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Interactions of B16F10 Melanoma Cells Aggregated on a Cellulose Substrate

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Abstract There is evidence that the shape of cells and their contact with a matrix direct the growth and the differentiation of both normal and cancer cells. Cells in 3D culture resemble the in vivo situation more closely than do those in conventional 2D cultures. We have studied the interactions and functions of B16F10 mouse melanoma cells, which spread and grow well on tissue culture polystyrene (tPS), when they were made to aggregate on cellulose-coated Petri dishes (CEL). This aggregation of melanoma cells on CEL was Ca^{2+} dependent and mediated by N-cadherins. The levels of N-cadherin and β-catenin transcripts in cells cultured on CEL and tPS were similar, but those on CEL contained less β-catenin protein. Immunoprecipitation and immunostaining showed that both N-cadherins and β-catenins were present at the membranes of cells on CEL. Cells proliferated significantly more slowly after 48 h on CEL and the cellulose coating caused most of them to arrest in G_1. We also compared the melanin contents and tyrosinase activity of cells on CEL and controls grown on tPS. Melanogenesis was induced in cells aggregated on CEL. A cellulose substrate thus appears to be an outstanding tool for studying cell–cell interactions and cell functions in 3D cultures. J. Cell. Biochem. 99: 96–104, 2006.

Key words: cell aggregation; cellulose substrate; N-cadherin; β catenins; melanogenesis; melanoma cells

The physical properties of a substrate and the density of extracellular matrix (ECM) molecules can prevent or encourage cell spreading [Mooney et al., 1992; Hohn and Denker, 1994; Hohn et al., 1996]. Hence, cell shape and contact with the matrix direct the growth and differentiation of several types of normal and cancer cells [Archer et al., 1982; Mooney et al., 1992; Hohn and Denker, 1994; Hohn et al., 1996; Bae et al., 1999; Dike et al., 1999; El-Sabban et al., 2003]. Cells in 3D cultures resemble more closely the in vivo situation with regard to cell shape and cell environment than do cells in conventional cultures [Mueller-Klieser, 1997]. Swiss 3T3 fibroblasts grown on cellulose substrates that poorly adsorb serum adhesive proteins, adopt a rounded shape of cells in aggregates attached to the substrate [Faucheux et al., 1999, 2004]. They proliferate more slowly and programmed cell death begins 24 and 48 h post-seeding [Gékas et al., 2004]. Highly metastatic B16F10 murine melanoma cells also aggregate on cellulose (CEL), but no focal contacts or stress fibers are formed, although these cells form well-defined focal adhesion complexes and stress fibers when they are spread on tissue culture polystyrene (tPS). This reflects the effective "outside-inside" transmembrane signaling produced by the attachment of integrins to substrate-adsorbed proteins [Pankov and Yamada, 2002; Wierzbicka-Patynowski and Schwarzbauer, 2003]. Our previous study [Hindié et al., 2005] focused on cell/matrix relationships when melanoma cells are aggregated on CEL for 48 h. We found that integrins interact with the fibronectin secreted at the surface of the aggregates. The present work

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analyses the cell–cell interactions and functions of melanoma cells aggregated on CEL for 48 h. Cadherins, the molecules involved in the cell–cell communication, belong to a large, diverse family of cell surface glycoproteins that promote calcium-dependent cell–cell adhesion [Vleminckx and Kemler, 1999]. They are also the transmembrane components of a number of cell junctions, and alterations in cadherin function have been implicated in tumorigenesis [Wheelock and Johnson, 2003]. N-cadherins mostly are expressed in melanoma [Perlis and Herlyn, 2004]. We first verified the part played by cadherins in the aggregation of B16F10 cells and assessed the synthesis and expression of N-cadherins. Cadherins mediate cell adhesion by interacting with the actin of the cytoskeleton via the intermediate proteins, the catenins (Gumbiner, 2000). We also compared the relationships between cadherin and catenin, and the β-catenin-N-cadherin binding in aggregated cells on CEL and in control cells spread on tPS. β-catenins are involved in signaling pathways that regulate cell growth and differentiation [Pirinen et al., 2001; Ryu et al., 2002; Wheelock and Johnson, 2003; Larue et al., 2003a,b; Charrasse et al., 2004]. We therefore compared the proliferation and cell-cycle progression of cells aggregated on CEL to those of control cells spread on tPS. Melanogenesis is a major feature of the differentiation of melanocytes and melanoma cells [Hearing and Jimenez, 1989]. Melanin synthesis starts with the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (L-Dopa), and is followed by the oxidation of L-Dopa to dopaquinone by tyrosinase, the rate limiting step [Hearing and Tsukamoto, 1991; Mishima, 1994]. Differentiation was assessed by comparing the melanin contents and tyrosinase activity of cells aggregated on CEL for 24 and 48 h, and cells grown on tPS. We believe that the cell aggregation induced by CEL is potentially a useful 3D culture model for studying the cell/matrix and cell–cell interactions of normal and cancer cells, and cell functions that result from these interactions.

