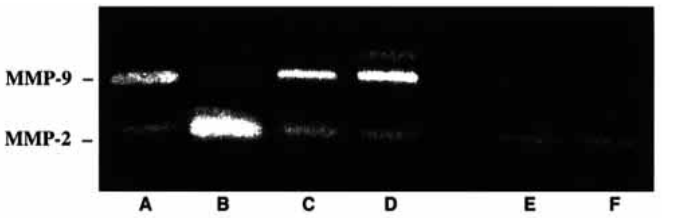

Fig. 4. Secretion of matrixmetalloproteinases MM-P2 and MM-P9. The secretion of matrixmetalloproteinases was assessed by gelatin zymography in several cell lines. The P_0-HeLa cells do not secrete MM-P2 or MM-P9 at the same levels as the control cells or the breast carcinoma cell lines (MDA-MB-231 and 453). Lane A, MDA-MB-231 cells; B, MDA-MB-453 cells; C, pSV2-neo cells + butyrate; D, pSV2-neo cells; E, P_0 Hela + butyrate; F, P_0 HeLa.

P_0 expressors do not form tumors when injected into athymic nude mice

One well-documented test of tumorigenicity of a cell line in vivo is to inject cells into athymic nude mice and observe tumor formation over a period of weeks (Celis et al., 1978; Freedman and Shin, 1974). Mice injected with cells harvested from highly aggressive cancer cell lines form more tumors in a shorter period of time. In order to assess how the P_0 expressors behaved in situ, we injected athymic nude mice with either P_0 or pSV2-neo expressing cells and evaluated gross and microscopic tumor growth over a period of 2 months. HeLa cells, including P_0 expressors, express high levels of cytokeratin 18 (Doyle et al., 1995). Thus, we could specifically identify even small micrometastases within the liver using antibodies to this protein.

Six mice from each group were sacrificed at 3 and 8 weeks. All of the mice injected with control HeLa cells exhibited pronounced ascites, which was found to contain viable pSV2-neo HeLa cells that were then cultured. Small tumors were found at the injection site and within the peritoneal cavity. Two mice had small tumor lesions attached to the outer surface of the liver (Fig. 5A). High power examination of the livers in three other mice revealed single cells or small groups of cells within the liver parenchyma that were cytokeratin positive (Fig. 5B) and thus that pSV2-neo cells had metastasized to this organ. By 7 and 8 weeks, four mice injected with the pSV2-neo cells died, presumably due to tumor overload, and surviving mice in this group were extremely sick with abdomens distended by ascitic fluid. These mice had multiple subcutaneous and peritoneal tumors, and had gross metastases on the outer surface and within liver. Micrometastases were found disrupting the normal architecture of the liver parenchyma (Fig. 5C,D).

The mice injected with the P_0 expressors showed no physical or morphological signs of tumor formation. Mice did not have ascites and their organs were healthy and well-perfused. There were no gross tumors within the peritoneal cavity or on any organs, including the liver. In order to verify that there were no micrometastases, the organs were analyzed immunohistochemically with cytokeratin antibody and P_0 antibody. The liver parenchyma had no disruption in its normal architecture at either 3 or 8 weeks (Fig. 5E,G). The cytokeratin antibody reveals that the liver parenchyma was free of any

