Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies

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Abstract. In a recently developed human breast cancer model, treatment of tumor cells in a 3-dimensional culture with inhibitory β1-integrin antibody or its Fab fragments led to a striking morphological and functional reversion to a normal phenotype. A stimulatory β1-integrin antibody proved to be ineffective. The newly formed reverted acini re-assembled a basement membrane and re-established E-cadherin–catenin complexes, and re-organized their cytoskeletons. At the same time they downregulated cyclin D1, upregulated p21cip,waf-1, and stopped growing. Tumor cells treated with the same antibody and injected into nude mice had significantly reduced number and size of tumors in nude mice. The tissue distribution of other integrins was also normalized, suggesting the existence of intimate interactions between the different integrin pathways as well as adherens junctions. On the other hand, nonmalignant cells when treated with either α6 or β4 function altering antibodies continued to grow, and had disorganized colony morphologies resembling the untreated tumor colonies. This shows a significant role of the α6/β4 heterodimer in directing polarity and tissue structure. The observed phenotypes were reversible when the cells were disassociated and the antibodies removed. Our results illustrate that the extracellular matrix and its receptors dictate the phenotype of mammary epithelial cells, and thus in this model system the tissue phenotype is dominant over the cellular genotype.

The extracellular matrix (ECM) mediates both biochemical and biomechanical signaling events (Roskelley et al., 1994), and that cell shape in turn can profoundly influence the phenotypic response of cells to the ECM (Dhawan and Farmer, 1994; Ingber et al., 1995). However, how alterations in these pathways can lead to mammary tumors is at present unknown. Because tumor cells have an altered response to the ECM and their microenvironment, we have argued that ECM-signaling pathways contain tumor suppressor checkpoints which impinge upon, and direct, cell and tissue architecture (Petersen et al., 1992; Howlett et al., 1994, 1995). If this were so, then the context and integration of adhesion-derived signaling networks could dictate the final tissue phenotype (Bissell et al., 1982).

Epithelial tissue architecture and function are orchestrated and maintained through multimeric adhesion complexes known to interact directly with elements of the actin and intermediate filament cytoskeleton, as well as with kinases and phosphatases (Schmidt et al., 1993; Hodivala and Watt, 1994; for review see Gumbiner 1996; Drubin and Nelson, 1996; Giancotti and Mainiero, 1994; Dedhar, 1995; Ashkenas et al., 1996). The mechanism whereby these ad-
hesive interactions control the spatio-temporal fate of cells, and direct higher order tissue structure is poorly understood, although a dynamic competition between integrins and cadherins for polymerized actin microfilaments, and a modulation of this interaction by tyrosine kinases appears likely (Adams and Watt, 1993; Drubin and Nelson, 1996). Thus, the coordinated regulation of integrins and adherens junctions has been described during keratinocyte differentiation (Hodivala and Watt, 1994; Braga et al., 1995; Hotchin et al., 1995), while cell–cell adhesion may be modulated by molecules such as fascin, an actin bundling/motility-associated protein (Tao et al., 1996). Furthermore, the importance of β1-integrins in directing cell–cell and cell–ECM polarity and differentiation has been shown by studies in kidney cells (Ojajian and Schwimmer, 1994; Schoenberger et al., 1994) and keratinocytes (Symington et al., 1993; Watt et al., 1993). Also, the redistribution of α6/β4-integrins has been reported to be associated with the establishment of epithelial cell polarity (Giancotti, 1996; Borradori and Sonnenberg, 1996), and integrin knockout studies have documented profound tissue degeneration and disorganization in β4-integrin null mice (Dowling et al., 1996). Thus, there is evidence which suggests that integrin and adherens receptor pathways directly control the organization of epithelial tissue structure. However, how these events are coordinated, and whether there is a direct reciprocity or hierarchy in these interactions is not known.

A common feature of mammary epithelial tumors in vivo is a disruption of tissue organization and polarity. Consistent with their structural function, cadherin–cytoskeletal interactions have been shown to be frequently altered in breast cancers, and loss of cell–cell interactions is associated with altered tissue organization and increased tumor invasiveness (for review see Takeichi, 1993; Sommers, 1996; Alford and Taylor-Papadimitriou, 1996). Although cadherins and catenins are often downregulated or absent in invasive breast cancers, there are many examples of mammary carcinoma in situ and aggressive, metastatic breast tumors in vivo that express the full complement of adherens proteins. Instead, the cells in these tissues have lost their ability to assemble adherens junctions (Birchmeier and Behrens, 1993; Moll et al., 1994; Sommers, 1996). While the precise cause(s) of this perturbation have yet to be determined, it has been shown that the assembly of adherens junctions can be disrupted by growth factors and oncogenes, and also modified by kinases and phosphatases (Warren and Nelson, 1987; D’Souza and Taylor-Papadimitriou, 1994; Hoschuetzky et al., 1994; Ochiai et al., 1994; Kinch et al., 1995). This suggests that connections exist between adherens junctions and signal transduction pathways and imply the potential for structural plasticity.

The disrupted tissue architecture observed in mammary adenocarcinoma is also frequently associated with alterations in integrin heterodimer profiles (D’Ardenne et al., 1991; Natali et al., 1992; Zutter et al., 1993; Gui et al., 1995; Howlett et al., 1995). Changes in β1-, β4-, α2-, α3, and α6-integrins have been reported for mammary tumor cell lines and in tissue sections, and were shown to be associated with tissue disorganization, loss of polarity, increased tumor aggressiveness, and metastasis (Natali et al., 1992; Berdichevsky et al., 1994; Rossen et al., 1994; Gui et al., 1995; Zutter et al., 1995). A relationship between altered signal transduction via integrins and the adherens junction pathways, and its relevance to the origin of the tumor phenotype has not been directly examined. This is mainly due to the lack of appropriate model systems in which such changes can be studied (Weaver et al., 1995).