MATERIALS AND METHODS

Cellulose Substrate

All studies were performed with cellulose derivatives E4M hydroxypropylmethylcellulose (HPMC) and 7LF carboxymethylcellulose (CMC) pharmaceutical grade products supplied by Colorcon (Dartford, UK) and the Benacel Company (Hercules International Limited, Rijswijk, The Netherlands). Bi-layers with HPMC 0.2 % (w/v) and CMC 0.2% (w/v) (CTTM, France) were prepared using polystyrene activated by glow discharges. The process is described in French patent no 2862979 (03-06-2005).

Cell Culture

The highly metastatic mouse melanoma cell line B16F10 was a generous gift from Dr. L. Larue (Institut Curie, Orsay, France). Cells were routinely cultured as monolayers at 37°C in RPMI 1640 (Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), in an atmosphere of 90% air, 10% CO_2. All tPS and cellulose-coated Petri dishes (CEL) were sterilized by incubating them for 1 h with penicillin (200 U/ml), and streptomycin (200 μg/ml), and washing them three times with ultra pure water.

Cell Aggregation and Calcium Deprivation

Cells were removed with 0.12% trypsin 0.05% EDTA, washed twice with Earle's balanced salt solution (EBSS), and the resulting single cells seeded at 2.5×10^4 cells/cm^2 on cellulose substrates. They were incubated in EBSS or EBSS plus 2 mM $CaCl_2$, both supplemented with 2% bovine serum albumin, for 1, 3, 24, and 48 h at 37°C in a humidified 90% air/10% CO_2 atmosphere. Photographs were then taken of three randomly chosen areas of each Petri dish to assess the part played by Ca^{2+} in cell aggregation. The numbers of single cells and cell aggregates (as defined by more than one cell per cluster) were counted (at least 1,500 cells were counted) and the data analyzed by the Tukey–Kramer test.

Immunostaining of Cadherins and β-Catenin

Cells were removed by trypsinisation, seeded on substrate at 10^4 cells/cm^2 and incubated for 48 h at 37°C. The aggregated cells were collected by centrifugation and washed in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. They were then cytocentrifuged (Shandon Cytospin®), fixed by incubation in 4% para-formaldehyde at room temperature for 15 min and permeabilized with phosphate buffered saline (PBS) containing 1% Triton X-100 (Promega) for 5 min at room temperature. The non-specific binding sites were

blocked by incubating the slides in PBS containing 1% BSA for 1 h at room temperature. The slides were then incubated with a rabbit polyclonal antibody raised against pan-cadherins (1:100 dilution, Sigma C3678) or with a mouse monoclonal antibody raised against β-catenin (1:100 dilution, Transduction Laboratories 610154). Bound specific antibodies were revealed by incubation with a Cy2-conjugated anti-rabbit antibody and Cy3-conjugated anti-mouse antibody (1:100 dilution, Jackson Immunoresearch Laboratories). Antibodies were diluted in PBS containing 0.1% BSA and labelings were carried out for 1 h at room temperature. Negative controls were performed without primary antibodies. The samples were then washed in PBS, mounted in mowiol and examined using a TCS-SP confocal Leica microscope (63× oil immersion objective).

Cadherin and β-Catenin Synthesis and Expression

Cadherin and β-catenin synthesis was assayed by RT-PCR. Total RNA was extracted from cells cultured for 48 h using RNABle (Eurobio); genomic DNA was removed with the RNeasy fibrous tissue mini kit (Qiagen) with RNase-free DNase (Qiagen). cDNA was obtained by reverse transcription (RT) of RNA (1 μg) using Moloney murine leukemia virus reverse transcriptase (SuperScript^{TM} RNase H^{-}, MMLV-RT Invitrogen) and random hexamers (Applied Biosystems) to prime the synthesis in conditions specified by the manufacturer.