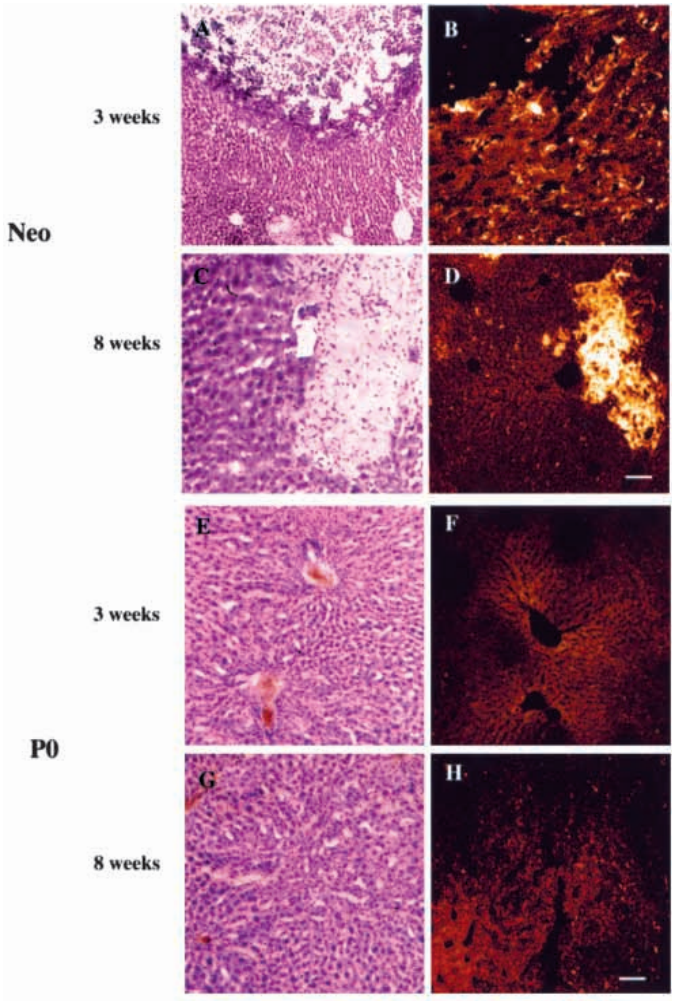

Fig. 5. Nude mice were injected with either pSV2-neo HeLa (A-D) or P_0 HeLa (E-H). Nude mice injected with pSV2-neo cells had small tumors attached to the outer surface of the liver capsule by 3 weeks (A) and the tumors had metastasized within the liver parenchyma by 8 weeks (C). Cytokeratin immunofluorescence showed that isolated cells can be identified within the liver parenchyma at 3 weeks, indicating that tumors cells have begun to metastasize at this early time point (B). By 8 weeks the tumor cells have formed nodules that disrupt the architecture of the liver parenchyma (D). In contrast, mice injected with P_0 expressors do not exhibit tumor formation within the peritoneum or metastatic lesions in any organ. At 3 weeks (E) and 8 weeks (G) the liver parenchyma showed no evidence of tumor cells. Cytokeratin immunofluorescence confirmed that there were no micrometastases in these livers at 3 (F) or 8 weeks (H). Bars, 30 μm (D); 5 μm (H).

micrometastases at both 3 and 8 weeks (Fig. 5F,H); results are identical with the P_0 antibody (data not shown). The spleens of the P_0 mice were 1.5-2 times larger that the pSV2-neo injected mice. The spleens from both animals had no abnormalities in the architecture in the spleens from either group (data not shown) and were tumor-free. All other organs are tumor-free as well (data not shown). In summary, 0/14 mice injected with P_0 expressors developed tumors and all of the mice with pSV2-neo cells formed tumors and metastasised. P_0 is not a promiscuous adhesion molecule and can only act homophilically to produce cell adhesion. We also considered that P_0 cells might be able to 'home' to myelinated peripheral nerves where in the normal animal P_0 is found exclusively. Thorough histological examination of the sciatic nerves revealed no tumors.

Taken together, our data reveal that the P_0-expressing HeLa cells have made the reverse transition from a carcinoma to a functioning epithelial-like cell line. It appears that P_0 expression re-engages a dormant, but undamaged program in this cell line that effectively suppresses the carcinoma phenotype.

DISCUSSION

P_0 is solely the natural product of Schwann cells, where it adheres a single plasma membrane surface to itself to generate compact myelin. Atomic structural modeling predicts that individual P_0 molecules emanate from the plasma membrane as tetramers that interdigitate to yield a virtually infinite, highly adhesive lattice network (Shapiro et al., 1996). Membrane surfaces are brought within 5 nm of each other, thus allowing very close apposition. In order to maintain compact myelin, P_0 lattice interactions are highly adhesive, and once formed are probably not subject to modulation (Colman et al., 1996). The molecular mechanisms that mediate P_0 adhesion are general and 'obligatory', since when expressed in a variety of cell lines, P_0 mediates intercellular adhesion (D'Urso et al., 1990; Doyle et al., 1995; Filbin et al., 1990; Schneider-Schaulies et al., 1990).

When the plasma membranes of HeLa are brought into close apposition by P_0, the eventual consequence is 'epithelialization'. P_0 HeLa exhibit adhesion-dependent growth properties leading to the acquisition of contact inhibition and the loss of anchorage-independent growth. Additionally, P_0 HeLa are unable to invade an artificial matrix and have decreased secretion of matrix-degrading enzymes. It appears that changes induced by initial P_0 expression are sustained; once this program is initiated, it is not dependent upon the maintenance of P_0-based adhesion. We have shown that individual P_0 cells injected into nude mice do not form tumors or metastatic lesions as control cells do. P_0 adhesion triggers complex, dormant but intact pathways that lead to these observed changes in HeLa morphology and physiology sustained within each individual cell.

Multiple complex and interacting signaling pathways involving specific gene transcription and translation lead to the regression from carcinoma to the epithelial phenotype. In epithelia, members of the classic cadherins are thought to initiate molecular pathways involving the catenins, which lead to the organization of epithelial junctions and the physiological properties of epithelia: adhesion mediated growth control, lack of MMP secretion and absence of tumorigenicity and invasion (Frixen et al., 1991; Miyaki et al., 1995; Vleminckx et al., 1991; Watabe et al., 1994).