We have thus taken advantage of a unique epithelial cell model of breast cancer developed by Briand and coworkers (Briand et al., 1987, 1996). The HMT-3522 breast cancer series was established under chemically defined conditions from a breast biopsy of a woman with a nonmalignant breast lesion (Briand et al., 1987; Nielsen and Briand, 1989). A subline of these cells, generated from passage 118, became spontaneously tumorigenic after an additional 120 passages (passage 238), while the parental line remained nontumorigenic for greater than 400 passages (Nielsen et al., 1994; Briand et al., 1996). In this study we used nontumorigenic S-1 cells at passage 50 (“normal”; S-1) and the tumorigenic progeny at passage 238 (T4-2; referred to as mt-1 in Briand et al., 1996). The latter cells were shown to have a trisomy of chromosome 7p in addition to other genetic alterations such as a p53 mutation and a c-myc amplification (Madsen et al., 1992; Moyret et al., 1994; Nielsen et al., 1994; Briand et al., 1996). These two cell lines, one originating from the other by spontaneous genetic events, therefore, provide a unique tool for addressing the genetic alterations involved in malignant conversion in the breast. In this paper, we postulated that if there were a cause and effect relationship between perturbed tissue organization, loss of cell–cell interactions and altered ECM-signal through integrins on the one hand, and tumor formation on the other hand, it should be possible to modify morphology and behavior of these malignant cells by altering cell–ECM interactions.

Here we show that modification of cell surface β1- and β4-integrins in a 3-dimensional (3-D) basement membrane (BM) assay (Petersen et al., 1992), influences mammary tissue morphogenesis and also regulates cell growth and signal transduction. Furthermore, cellular integrins, when normalized, promote the assembly of adherens junctions and influence the cytoskeleton of these cells, thereby implying that these two adhesion systems may be interconnected. Finally, our results suggest that growth as well as malignant behavior is regulated at the level of the tissue (acin) organization, i.e., the tissue structure appears to determine the phenotype which in turn overrides the cellular genotype.

Materials and Methods

Substrates and Antibodies

Commercially prepared EHS matrix (Matrigel, Collaborative Research, Waltham, MA) was used for reconstituted basement membrane assays, and Vitrogen (rat tail collagen I), ~3 mg/ml (Vitrogen 100, Celtrix Laboratories), was used for thinning the surfaces of culture dishes. Antibodies used for biochemical analysis and immunostaining studies were as follows: for immunostaining, immunoblotting, and immunoprecipitation of E-cadherin, α-catenin, and β-catenin, we used clones 36, 29, and 14, respectively (Transduction Laboratories, Lexington, NY); for immunostaining of type IV collagen we used clone PHM-12 (Biogenex, San Ramon, CA); for immunostaining of β1- and α6-integrins we used clones AIIB2 and J1B5 (C. Damsky); for immunostaining of α3-integrin we used clone P1B5; for immunostaining and immunoprecipitation of β4-integrin we used clone 3E1; for immunoblot analysis of β1-integrin we used clone
Morphogenic reversion was scored positive by the deduced presence of bedment into EHS matrices as single cells (8.5 T4-2 cells to confluence as monolayers, followed by trypsinization and EGF. Three dimensional (3D) cultures were prepared by growing S-1 and International); for lin (5 min). Control sections were stained with second antibodies only. gated streptavidin or HRP-conjugated streptavidin (30 min). Nuclei were Specimens were embedded in sucrose, frozen in Tissue-Tek OCT com- mision was determined by directly examining the morphology, uniformity, and size of 2,000-4,000 colonies of S-1, T4-2, and b-1-inhibitory antibody- treated T4-2 cells (T4-b1). Morphology was assessed in situ by examining the degree of colony organization visually by phase contrast microscopy, and by measuring colony diameter using an eyepiece equipped with a mi- crometer spindle. Colonies were deemed disorganized if they were both irregular in shape and the colony length exceeded the width by greater than twofold. Colony organization was also suggested by a lateral and ra- dially distributed cytoarchitecture, which was visualized by immunostain- ing for the distribution of actin microfilaments and cytookeratin 18 inter- mediate filaments, and related to the distribution observed in acini formed by normal mammary epithelial cells. Polarity was indicated by the pres- ence of a basally organized BM as determined by collagen IV and laminin immunostaining, and by basally immunolocalized a6-, b4-, and b-integrins. Morphogenetic reversion was scored positive by the deduced presence of adherens junctions as determined by colocalization of E-cadherin and b- and b-catenins at cell–cell contacts.

Analysis of Cellular Growth and Apoptosis
The proliferative rate of cells in 3-D EHS was assessed by measuring the thymidine incorporation index. Cryosections (5 µm) of 3D cultures were pulse-labeled for 24 h with [H]Tdr (20 Ci/mmol. New England Nuclear Research Products, Dupont, MA). dNTPs were isolated using a kit (Boehringer Mannheim), and indices were calculated as above. Proliferative potential of 100–400 cells was as- sayed by immunostaining and measuring the Ki-67 labeling index in 5 µ cyrosections of 3D EHS cultures. Proliferative status was determined also by counting and immunostaining the number of cells per colony as well as the average colony size. The colony size was measured directly in 3-D cul- tures by use of a phase contrast microscope using an eyepiece equipped with a mi- crometer spindle, which had been previously calibrated with a ruler. Cell number per colony was determined by counting the number of DAPI stained nuclei per colony in cryosections of cells in 3-D. Cell cycle status was determined by immunoblotting equal amounts of total cell protein lysates for cyclin D1 and p21(WAF1/CIP1) protein levels after separating the proteins by reducing Laemmli gels. Apoptosis was assessed by detection of FITC-labeled 3'OH DNA ends using an in situ apoptosis kit (Boehringer Mannheim) in 5 µ cryosections (Boudreau et al., 1996). Cells were scored (150–200 cells) for the presence of DNA nicks and positively scored cells for each condition were expressed as a percentage of the total number of cells scored (apoptotic index).