Transcripts were assayed using the following primers: 5′-GCT GAG TAT GTC GTG GAG TC-3′ and 5′-TTG GTG GTG CAG GAT GCA TT-3′ for *GAPDH*, 5′-GCG GAG ACC TGT GAA ACT-3′ and 5′-GCC GTT TCA TCC ATA CCA-3′ for *N-cadherin*, and 5′-ATA TTG ACG GGC AGT ATG CA-3′ and 5′-TCA AAC TGC GTG GAT GGG AT-3′ for *β-catenin*. cDNA (1 μl) was amplified in a 20 μl reaction mixture containing 100 ng of each primers in 10 mM Tris-HCl pH 9.0; 50 mM KCl; 0.1% TritonX-100; 1.75 mM $MgCl_2$; 200 μM dNTP (Roche); and 1 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems). The reaction mixtures were heated at 94°C for 15 min, and amplified using sequential cycles at 94°C for 30 s, 58°C (GAPDH and N-cadherin) or 56°C (β-catenin) for 1 min, 72°C for 2 min 30 s, and 72°C for 7 min after the last amplification cycle. Amplified cDNA fragments were separated by electrophoresis on ethidium bromide-containing agarose gels.

The cadherin and β-catenin in cells cultured for 48 h were assayed by Western blotting. Cells (10^6) were lysed in 100 μl SDS–PAGE buffer (2% SDS, 0.5 M Tris-HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue), and heated to 100°C for 5 min. Lysates (20 μl) were electrophoresed in SDS–polyacrylamide gel, and the proteins transferred to nitrocellulose membranes (BioRad) using a semi-dry transfer cell (BioRad). The nitrocellulose membranes were stained with Ponceau red (Sigma) to check transfer efficiency. The membranes were incubated with mouse anti-β-catenin (Transduction Laboratories, diluted 1/500), mouse anti-N-cadherin (Zymed, diluted 1/1,000), or rabbit anti-pan-cadherin (Sigma, diluted 1/200) antibodies. Specific antigen-antibody binding was located by incubation with a peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (Uptima Interchim, diluted 1/2,000) and visualized with an enhanced chemiluminescence system (Amersham). The membranes were then washed with 0.5% Tween 20 in PBS, and incubated with mouse anti-actin antibody (C4, Chemicon, diluted 1/5,000) in order to quantify and normalize the results.

Immunoprecipitation was used to assess the binding of N-cadherin to β-catenin. Cultured cells were washed twice with ice-cold PBS, and lysed with lysis buffer (50 mM HEPES pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 100 μM sodium vanadate; 0.1 M NaF; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin) for 3 min at 4°C. Cell lysates were centrifuged at 16,000 g for 5 min, and the supernatants collected. Protein extracts (500 μg) were incubated with 2.5 μg monoclonal anti-β-catenin antibody (Transduction Laboratories) and 20 μl anti-mouse Ig-agarose beads (Sigma) on a rotary shaker for 2 h at 4°C. Supernatants were removed and the pellets washed three times with cold lysis buffer. The pellets were then suspended in SDS–PAGE buffer, boiled for 10 min, centrifuged, and the clear supernatants loaded on acrylamide gels.

Cell Proliferation and Cell Cycle Analysis

Cells were seeded on tPS and CEL at 10^4 cells/cm^2, and incubated at 37°C for 24 or 48 h. Cells were then removed from tPS by incubation with 0.12% trypsin—0.05% EDTA for 2 min at 37°C and 2 min at room temperature. The trypsin was neutralized with FBS, and the Petri dishes rinsed three times with medium. The cells on

CEL were collected by centrifugation for 5 min at 200*g* and suspended in 500 μl trypsin-EDTA for 5 to 10 min at 37°C to dissociate cell aggregates. Five hundred microliters FBS was added, and the viable cells counted by trypan blue exclusion. Cell proliferation is represented by the index N/N_0, where N is the total number of cells at $T = 24$ or 48 h, and N_0 the number at $T = 0$.

Cell cycle analysis was performed on cells grown on tPS or CEL for 48 h. They were removed by trypsinization, counted, harvested, and washed twice in PBS containing 5 mM EDTA (Prolabo). Cells were then suspended in 250 μl PBS-EDTA, fixed by adding 750 μl ice-cold absolute ethanol for 45 min at 4°C, and washed with PBS-EDTA. The washed cells were suspended in PBS-EDTA containing 0.1% Triton X-100 (Promega), mixed with 40 μg RNase A (Sigma), and 25 μg propidium iodide (Sigma), and incubated for 15 min protected from light. The stained samples were analyzed in an Epics XL-MCL flow cytometer (Beckman Coulter). Histograms were analyzed using Wincycle software.