How might P_0 act to induce these complex sets of events that ultimately lead to 'epithelialization' of this aggressive carcinoma? One hypothesis is that P_0 directly signals these physiological changes through its cytoplasmic domain via the catenins. In myelin, P_0 has no demonstrable outside to inside signaling properties because once P_0 is synthesized, it is

completely sequestered in PNS myelin and does not appear on Schwann cell surfaces. As myelin is compacted, cytoplasm is completely excluded from the wraps of Schwann cell membranes, allowing no signaling proteins or cytoskeletal elements to associate with the P_0 cytoplasmic domain. However, mutational analysis and truncation studies have demonstrated that the intracellular domain is needed to activate the adhesive properties of P_0 (Wong and Filbin, 1994, 1996) and so this domain may affect the conformation of the ectodomain, thereby modulating strength of adhesion during maturation of compact myelin; in effect, mediating *inside to outside* signaling.

It is perhaps most likely that, since P_0 lattices bring membranes very close together (Doyle et al., 1995; Shapiro et al., 1996), the small amount of endogenous N-cadherin molecules on opposing cell surfaces are able to engage. Cadherins only require a distance of about 20-30 nm to interact adhesively with one another to form adherens junctions and desmosomes (Farquhar and Palade, 1963; Schmidt et al., 1994; Shapiro et al., 1995). According to this scenario, N-cadherin interactions would trigger upregulation of N-cadherin synthesis as well as the catenins. The catenins are a distinct set of cytoplasmic proteins that interact with the cytoplasmic tail of cadherins (Gumbiner, 1993, 1996). These proteins coordinate extracellular adhesive signals of the cadherin with intracellular signaling pathways that ultimately induce or inhibit specific gene transcription and translation (i.e. genes responsible for transformation) and lead to the assembly of adherens junctions, desmosomes and functional tight junctions (Aberle et al., 1996; Bracke et al., 1996; Gumbiner and McCrea, 1993; Jiang, 1996; Ranscht, 1994; Watabe et al., 1994). In particular, it has been speculated that plakoglobin relays signals from classic cadherins to the desmosomal proteins to trigger desmosome formation (Lewis et al., 1994).

If the expression of any one of the catenins is disrupted, regardless of the levels of cadherin expression, this may lead to carcinomatous transformation (Andrews et al., 1997; Hao et al., 1997; Hiscox and Jiang, 1997; Krishnadath et al., 1997; Richmond et al., 1997; Shiozaki et al., 1994; Umbas et al., 1997; vanderWurff et al., 1997). In certain renal carcinomas that lack cadherin expression, overexpression of plakoglobin triggers epithelial characteristics: cells gain adhesion-mediated growth control and are less invasive and tumorigenic, but there are no changes in cell morphology probably due to the lack of cadherin expression (Simcha et al., 1996). One highly invasive lung carcinoma cell line (PC9 cells) and some ovarian carcinomas express normal levels of E-cadherin and β-catenin, but either lack α-catenin or have a mutant form (Bullions et al., 1997; Nagafuchi and Takeichi, 1988; Watabe et al., 1994). Once full-length α-catenin is transfected into these cells, cell to cell adhesion is restored, junctional complex proteins are redistributed to the appropriate position in the membranes, and cells are no longer invasive (Bullions et al., 1997; Nagafuchi and Takeichi, 1988; Watabe et al., 1994). The role of N-cadherin in epithelial junction formation and tumor suppression has not been fully elucidated. In SV40-transformed fibroblast 3T3 cells, overexpression of N-cadherin changes cell morphology to be more 'epithelial-like' but does not reduce their tumorigenicity (Simcha et al., 1996); plakoglobin expression is needed to decrease the invasive properties of these cells (Simcha et al., 1996). P_0 expression in HeLa cells significantly upregulates components of the cadherin/catenin pathway including N-cadherin, α-catenin, β-catenin and plakoglobin, suggesting that it works upstream of the cadherins. P_0 adhesion triggers these dormant pathways leading to the reverse transition from a carcinoma to an epithelium.

In conclusion, we have shown that P_0 expression in this cervical carcinoma cell line restores normal morphological features and, most interestingly, leads to the recovery of the normal physiology associated with epithelia. We conclude that, in general, obligatory adhesion molecules, such as P_0, may serve as inducers of dormant intracellular events in carcinoma cells that ultimately triggers these cells to regain epithelial characteristics. It is important to note that multiple pathways need to be engaged to elicit the growth parameters, polarity, morphologic features, and lack of invasion of an intact epithelium. It may be that there is a single 'master' signaling event triggered by adhesion which in turn induces all subsequent events, and if so, this would have significant implications for cancer therapeutics.

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