Indirect Immunofluorescence
H&E slides were fixed in 90% methanol-acetone at ambient temperature for 20 min or in 1:1 methanol-acetone at 20°C for 2–3 min. Specimens were embedded in su- crose, frozen in Tissue-Tek OCT com- pound (Miles Specimens, Elkhart, IN), and 5 µ frozen sections were prepared for immunostaining as described previously (Streuli et al., 1991). Sections were incubated with primary antibodies for 60 min followed di- rectly by either FITC or Texas red-conjugated secondary antibodies (45 min), or by biotinylated secondary antibodies (45 min), and either Texas red–conju- gated streptavidin or HRP-conjugated streptavidin (30 min). Nuclei were counterstained with di-amino-phenyl-indole (DAPI, Sigma) or hematoxy- lin (5 min). Control sections were stained with secondary antibodies only. Morphogenesis Criteria
Quantitative analysis of tumor cell conversion efficiency by b1-integrin func- tion blocking antibodies was determined by calculating the percentage of colonies converted to a “nontumorigenic morphology.” Percent conver- sion was determined by directly examining the morphology, uniformity, and size of 2,000–4,000 colonies of S-1, T4-2, and b-1-inhibitory antibody- treated T4-2 cells (T4-b1). Morphology was assessed in situ by examining the degree of colony organization visually by phase contrast microscopy, and by measuring colony diameter using an eyepiece equipped with a mi- crometer spindle. Colonies were deemed disorganized if they were both irregular in shape and the colony length exceeded the width by greater than twofold. Colony organization was also suggested by a lateral and ra- dially distributed cytoarchitecture, which was visualized by immunostain- ing for the distribution of actin microfilaments and cytookeratin 18 inter- mediate filaments, and related to the distribution observed in acini formed by normal mammary epithelial cells. Polarity was indicated by the pres- ence of a basally organized BM as determined by collagen IV and laminin immunostaining, and by basally immunolocalized a6-, b4-, and b-integrins. Morphogenetic reversion was scored positive by the deduced presence of adherens junctions as determined by colocalization of E-cadherin and b- and b-catenins at cell–cell contacts.
surface β1- and β4-integrins, cells were grown in 3-D EHS cultures for 10- 12 d and colonies were then released from the matrix by 60–90 min incuba- tion at 37°C with dispase (5,000 U/ml caspase activity; Collaborative Research), washed three times in fresh DMEM:F12 medium and once in ice-cold PBS. Released colonies were incubated and rocked at 4°C for 60 min with 1 mg/ml sulfo-NHS-biotin (Pierce, Rockford, IL) in PBS to label the cell surface proteins, washed twice in ice-cold PBS containing 50 mM glycine, and incubated for 10–15 min on ice in PBS/glycine to stop the reac- tion. Colonies were washed three more times in ice-cold PBS/glycine and lysed in 1% NP-40 lysix buffer also containing 120 mM sodium chlo- ride, 50 mM Tris-HCl and 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Pefabloc (Boehringer Mannheim), 10 μg/ml E64, 25 μg/ml aprotinin, and 0.5 mM benzamidine. Equal numbers of cells (determined by counting the cells in parallel cultures) or equivalent amounts of extracted proteins, were immunoprecipitated with anti-β1- or β4-integrin monoclonal anti- bodies, separated on nonreducing Laemmli gels, and immunoblotted for the presence of biotinylated proteins using HRP-conjugated streptavidin antibody and ECL detection. Irrelevant mouse or rat IgG's served as non- specific controls. Exposed films were scanned and subjected to densito- metric analysis for the determination of intensity differences and relative quantification.

Preparation of Fab Fragments

Rat monoclonal AIIB2 IgG1's were isolated using Affi-Gel Protein A Maps II system (Bio-Rad Labs, Hercules, CA). After purification of rat IgG1, the material was put through a 10DG desalting column, con- centrated by centrifugation and dialyzed against 100 mM sodium acetate buffer, pH 5.0. Fab fragments were generated after 20 h of papain diges- tion (20 μM enzyme/5–10 μg IgG protein, in 100 mM sodium acetate, pH 5.0, 50 mM cysteine, 1 mM EDTA) and reactions were terminated by ad- dition of iodoaceticamide. The presence of Fab fragments was assayed by Coomassie blue staining of acrylamide gels as well as by Western blotting using secondary antibodies. Completion of the reaction was monitored by the disappearance of high molecular weight intact antibody and the ap- pearance of low molecular weight proteins comigrating with commercial Fab's. Western blotting with anti-[Fab'] antibodies verified the absence of the latter fragments in the digest. Intact AIIB2 antibody was efficiently competed out by the Fab preparation, as shown by solution competition and sequential immunostaining of S-1 acini. Once the presence and activ- ity of the Fab's was established, samples were treated with sodium azide and dialyzed into DMEM:F12 before cell culture experiments.

Integrin Function-altering Assays

Antibodies and Fab's and control non-immune mouse and rat IgG's were introduced into the cell-embedded substratum at the time of EHS gela- tion. For the crude ascites of β1- and β4-integrins, we used a range of 25–200 μg ascites protein/ml; for purified β1- and α6-integrins and purified mouse or rat IgG's we used 2–25 μg/ml, ~10–150 nM. Cultures were scored visually after 12 d. 10–20 random fields were viewed by phase con- trast microscopy and the number of normal spheres or tumor-like colonies were counted in each field and related to the behavior of control cultures either without antibodies or in the presence of irrelevant antibodies. To compare the reversal efficiency of various β1-integrin antibodies and Fab fragments on T4-2 cells, the diameter of 200 S-1 nonmalignant acini, as observed directly by phase contrast microscopy, ±SEM was calculated and the ratio of the tumor colony diameter to the nonmalignant S-1 acini diameter ±SEM was determined.

Reversion Assay

Equal densities of cells were seeded into 3-D basement membranes and were subjected to either β1-inhibitory integrin or mock-antibody (nonspe- cific rat IgG) treatment. After 12 d, cells were photographed, released from their matrices, and propagated as monolayers. They were then recul- tured within the 3-D basement membrane assay with or without the β1-integrin inhibitory antibody treatment. These steps were repeated two more times.

Tumor Formation In Vivo

Tumor cells were propagated as monolayers, grown for 3–4 d in a 3-D-reconsti- tuted basement membrane in the absence of antibody, released by dis- pase (Collaborative Research), washed three times in DMEM:F12, and incu- bated with 100 μg/ml function blocking β1-integrin antibody (clone AIIB2), mock antibody (nonspecific rat IgG's), or no treatment (control group) for 3 h. Cell colonies were then washed three times in PBS and 1–4 × 10^6 cells were subcutaneously injected into the rear flanks of 6–8-wk-old Balb/C nu/nu mice, without further antibody treatment. Nontreated, non- malignant S-1 cells were also injected into nude mice as negative controls. Statistics were done by “unistat V 4.0 for windows.”

Experimental Subjects

In conducting research using animals, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals,” prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publica- tion no. 86-23, revised 1985). In the conduct of research where human specimens were used, the investigators adhered to the policies regarding the protection of human subjects as prescribed by 32 CFR 219 and sub- parts B, C, and D.