Cell Differentiation

The melanin content of cells was evaluated by the method of De Pauw-Gillet et al. [1990]. Briefly, cell aggregates grown for 48 h on CEL were collected, centrifuged at 200*g* (5 min) and dissociated by incubation with 0.12% trypsin-0.05% EDTA at 37°C for 6–10 min. An equal volume of FBS was added and the cells were collected by centrifugation. They were then gently suspended in PBS containing 10 mM glucose, 0.1% BSA, 30 mM HEPES, and counted. The cell pellets (5×10^5) were dissolved in 1 ml 1 N NaOH 10% DMSO and incubated for 2 h at 60°C. Melanin was assayed by measuring the absorbance at 450 nm with a Hitachi spectrophotometer and amounts were calculated from a standard curve prepared with commercial melanin (Sigma). Absorbance was linear between 1 and 50 μg/ml. Control cells were cultured on tPS. They were rinsed with PBS, dissociated with trypsin-EDTA, and melanin measured as above. The cell melanin content was calculated by the index M/M_0. M = melanin (μg/5×10^5 cells) in cells cultured on tPS and CEL for 48 h; M_0 = melanin (μg/5×10^5 cells) in cells before seeding (T_0). Tyrosinase activity was assayed by the modified method of Steinberg and Whittaker [1976]. Cell aggregates were collected by centrifugation at 200*g* for 5 min, rinsed with PBS and dissociated with 0.12% trypsin-0.05% EDTA. An equal volume of FBS was added to each cell suspension and the mixtures were centrifuged (200*g*, 5 min). The cell pellets were suspended in PBS containing 10 mM glucose, 0.1% BSA, 30 mM HEPES, and counted. The cell concentrations were adjusted to 5×10^5 cells per ml of PBS containing 1% Triton at 4°C for 2 h. The resulting cell lysates were clarified by centrifugation at 3,000*g* for 10 min at 4°C. The supernatants were incubated with 1.5 ml sodium phosphate buffer (0.1 M, pH 6.8) containing 0.04% L-3,4-dihydroxyphenyl-alanine (L-Dopa, Sigma) for 10 and 60 min at 37°C, and absorbance read at 450 nm. The tyrosinase activity was calculated by the index A/A_0. $A = \Delta OD$ at 10 and 60 min (5×10^5 cells) of cells before seeding (T_0). The results are the means of three determinations performed in triplicate.

Statistical Analysis

Data are presented as the means of triplicate measurements. One-way ANOVA and the Kruskal–Wallis test were used to test for differences between the groups, followed by the Tukey–Kramer test for multiple comparisons. All statistical computations were performed with Graphpad Instat® 2.00 software. Values were considered significantly different if the P was <0.05.

RESULTS

Cell–Cell Interactions on a Cellulose Substrate

The kinetics of melanoma cell aggregation on CEL for 3, 24, and 48 h under normal culture conditions (culture medium supplemented with 10% FBS) are shown in Figure 1a–c and referred to cell spreading on control tPS (d-e-f). Cell aggregation was already well developed 3 h post-seeding on CEL (Fig. 1a). The aggregates were attached to the substrate at a few points and gradually coalesced to form a single huge aggregate 48 h post-seeding (Fig. 1c). Control cells on tPS were well spread at all times (Fig. 1d–f).

Cadherins promote calcium-dependent cell–cell adhesion and N-cadherins are most frequent in melanoma cells. We first verified the part played by Ca^{2+} in the aggregation of B16F10 cells.

Cells were deprived of Ca^{2+} and serum by incubation in EBSS supplemented with 2%

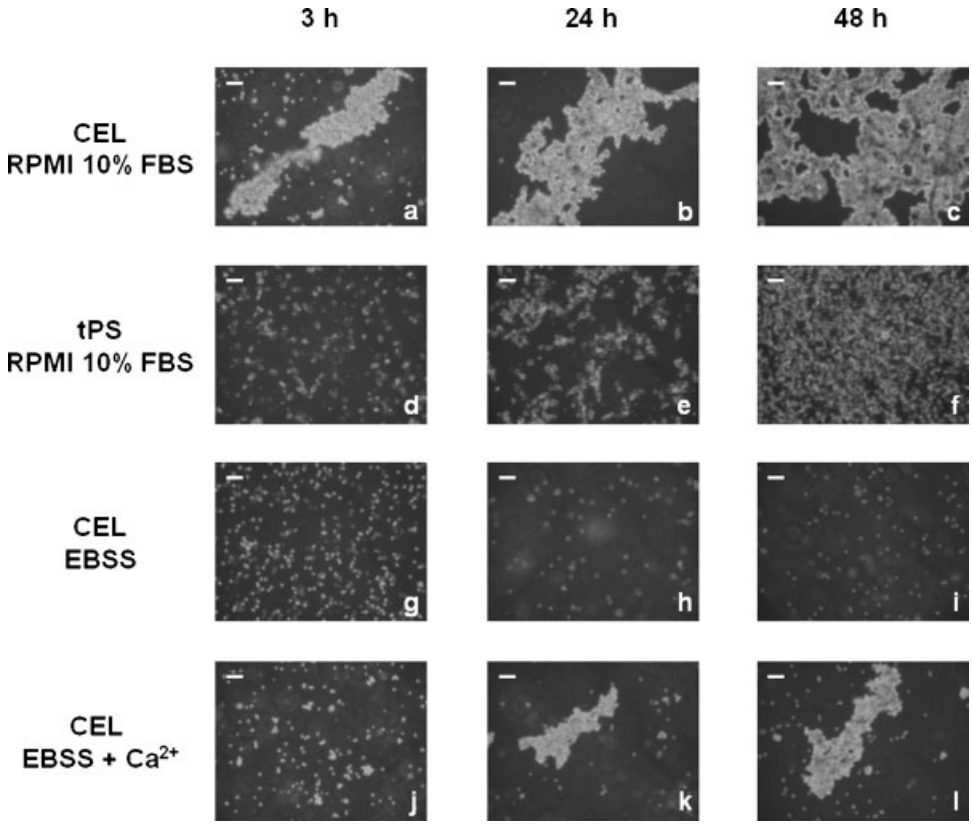

Fig. 1. Aggregation assays. B16F10 cells were incubated on CEL or tPS for 3, 24, and 48 h in the presence (**a-b-c**, **d-e-f**, **j-k-l**) or absence of Ca^{2+} or serum (**g-h-i**). Microphotographs illustrate the aggregation of cells grown on CEL plus Ca^{2+} as soon as 3 h (a-b-c, j-k-l). Cells incubated without Ca^{2+} do not aggregate on CEL (g-h-i). Control cells on tPS spread (d-e-f). Bar 100 μm.

BSA. Cells on CEL for 3, 24, and 48 h, but without Ca^{2+} aggregated much less (Fig. 1g–i) than control cultures in EBSS with Ca^{2+} (Fig. 1j–l). The percentage of aggregated cells in cultures on the cellulose substrate without Ca^{2+} for 60 and 180 min was assessed by counting clustered and single cells. It increased by $16.4\% \pm 6.2$ without Ca^{2+} and by $59.8\% \pm 19.8$ with Ca^{2+} after 1 h ($P < 0.01$), and by $35.5\ \% \pm 3.3$ without Ca^{2+} and by $89.1\% \pm 6.5$ with Ca^{2+} after 3 h ($P < 0.001$). Cell trypsinization broke down cell surface cadherins, but they gradually reappeared after 1 and 3 h in cells on CEL (Fig. 2a).

RT-PCR and Western blotting of extracts of cells aggregated on cellulose and control cells spread on tPS for 48 h showed N-cadherin mRNA and protein (Fig. 2b). The amount of *N-cadherin* mRNA seemed to be decreased and that of N-cadherin protein increased in cells on CEL. E-cadherin protein was not found in any cells (results not shown). The amounts of *β-catenin* mRNA in the aggregated and control cells appeared to be similar, but there was less β-catenin protein in the aggregated B16F10 cells (Fig. 2b). Immunoprecipitation of the N-cadherin β-catenin complex showed that β-catenins were bound to N-cadherins (Fig. 3a). Double immunostaining with anti β-catenin and anti pan-cadherin antibodies showed that cells on CEL were labeled subjacent to the external cell layer of the aggregate (Fig. 3b,c). The outer cell surface of the aggregate was negative. Labeling was observed only when cells were in contact, in deeper layers. The core of the aggregate was not labeled, probably because antibodies were unable to reach it.

Catenins and cadherins were both located close to cell membrane, at cell–cell contacts in the aggregates on CEL (Fig. 3b,c), suggesting that catenins bound to cadherins formed a functional junctional complex.