Results

S-1 and T4-2 Cells in a 3-D Basement Membrane Culture Assay Recapitulate the Phenotypic Characteristics of Normal and Malignant Breast Tissue In Vivo

Only small differences in morphology and growth rates could be observed between S-1 and T4-2 cells on tissue culture plastic (not shown), but profound differences be- came evident after only 4 d following their culture within a 3-D reconstituted basement membrane. Within 10 d, S-1 cells underwent morphogenesis and formed organized acini reminiscent of those formed by cells from reduction mam- moplasty, while T4-2 cells formed large, loosely disorga- nized invasive colonies of cells similar to primary tumor cells tested in this assay previously (Fig. 1, a and a'; Petersen et al., 1992). In addition, S-1 cells were able to basally deposit and organize a basement membrane, as shown by immu- nostaining of type IV collagen (Fig. 1 b, and laminin, not shown), thereby demonstrating that the cells were able to form polarized structures. The irregular T4-2 colonies, while staining for basement membrane components had no discernible organized basement membrane (Fig. 1, b and b'). The failure of T4-2 cells to undergo morphogene- sis was also indicated by their compromised cell–cell adhe- sion. This was shown by the absence of lateral E-cadherin immunostaining (Fig. 1, c and c'), an increase in cytoplasmic localization of E-cadherin (cell fractionation and ex- traction studies, not shown) and reduced interaction of α- and β-catenins with E-cadherin (co-immunoprecipita- tion, Fig. 1 d). Nevertheless, the two cell types expressed essentially the same levels of the three cell adhesion proteins (Fig. 1 e) indicating that the malignant conversion was associated with compromised assembly of adherens junctions rather than with down-regulation of these adhe- sion proteins. Coincident with the formation of acini, S-1 cells became growth arrested and exited the cell cycle, as demonstrated by negligible thymidine incorporation and low immunostaining for Ki-67. Consistent with their loss of structural organization, the tumorigenic T4-2 cells failed to grow arrest by these criteria (Fig. 1, f and g).

S-1 and T4-2 Cells Differ in the Localization and Expression of ECM Integrin Receptors

We have postulated previously that tumor cells have lost their ability to respond correctly to ECM-induced signals
To determine if the loss of structural organization and growth control exhibited by the tumor cells was related to an alteration in their integrins, we characterized the integrin receptors for laminin in the two cell lines. While both S-1 and T4-2 cells expressed integrins $\beta_1$, $\beta_4$, $\alpha_3$, and $\alpha_6$, their distribution patterns were radically different in 3-D cultures (Fig. 2, a and a’ through d and d’). S-1 acini had basally distributed $\beta_1$-, $\beta_4$-, and $\alpha_6$-integrins and basolateral $\alpha_3$-integrin, consistent with their polarized phenotype. In contrast, all of these integrins were found to be randomly distributed and disorganized in the T4-2 colonies. Western blot analysis of the tumor colonies showed over-expression of both $\beta_1$- and $\beta_4$-integrins relative to the levels observed in the S-1 acini (Fig. 3, a and b). Cell surface labeling and immunoprecipitation experiments revealed that the surface levels of $\beta_1$-integrins were slightly higher (12.5%) in the T4-2 cells (Fig. 3 c) whereas the surface $\beta_4$-integrin levels were much lower (60%) in T4-2 than in S-1 colonies (Fig. 3 d). However, the ratio of $\beta_1$- to $\beta_4$-integrins at the cell surface was increased by more than 2.8-fold in the T4-2 cell colonies than in the S-1 acini (Fig. 3 e).

Figure 1. Characterization of the HMT-3522 human breast cancer model. (a and a’) Phase contrast micrographs of nonmalignant S-1 cell colonies (a) and tumorigenic T4-2 cell colonies (a’) viewed directly inside EHS for morphology: S-1 cells formed spherical structures reminiscent of true acini (a), whereas T4-2 cells formed large irregular colonies (a’). (b and b’) Immunostaining for basement membrane components: S-1 acini (b) stained for basement membrane proteins at the cell-ECM junctions as expected, while basement membrane deposition in tumor colonies (b’) was clearly disorganized and no longer polarized. Comparable results were obtained for laminin (not shown). (c and c’) Confocal fluorescence microscopy of cryosectioned colonies immunostained for E-cadherin: S-1 acini (c) stained for E-cadherin primarily at the cell–cell junctions as is typically observed in normal breast sections. The T4-2 colonies (c’) showed punctate, dispersed membrane and intracellular staining. (d) The $\beta$-catenin–E-cadherin interaction ratio as a measure of adherens junction assembly: There was a 50% decrease in $\beta$-catenin protein co-precipitating with E-cadherin in T4-2 cells as compared to S-1 acini. Data are expressed as a ratio of $\beta$-catenin to E-cadherin densitometric measurements from duplicates of two separate experiments. Similar results were observed for $\alpha$-catenin (not shown). (e) Immunoblot of total levels of E-cadherin, $\alpha$-catenin, and $\beta$-catenin: There were comparable levels of all three adherens proteins in S-1 acini and T4-2 colonies. (f and g) Percent of thymidine and Ki-67 labeling in S-1 acini and T4-2 colonies, expressed as thymidine and Ki-67 labeling indices from 3–4 separate experiments. Greater than 90% of the S-1 acini were growth arrested (f), and had exited the cell cycle (g) whereas T4-2 colonies were still actively growing (f) and continued to cycle (g). All cultures were analyzed after 10–12 d inside EHS. Bars: (a and a’) 16 μm; (b and b’) 25 μm; (c and c’) 8 μm.
Figure 2. Immunofluorescence characterization of integrins in the HMT-3522 cells in 3-dimensional cultures. (a–d) Cryosections of S-1 acini and (a’–d’) T4-2 colonies, immunostained and examined by confocal fluorescence microscopy for localization of $\beta_1$- (a and a’), $\beta_4$- (b and b’), $\alpha_6$- (c and c’), and $\alpha_3$-integrin (d and d’) localization: $\beta_1$, $\beta_4$, and $\alpha_6$-integrins were targeted to the cell-ECM junction in the S-1 acini (a–c), in contrast, in T4-2 colonies (a’–c’) this polarized-basal distribution was lost. S-1 acini exhibited basolateral $\alpha_3$ integrins (d), whereas T4-2 colonies (d’) demonstrated disorganized plasma membrane and cytosolic expression of this integrin. All cultures were analyzed after 10–12 d inside EHS. Bars: (a–d and a’–d’) 16 μm.
Function Blocking $\beta_1$-Integrin Antibodies Cause Dramatic Phenotypic Reversion of the T4-2 Cells