Cell Functions

β catenins are involved in signaling pathways that regulate cell growth and differentiation. We compared cell counts and cell cycle progression of B16F10 melanoma cells cultured on CEL

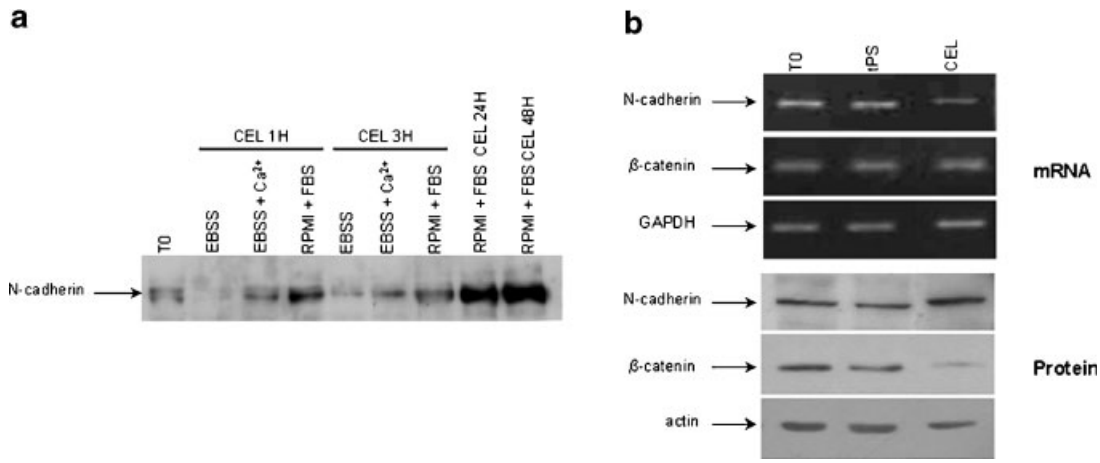

Fig. 2. **a**: N-cadherin analyzed by immunoblotting after incubation for 0, and 1, 3, 24, and 48 h with or without Ca^{2+}. Cadherin re-appears within 1 h at the cell membrane of cells grown on CEL with Ca^{2+}. Cells grown without Ca^{2+} contain little or no cadherin. **b**: N-cadherin and β-catenin in B16F10 cells cultured on tPS or CEL for 48 h. N-cadherin and β-catenin mRNAs analyzed by RT-PCR and proteins by immunoblotting. T_0 = B16F10 cells before seeding on tPS or CEL. *GAPDH* gene expression and actin protein were used as controls for gel loading.