Since T4-2 cells had both a higher total level and an elevated ratio of cell surface $\beta_1$-to $\beta_4$-integrins, we wondered whether the aberrant malignant behavior may be a reflection of the changes in these integrins. Accordingly, we examined the consequences of treatment in 3-D with varying concentrations of a previously characterized rat monoclonal $\beta_1$-integrin antibody (clone AIIB2) which has been shown to inhibit ligand binding (Werb et al., 1989). This antibody caused massive apoptosis in S-1 cells, as shown previously (Howlett et al., 1995), while T4-2 cells were refractory (Fig. 4). Remarkably however, in addition to resistance to apoptosis, almost all the antibody-treated T4-2 tumor cells assumed a morphology which was indistinguishable from that observed in S-1 cultures and was discernible as early as 4 d after incubation. When examined by light microscopy after 12 d, these cultures appeared as if they had truly reverted to a “nonmalignant” phenotype. We therefore cryosectioned the colonies and examined their morphology by immunofluorescence confocal microscopy. As markers of normal acinar formation, we examined both actin cytoskeleton and superimposition and distribution of cadherins and catenins. Sections of S-1 acini revealed uniform and polarized nuclei (stained with propidium iodide; red), well-organized filamentous actin (FITC phalloidin; green) (Fig. 5a), and uniformly superimposed E-cadherin and $\beta$-catenin at the lateral cell–cell junctions (Fig. 5b). In contrast, untreated or IgG-treated tumor cells had polymorphic nuclei and a grossly disorganized actin cytoskeleton, visualized as random, hatched bundles (Fig. 5a’). Additionally, E-cadherin and $\beta$-catenin were no longer colocalized (Fig. 5b’). In contrast, $\beta_1$-treated T4-2 cells (referred to as T4-$\beta_1$) revealed striking rearrangements of cytoarchitecture as demonstrated by their well-organized actin filaments (Fig. 5a’’), and uniformly superimposed E-cadherin–$\beta$-catenin at the lateral cell–cell junctions (Fig. 5b’’). In contrast, untreated or IgG-treated tumor cells had polymorphic nuclei and a grossly disorganized actin cytoskeleton, visualized as random, hatched bundles (Fig. 5a’). Additionally, E-cadherin and $\beta$-catenin were no longer colocalized (Fig. 5b’). In contrast, $\beta_1$-treated T4-2 cells (referred to as T4-$\beta_1$) revealed striking rearrangements of cytoarchitecture as demonstrated by their well-organized actin filaments (Fig. 5a’’), and uniformly superimposed E-cadherin–$\beta$-catenin at the lateral cell–cell junctions (Fig. 5b’’).
mock-treated T4-2 cells (Fig. 5 c). Viability and growth assays conducted on cells grown as monolayers ruled out toxicity (not shown). Interestingly, while treatment with the inhibitory β1-integrin antibody reduced cell adhesion and retarded the rate of cell proliferation when T4-2 cells were grown in two-dimension, culture in a three-dimensional reconstituted basement membrane was required for complete expression of the reverted phenotype (not shown).

An examination of markers of proliferation and cell cycle status in T4-β1 cells revealed that they were growth arrested as indicated by both a decrease in thymidine incorporation and the size of the acini which was now composed of only 6–8 cells, similar to that observed for S-1 cultures and marked contrast to the average of 18–22 cells observed for nontreated T4-2 tumor colonies and the high mean number of T4-2 nuclei which incorporated [3H]thymidine (Fig. 5, d and e). T4-β1 colonies also had dramatically decreased cyclin D-1 levels (Fig. 5 f) again comparable to that seen in S-1 cultures and markedly reduced Ki-67 levels (not shown), suggesting...
that most reverted cells had exited the cell cycle and therefore had a reduced propensity to proliferate.

Cryosections of T4-β1 colonies incubated with antibodies against either collagen IV (Fig. 5 g') or laminin (not shown) revealed deposition of a basally distributed, almost continuous basement membrane, with characteristics similar to that observed in the S-1 acini (Fig. 1 b). In contrast, punctate and inversely polarized collagen IV (Fig. 5 g) and laminin immunostaining (not shown) were observed in the mock-treated tumor colonies. Thus, these tumor cells had retained the ability to deposit a basement membrane and to form polarized structures if the correct structural cues could be received.

The Inhibitory β1-Integrin Activity Is Necessary for Phenotypic Reversion

The AIIB2 monoclonal antibody (mAb) recognizes a region connecting the two putative extracellular ligand binding sites of the β1-integrin receptor, thereby inducing a conformational change consistent with inhibition of receptor activity (Takada and Puzon, 1993). To distinguish between integrin binding per se or a requirement for inhibition of signaling, we examined whether the T4-2 cells would phenotypically revert upon treatment with a nonfunctional (neither activating nor inhibiting) anti–β1-integrin mAb (clone DF5; Korhonen et al., 1991), an alternate inhibitory anti–β1-integrin mAb (clone JB1a or J10; Stupack et al., 1991), and an activating anti–β1-integrin mAb (clone TS2/16; Weitzman et al., 1995), whose epitope was mapped to the same region as the AIIB2 antibody (Takada and Puzon, 1993). Only the β1-integrin function blocking antibodies were found to be capable of inducing phenotypic reversion of the T4-2 cells (Table I), implying that overexpression or altered signaling through a β1-integrin–specific pathway is at least in part responsible for the malignant phenotype of these tumor cells.

To investigate the possibility that the inhibitory antibodies may have facilitated reversion by inducing clustering of the integrin receptors by divalent mAb-mediated cross-bridging, we purified the rat IgG1 AIIB2 mAb and made AIIB2 Fab fragments (Miyamoto et al., 1995a,b). Significantly, both the purified AIIB2 mAb and its Fab fragments were capable of inducing the same phenotypic reversion of the T4-2 tumor cells (Table I), indicating that it was the β1-integrin function at the cell surface that needed to be reduced for reversion to occur, rather than augmentation of integrin signaling by receptor clustering per se.

The Reversion Is Phenotypic and Reversible

To distinguish between a reversible, microenvironmentally induced change as opposed to selection of possible nonmalignant contaminants or genetically altered mutants, we undertook a series of “reversion rescue” studies. Despite two rounds of reversion, rescue, monolayer propagation, and reculturing in 3-D, these cells were able to resume their original tumorigenic phenotypes when cultured in the absence of antibody (Fig. 6).

Treatment with β1-Inhibitory Antibody Is Sufficient to Significantly Reduce Malignancy In Vivo

To find out whether phenotypic reversion of tumor cells would be sufficient to reduce tumorigenicity in vivo, we injected tumor cells treated in suspension with β1-integrin blocking mAb, mock mAb, or no treatment for 3 h, as well as S-1 cells into nude mice. Within two weeks small nodules were observed in all injected sites including the S-1 controls (not shown). Whereas these nodules regressed rapidly in the S-1 and T4-β1 groups, actively growing tumors were observed in greater than 75–90% of the mock mAb or vehicle-treated T4-2 mice. Upon sacrifice we observed both a significantly reduced tumor number and tumor size in the T4-β1 group (Table II). These data suggest that “normalization” of the tumor cell phenotype in culture has a counterpart in vivo where the malignant potential is reduced or lost.

Table I. Comparison of Various β1-Integrin Antibody Treatments on Colony Size in 3-Dimensional Culture

<table>
<thead>
<tr>
<th>Description</th>
<th>Mean colony size (μm)</th>
<th>Relative size ratio of T4-2/S-1 colonies†</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1 50 cells</td>
<td>24.5 ± 0.7</td>
<td>N/A</td>
</tr>
<tr>
<td>Control T4-2 cells</td>
<td>74.3 ± 2.4</td>
<td>3.03 ± 0.03</td>
</tr>
<tr>
<td>Inhibitory β1-antibody-treated cells, clone AIIB2 crude ascites</td>
<td>24.1 ± 1.0</td>
<td>0.98 ± 0.10</td>
</tr>
<tr>
<td>Inhibitory β1-antibody-treated cells, clone AIIB2 purified IgG1</td>
<td>28.2 ± 1.1</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td>Inhibitory β1-antibody-treated cells, clone AIIB2 Fab fragments</td>
<td>24.5 ± 1.4</td>
<td>1.04 ± 0.04</td>
</tr>
<tr>
<td>Inhibitory β1-antibody-treated cells, clone JB1a crude ascites (alternate inhibitory mAb)</td>
<td>32.3 ± 1.4</td>
<td>1.32 ± 0.06</td>
</tr>
<tr>
<td>Activating β1-antibody-treated cells, clone TS2/16 crude ascites (stimulatory mAb)</td>
<td>61.7 ± 3.3</td>
<td>2.52 ± 0.13</td>
</tr>
</tbody>
</table>

†The diameter of 200 S-1 acini as observed directly by phase contrast microscopy, were measured after 12 d in 3-D culture using an eyepiece equipped with a micrometer spindle. The same measurements were made on 100–400 T4-2 colonies in the presence of 100 μg antibody ascites protein or 25 μg purified IgG/ml EHS matrix of various β1-integrin antibodies (listed above). The average measurements of each of these groups ± SEM were calculated and the ratio of T4-2/S-1 diameters ± SEM were determined.

Table II. Effect of β1-Inhibitory Antibody Induced Phenotypic Reversion on Tumorigenicity In Vivo

<table>
<thead>
<tr>
<th>Treatment description</th>
<th>Total number of tumors per group</th>
<th>Large tumors*</th>
<th>Small tumors†</th>
<th>Mice with no tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>15/16</td>
<td>14/16</td>
<td>1/16</td>
<td>1/16</td>
</tr>
<tr>
<td>T4-2 cells</td>
<td>14/16</td>
<td>11/16</td>
<td>3/16</td>
<td>2/16</td>
</tr>
<tr>
<td>Non-immune rat IgG-treated T4-2 cells</td>
<td>7/16</td>
<td>5/16</td>
<td>2/16</td>
<td>9/16</td>
</tr>
</tbody>
</table>

* tumors >5 <300 mm3.
† tumors <5 mm3.
‡ P < 0.005 by X2 test.
Figure 6. Phenotypic reversion as opposed to selection. Phase contrast micrographs of T4-2 cells grown in the presence of anti-β1-integrin function blocking antibody (T4 β1), mock antibody (nonspecific IgG's) (T4-2 IgG) or no antibodies (T4-2) viewed directly inside EHS. Despite two rounds of treatment, these antibody reverted cells were able to resume their original tumorigenic phenotypes when cultured in the absence of antibody. All cultures were analyzed after 10-12 d inside EHS. Bar, 50 μm.
Malignant Conversion Is Associated with Alterations in α6- and β4-Integrins

The ability of T4-β1 cells to re-form a basally organized basement membrane (Fig. 5 g’) indicated the re-establishment of acinar polarity. Polarity has been shown to be associated with a basal localization of α6/β4-integrin heterodimers in keratinocytes (DeLuca et al., 1994). T4-β1 reverted acini had polar, basally localized α6- and β4-integrins (compare Fig. 7, a–7 a’), increased cell surface β4-integrin heterodimers (Fig. 7 b), and higher p21cip1,waf1 levels (Fig. 7 c). These findings showed that the β1-integrin signaling pathway was not only interconnected with catenin-E-cadherin adherens junction assembly, but was also connected to the α6/β4-integrin signaling pathway.