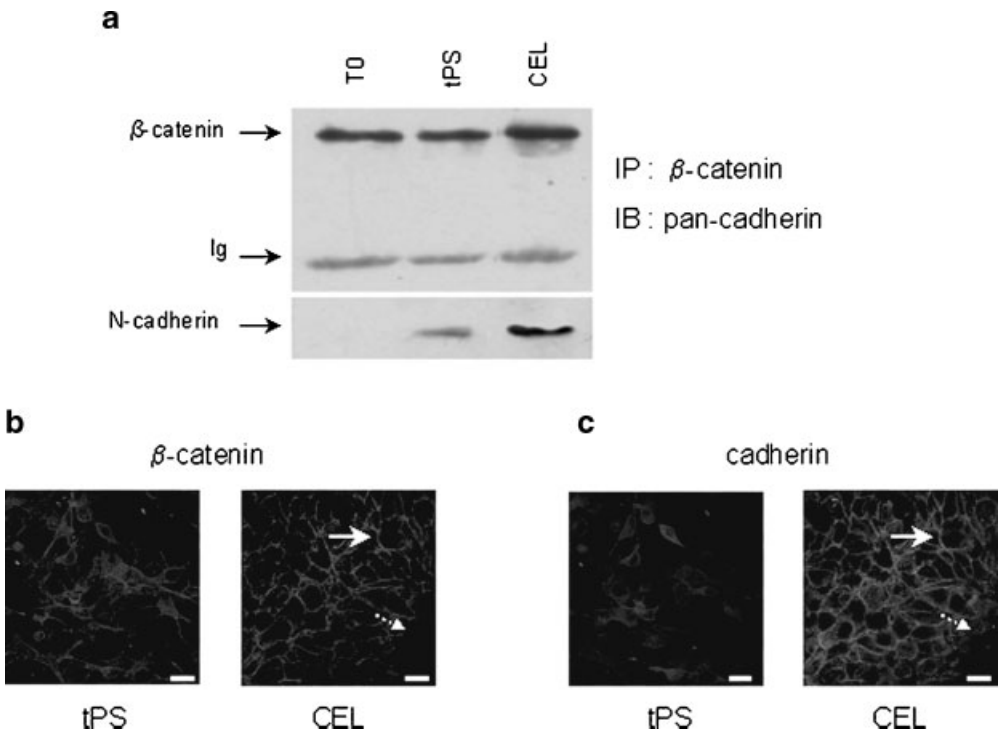

Fig. 3. Distributions of N-cadherin and β-catenin in B16F10 cells. **a**: Immunoprecipitation of B16F10 cell lysates with anti β-catenin antibody. The immunoprecipitates were Western blotted to detect β-catenin and N-cadherin. No N-cadherin was found in the T_0 samples because of cell trypsinization. Immunofluorescence of aggregated B16F10 cells stained for β-catenin (**b**) and cadherins (**c**) examined using a TCS-SP confocal Leica microscope, 63× oil immersion objective. Both β-catenin and cadherins are located at the cell–cell contacts on CEL (plain arrows). On tPS, β-catenin, and cadherin are diffused through cytoplasm (dotted arrows). Bar 20 μm.

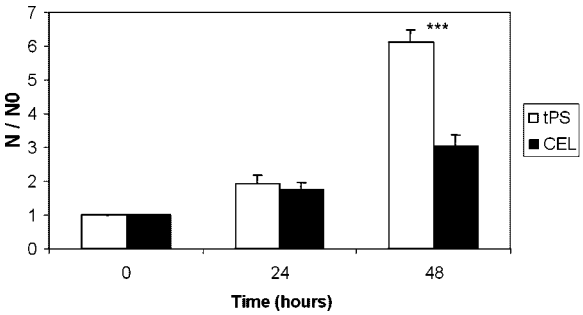

Fig. 4. Proliferation of B16 F10 cells on tPS and CEL. Proliferation of B16F10 cells on tPS and CEL after 24 and 48 h. Cell proliferation is represented by the index N/N_0, where N is the total number of cells at $T = 24$ or 48 h and N_0 the number at $T = 0$. Results are mean ± SD of three experiments each performed in triplicate. Asterisks (***) indicate significant differences in the proliferation of cells on tPS and CEL (Tukey–Kramer test) with $P < 0.001$.

and tPS. We also checked to see if melanogenesis, a major feature of the differentiation of melanoma cells, was increased in cells on CEL.

Cell proliferation was dramatically reduced on CEL (Fig. 4). The cell-cycle progression of cells aggregated on CEL and those spread on tPS for 48 h was analyzed by FACScan (Table I). There were significantly more cells in the G_1 phase in the cultures on CEL than in the controls, while there were many fewer in S.

Cells aggregated on CEL looked darker than controls after 48 h in culture. The cells on CEL contained more melanin (2.19 ± 0.74) than did the control cells on tPS (1.07 ± 0.21, Tuckey–Kramer test: $P < 0.01$). Tyrosinase activity was increased in parallel with the melanin (1.095 ± 0.35 in cells on CEL and 0.66 ± 0.28 in cells on tPS, Tuckey–Kramer test: $P < 0.01$).

DISCUSSION

The aggregation of B16F10 melanoma cells grown for 3, 24, and 48 h on CEL demonstrates how effectively the cellulose substrate provides full 3D cultures after 24 and 48 h. The results obtained with cells grown without Ca^{2+} and serum suggest that the aggregation of melanoma cells on CEL is Ca^{2+} dependent. Blocking vitronectin and fibronectin receptors with the disintegrin echistatin (a potent inhibitor of the binding of all RGD-dependent integrins to their natural ligands [Smith et al., 2002]) or the peptide RGD does not modify the aggregation of cells seeded on CEL (results not shown). Therefore, the aggregation of melanoma cells on CEL appears to be mediated by Ca^{2+} and does not involved RGD-dependent integrins. Li et al. [2001] and McGary et al. [2002] examined the role of N-cadherin in the development and progression of melanoma and found that N-cadherin mediated the homotypic aggregation of melanoma cells and their heterotypic adhesion to dermal fibroblasts and vascular endothelial cells. A loss of E-cadherin is associated with a change in the concentration and distribution of the transcription regulator β-catenin in vivo [Johnson, 1999], and melanoma cells are rich in N-cadherins in vitro [Tang et al., 1994; Hsu et al., 1996; Scott and Cassidy, 1998; Li et al., 2001]. Jouneau et al. [2000] demonstrated N-cadherins by RT-PCR, Northern blotting and Western blotting using B16 F10 melanoma cells and E cadherins only by RT-PCR. M, P, R, or 11 cadherins were not detected by any of the methods used.