We therefore examined whether an alteration of α6/β4 signaling in the nonmalignant cells might lead to abrogation of growth control, disruption of morphogenesis and loss of polarity. We used clone 3E1 (Hessle et al., 1984), a β4-integrin mAb that was shown to influence the function of colon adenoma and prostatic carcinoma cells (Jewell et al., 1995), and a protocol similar to that described for the β1-inte-

Figure 7. Alteration of α6/β4-integrin signaling in S-1 cells leads to the formation of disorganized colonies. (a and a’) Confocal fluorescence microscopy of double immunostaining for α6- (Texas red) and β4-integrins (FITC): T4-β1 revertant acini (a’) showed basally polarized α6- and β4-integrins, while T4-2 mock–treated (T-4 IgG) colonies (a) demonstrated disorganized, nonpolarized expression. (b) Cell surface expression of β4-integrin heterodimers using biotinylation: Tumor colonies had lower cell surface levels of β4-integrin heterodimers (60% lower) relative to the S-1 acini (see Fig. 3 d), which were restored in the T4-β1 reverted acini (40% higher) relative to the T4-2 colonies. (c) Immunoblot of p21cip1,waf1 levels in T4-2 IgG and T4-β1 colonies: The level of p21 protein was clearly increased in the T4-β1 reverted acini. (d and d’) Phase contrast micrographs of S-1 nonmalignant acini (d) and tumorigenic T4-2 colonies (d’) viewed directly inside EHS: S-1 cells formed spherical structures (d), whereas T4-2 cells formed large, irregular colonies (d’). (e and e’) Phase contrast micrographs of S-1 nonmalignant cells treated with function altering β4-integrin antibodies (e) and tumorigenic T4-2 cells treated with function blocking β1-integrin antibodies (e’) viewed directly inside EHS: S-1 cells treated with β4-integrin antibodies formed large, irregular colonies (e), while T4-2 cells treated with β1-integrin function-blocking antibody (e’) formed spherical structures similar to S-1 acini (see Fig. 1 a). All cultures were analyzed after 10–12 d inside EHS. Bar, 16 μm.
grin inhibitory antibody studies. Treatment of S-1 cells with this antibody led to the abrogation of normal morphogenesis and loss of growth control. Thus, β4-integrin mAb-treated S-1 cells formed disorganized, large structures similar to T4-2 colonies (Fig. 7 e vs 7 d'). Interestingly the treatment had little or no effect on T4-2 colonies (not shown). The heterodimer partner for β4 is the α6-integrin (Sonnenberg et al., 1988a). Treatment of nonmalignant cells with GoH3 (Sonnenberg et al., 1988b), an α6-integrin function-blocking antibody, led to similar results (not shown). These findings suggest that the α6/β4 pathway not only influences morphogenesis but is also involved in growth regulation in nonmalignant MEC's. Furthermore, these results support the notion that the tumorigenic conversion of the HMT-3522 cells may be associated with an alteration in the α6/β4 signaling pathway. If this were so, then treatment of T4-β1 acini with β4 function-altering antibodies (or simultaneous treatment with β1 and β4 function-altering antibodies) should not alter the T4-β1 reverted phenotype. This in fact was found to be the case (not shown).

Discussion

The use of a 3-D BM assay and two related human MEC lines, one phenotypically “normal” and the other malignant, have allowed us to examine the fundamental role of integrins and their intimate relationship to adherens junctions in the regulation of growth and tissue morphogenesis. The results described in this paper demonstrate not only that misregulation of integrins may play a causal role in the expression of malignancy in human epithelial breast cells, but also that manipulations from the cell surface (such as modification of the transmembrane signaling receptors), can restore tissue form and function and reduce tumorigenicity in vivo. Thus, analogous to the effects following retinoid treatment of leukemias, cell surface ECM-receptor manipulations may offer a plausible alternative therapeutic modality for solid epithelial tumors.

Our results showed that the T4-2 tumorigenic cells have increased β1-integrin expression and cell surface levels which are associated with loss of growth control and perturbed morphogenesis. Moreover, a reduction of β1-cell surface integrin activity was found to be sufficient to revert this tumor phenotype. These observations are consistent with the reciprocal relationship shown to exist between proliferation and differentiation and changes in levels of β1-integrins in keratinocyte cells (Jones et al., 1995), and the anchorage-free growth described for kidney cells over-expressing a downstream integrin signaling protein (Frisch et al., 1996). Overexpression and perturbed signaling through β1-integrin heterodimers have previously been reported in primary and metastatic cancers of the breast, colon, skin, and prostate (Cress et al., 1995; Fujita et al., 1995; Leppa et al., 1995; Mortarini et al., 1995; Shaw et al., 1996).Enhanced β1-integrin expression was also shown to be associated with increased tumor aggressiveness, invasiveness, and metastatic potential, and was found to correlate with decreased patient survival (Friedrichs et al., 1995). However, a direct effect of the altered integrin expression on the tumor phenotype was not demonstrated in these studies, although our results may offer a plausible explanation for this relationship. Furthermore, the reported inhibition of invasion and metastasis of bladder and gastric carcinoma cells, by inhibitory β1-integrin antibodies or Arg-Gly-Asp peptides may be by a mechanism similar to that described in this paper (Saiki et al., 1990; Fujita et al., 1992).

Despite dramatic differences in morphology, both the nonmalignant and tumorigenic HMT-3522 cell lines expressed comparable levels of cell–cell adhesion proteins, but the ability to form cytoskeletal-associated adherens complexes was found to be compromised in the T4-2 cells. This is a condition which has been shown to characterize mammary carcinoma in situ and has been described for several aggressive metastatic breast cancers in vivo and cell lines in culture (Takeichi, 1993; Birchmeirer and Behrens, 1994; Sommers, 1996). By inhibiting tumor cell β1-integrins we were able to reduce adherens junction assembly and revert the tumor phenotype of the T4-2 cells. These results are in agreement with the metastasis-suppressor/epithelial-promoting role of enhanced cell–cell interactions previously shown in metastatic MB-435S/1 breast cells (Frixen et al., 1991) and in HeLa cells (Doyle et al., 1995) transfected with cell adhesion molecules. These observations also emphasize the plasticity of cell–cell junction perturbations, and establish a definite link between integrin signaling, adherens junction assembly and the coordinated formation of a differentiated tissue structure, as has been previously implied by studies in keratinocytes and kidney cells (Hodivala et al., 1994; Ojakian and Schwimmer, 1994; Schoenberger et al., 1994; Braga et al., 1995). While our findings suggest an integrated mechanism, the key intermediates still need to be identified, although perturbed adherens junction assembly can be restored by kinase inhibitors suggesting down-stream signal transduction molecules are involved (Kinch et al., 1995). The possibility that cytoskeletal proteins such as fascin, a new β-catenin–binding protein which competes against junction assembly, and is thought to interact with the actin cytoskeleton to promote filament bundling and enhance cell motility, may play a role in intriguing and is under investigation (Tao et al., 1996). Also, whether a manipulation of the catenin-E-cadherin pathway would reciprocally affect integrins, or whether direct restoration of adherens assembly would be sufficient to lead to phenotypic reversion and inhibition of tumor formation in this system remain important questions.

Polarity is associated with a basal localization of α6/β4 integrin heterodimers in keratinocytes (DeLuca 1994), which has been shown to direct intermediate filament organization (Spinardi et al., 1993; Guo et al., 1995), while its activity correlates with an increase in the level of p21^cip,waf-1 (Clarke et al., 1995), a gene important in growth arrest (Harper et al., 1993). Our data showing reorganized intermediate filaments, increased levels of p21^cip,waf-1 and polarized α6- and β4-integrins in T4-β1 acini, are consistent with these findings and emphasize the existence of coordinate β1–β4-integrin pathway interactions. Since we showed that α6- and β4-integrin perturbing antibodies were able to disrupt normal MEC cell morphogenesis, this also strongly implicates α6/β4-integrins in establishing mammary gland morphogenesis and tissue organization, as has been previously reported for kidney cells (Sorokin et al., 1990; Matter and Laurie, 1994) and in β4-integrin knockout mice (Dowling et al., 1996). How these interactions occur...
has yet to be determined although a role for actin and intermediate filament-associated proteins, interacting with the β4-integrin cytoplasmic tail, such as that described for neural BPAG1 should be considered (Yang et al., 1996). Finally, because we were unable to perturb the morphogenesis induced by β1-integrin inhibition in the T4-β1 revertant acini either by sequential or simultaneous incubation with function-altering β4-integrin antibodies, this indicates to us that a genetic defect in a facet of α6/β4-integrin signaling pathway may indeed be responsible for the observed loss of growth control and tissue organization observed in the T4-2 cells. Further experiments will be required to clarify the validity of this argument.

We used a well studied parameter of cell cycle progression, cyclin D1, and of quiescence, p21^cip1,waf1, in addition to standard DNA replication and Ki-67 assays, to determine how the reverted tumor cells compared to functionally normal acini. By all these criteria, T4-β1 revertant colonies behaved like growth-arrested S-1 acini. It was also clear from these studies that the formation of a BM accompanied the cessation of growth confirming our previous findings of an inverse relationship between the presence of a BM and proliferation (Petersen et al., 1992; Howlett et al., 1994; Desprez et al., 1995). It is therefore plausible that the repression of tumorigenicity in vivo, as documented in these studies may have been directly related to the observed growth arrest in culture. Thus, it is reasonable to postulate that it is the balance of signals transduced by the β1- and β4-integrin receptors (or the corresponding α-integrin subunits) that allows these breast cells to sense their microenvironment appropriately and drive tissue organization and function. Therefore, integrins must function in concert with growth factor signaling pathways to determine the decision to grow or to undergo morphogenesis. Very recently, Sastry et al. (1996) overexpressed chimeric α5- and α6/β4-integrins in quail muscle cells and concluded that it is the cytoplasmic domain of these integrins which determines proliferation or growth arrest. Clearly then both direct and indirect evidence in the literature points to the fact that integrins, much like other biological parameters, are cell and tissue context specific. While the detailed molecular mechanism of β1-integrin signaling in breast cells remains to be elucidated, it is safe to conclude that a change in the surface-associated β1-integrin sets into motion global changes in growth and higher order tissue organization. The model system described here offers the opportunity to elucidate how β1- and α6/β4-integrin heterodimers, catenin-cadherin adherens junction proteins, and growth factor pathways communicate within and between epithelial cells in the 3-D tissue.

The demonstrated plasticity of T4-2 cells, their ability to revert to a near normal phenotype and remain quiescent for up to one month in culture, as well as their reduced tumorigenicity in vivo is reminiscent of the “reversion” of mouse teratocarcinoma cells by a normal embryonic microenvironment. In these seminal experiments, embryonal carcinoma cells which were fused with normal blastocysts were able to give rise to phenotypically normal, genetically mosaic mice (Mintz and Illmensee, 1975; for review see Mintz and Fleischman, 1981). While the conclusion of these studies was that the teratocarcinoma cells did not possess cancer-associated genetic defects, we show in our system that despite the presence of genetic defects they could be restrained by the normal tissue structure. A similar dominant, tumor suppressor role of the embryonic microenvironment was shown by our previous work in which active pp60^src constructs attached to a lacZ gene, packaged in a replication-defective virus, when injected into stage 24 chick embryos appeared as apparently normal blue cells in a well formed background of feathers and tissues (Stoker et al., 1990). Significantly, once these cells were removed from the embryos, trypsinized and placed in culture, they became rapidly transformed (Boudreau et al., 1995a). These findings may also provide a possible mechanistic explanation for the poorly understood phenomenon of both spontaneous breast tumor reversion, tumor cell dormancy, and the sporadic occurrence of an auxiliary nodal metastasis in which a true ductal carcinoma in situ (DCIS), complete with a central necrosis and a surrounding intact basement membrane is recapitulated (Barsky et al., 1994).

In conclusion, our results show that changes in tissue structure are critical for the expression of the malignant phenotype and that ECM receptors are important modulators of this effect. Furthermore, these data underscore the concept that it is the relative distribution and proportion of cell surface integrins (undoubtedly in cooperation with other receptors at the cell surface) and their integrated down-stream events which maintain the structural homeostasis of breast and possibly other epithelial tissues. Therefore, tumor cells need not have lost their ability to respond to ECM-generated signals, but rather alterations in the level of the integrated signals can lead to aberrant behavior. Our results may also have a bearing on why some tumor cells retain strong cell-ECM interactions; integrin switching may promote enhanced survival in alternate microenvironments encouraging metastasis.

Most fundamentally, these studies show that despite a number of prominent mutations, amplifications, and deletions, signaling events which are linked to the maintenance of normal tissue architecture are sufficient to abrogate malignancy and to repress the tumor phenotype. It is thus fair to state that cellular and tissue architecture act as the most dominant tumor-suppressor of all, and that the phenotype can—and does—override the genotype as long as the tissue architecture is maintained. These results may explain why breast cancer takes so long to develop even in individuals with inherited susceptibility. Finally, these studies should further aid in the development of therapeutic peptides and antibodies for the treatment of breast and other solid tissue malignancies.

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