TABLE I. Cell Cycle Distribution of B16F10 Cells Cultured on tPS or CEL for 48 h

	tPS	CEL
G_1	62.9 ± 1.05[a]	84.1 ± 4.9***
S	28.3 ± 3.5	10.2 ± 5.1***
G_2/M	8.8 ± 3.4	5.7 ± 1.2 (NS)

Results are means ± SD of three experiments performed in triplicate. Asterisks (***) indicate significant difference (Student's paired t-test: $P < 0.0001$) between tPS and CEL. NS, non significant.
[a]Percentage of cells.

Our results indicate that N-cadherins are synthesized by both cells aggregated on CEL for 48 h and by cells spread on tPS. Nevertheless, the decreased N-cadherin mRNA concentration in cells on CEL, together with the increased N-cadherin protein, suggests that the N-cadherin gene is downregulated in cells on CEL. Recent observations have shown that N-cadherin synthesis is regulated by the loss of E-cadherin due to NFkappaB activity [Kuphal and Bosserhoff, 2006]. Further investigation is now required using longer culture times on CEL to assess the E-cadherin and NFkappaB status of these cells. B16F10 cells on CEL seem to contain less β-catenin than do control cells. 3D spheroid anchorage-independent B16F10 cultures also seem to contain less alpha and gamma catenins than their 2D counterparts [Shiras et al., 2002].

The synthesis of cadherin and catenin varies greatly and depends on the cell environment as well as their transformation status [Jouneau et al., 2000]. Demunter et al. [2002] recently

used a combination of immunohistochemistry and RT-PCR to show that a reduction in membrane-bound β-catenin is associated with increased amounts of *β-catenin* mRNA in primary and metastatic melanomas. They suggest that postranslational events are responsible for the altered distribution of β-catenin in cutaneous melanomas. Our results suggest that the decreased β-catenin content is also due to a postranslational event. The immunoprecipitation of β-catenin with N-cadherins demonstrates the integrity of the junctional complex in cells aggregated on CEL. Catenins are also involved in the Wnt signaling pathways that regulate cell proliferation and differentiation. We find that cell proliferation is significantly decreased after 48 h on CEL and the cellulose coating causes cells to arrest in G_1. Widlund et al. [2002] have shown that β-catenin is a potent mediator of the growth of melanoma cells, with MITF as a critical downstream target: disruption of the Wnt pathway abrogates the growth of melanoma cells and the constitutive overproduction of MITF overrides this blockade. Endogenous β-catenin has been found at the MITF-promoter in B16 melanoma cells [Widlund et al., 2002]. Further studies are now required to assess the status of the Wnt pathway in our experimental conditions.

We also compared melanin synthesis and the tyrosinase activity in cells aggregated on cellulose and controls grown on tPS, as catenins are known to participate in signaling pathways that regulate cell differentiation [Ryu et al., 2002]. We find that melanogenesis is induced in cells aggregated on CEL, in agreement with De Pauw-Gillet et al. [1988] for B16 melanoma spheroids. 3D spheroid anchorage-independent cultures [Shiras et al., 2002] have been suggested as an experimental model for tumorigenesis, and melanoma multicellular aggregates in suspension have been used by several authors [De Pauw-Gillet et al., 1988; LaRue et al., 2004; Ghosh et al., 2005; Jorgensen et al., 2005]. Cells proliferate relatively rapidly [De Pauw-Gillet et al., 1988], depending on culture conditions. Aggregates on CEL are anchored to the substrate by a few cells under our experimental conditions. We find a greater percentage of cells on CEL are arrested in G_1 at 48 h than do others who have used human melanoma cells and mouse mammary carcinoma spheroids [LaRue et al., 2004]. We believe that 3D cultures on cellulose substrates, useful for studies on cell–cell communication [Faucheux et al., 2004] and apoptosis [Gékas et al., 2004], are also an excellent model for studies on the mechanisms by which cell proliferation and differentiation are modulated in melanoma cells. They may also provide a novel diagnostic tool for evaluating the residual capacity of human melanoma cells to regulate proliferation, differentiation, and apoptosis.

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