E-cadherin Regulates Anchorage-independent Growth and Survival in Oral Squamous Cell Carcinoma Cells*

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Integrin-basement membrane interactions provide essential signals that promote survival and growth of epithelial cells, whereas loss of such adhesions triggers programmed cell death. We found that HSC-3 human squamous carcinoma cells survived and grew readily as monolayers, but when they were suspended as single cells, they ceased proliferating and entered into the apoptotic death pathway, characterized by DNA fragmentation. In contrast, if the suspended carcinoma cells were permitted to form E-cadherin-mediated multicellular aggregates, they not only survived but proliferated. However, aggregated normal keratinocytes were unable to survive in suspension culture and became apoptotic. Anchorage independence and resistance to apoptosis of HSC-3 cell aggregates required high levels of extracellular Ca\(^{2+}\) and was inhibited with function-perturbing anti-E-cadherin antibody. Resistance to suspension-induced apoptosis in cell aggregates paralleled the up-regulation of Bcl-2 but occurred in the absence of focal adhesion kinase activation. Analysis of suspension-induced death in a set of clonal squamous epithelial cell lines with different levels of E-cadherin expression revealed that receptor-positive cell clones evaded apoptosis and proliferated in three-dimensional aggregate culture, whereas cadherin-negative clones failed to survive. Collectively, these observations indicate that cadherin-mediated intercellular adhesions generate a compensatory mechanism that promotes anchorage-independent growth and suppresses apoptosis.

In stratifying normal epithelium, proliferation is largely confined to the basal layer of cells attached to the basement membrane, and as the cells move to the suprabasal layers, they undergo terminal differentiation. This anchorage-dependent growth requires integrin-mediated signaling generated by cellular contact with extracellular matrix (ECM)\(^1\) ligands (1). When anchorage is lost, normal epithelial cells undergo programmed cell death (2). Apoptosis is a genetically controlled response for cells to commit suicide. The terms apoptosis and programmed cell death are often used interchangeably to describe a mechanism of cellular death that is believed to play an important role in a wide variety of physiological situations and that, when dysregulated, can contribute to the pathogenesis of many diseases.

However, in certain pathologic conditions such as psoriasis, dysplasia, and neoplasia, there is evidence of suprabasal proliferation when cells are no longer in direct contact with the basement membrane (3–5). This raises the question: what factors promote the growth of tumor cells in the absence of ECM anchorage? One possibility is cadherin-mediated cell-cell contacts. The cadherins are cell-surface membrane proteins that mediate homophilic calcium-dependent cell-cell adhesion, which is crucial for the structural organization and differentiation of cells (6–8).

It is interesting that integrin and cadherin receptors have many parallels beyond their common role in regulating cell adhesion: both couple to the actin cytoskeleton to form adhesion plaque structures, both require divalent cations for activity, and both transduce intracellular signals when engaged. Several studies have examined epithelial anchorage dependence in terms of connections between integrin signaling and apoptosis-regulating genes (9, 10). Peluso et al. (11) reported that N-cadherin-mediated cell contacts inhibit granulosa cell apoptosis. However, to date, little is known about whether cadherin-mediated cell-cell contacts can regulate cell growth. In view of the established role for E-cadherin in morphology and function and our initial observations that suspended squamous epithelial cells form compacted multicellular aggregates, we hypothesized that cadherin-mediated cell-cell contacts could substitute for integrin-ECM adhesion to prevent apoptosis and promote growth in anchorage-deprived cells.

The overall goals of the present study were to determine whether epithelial cell-cell adhesion can prevent suspension-induced apoptosis and whether these interactions can facilitate anchorage-independent growth. We show that squamous carcinoma cells can use either cell-substrate or cell-cell adhesion for survival and growth. In the case of cell-substrate interactions, growth information is transmitted via integrin receptors, whereas cell-cell contacts appear to generate survival and proliferation cues through cadherins. Presumably, the overlapping information transduced by both types of adhesion receptors requires receptor-induced cytoskeletal organization and concurrent change in cell shape (i.e., spreading and compaction, respectively). The signals transduced by integrins are just now being identified, but cadherin-based signals are yet to be defined. Whether cells are susceptible or resistant to apoptosis induced by loss of anchorage is a potentially crucial step during tumor progression. We conclude that cadherin-mediated cell-cell adhesion is a novel mechanism by which epithelial cells can regulate cell growth and programmed cell death.

**EXPERIMENTAL PROCEDURES**

Cell Culture—The human oral squamous carcinoma cell lines HSC-3 and HOC-313 have been described previously (12). Both cell lines are...
tumorogenic in nude mice (13). Cells were cultured in serum-free Dulbecco’s modified Eagle’s medium supplemented with insulin, transferrin, and selenium (Life Technologies, Inc.). HOC-313 cells were cloned by serial dilution of a single-cell suspension plated in 96-well plates. Single-cell clones were randomly selected for further culture. Human skin keratinocytes (HSC-3) were cultured as described previously (14). The keratinocytes were plated in tissue culture dishes and maintained in serum-free keratinocyte growth medium (Clonetics Corp., San Diego, CA). The medium was changed every 3 days. Cells from passages 2–5 were used for the experiments.

Cell monolayer cultures were prepared by seeding 6 × 10³ tissue culture dishes (10 cm; Falcon) for culture of HSC-3 and 6 × 10³ HOC-313 cells in suspension, monolayers were briefly treated with EDTA to prepare single cells. To generate multicellular aggregates (MCAs), cells were then plated on polyhydroxyethylmethacrylate (poly-HEMA)-coated 60-mm dishes (6 × 10⁵ cells/dish) in the presence of Dulbecco’s modified Eagle’s medium supplemented with insulin, transferrin, and selenium/Dulbecco’s modified Eagle’s medium containing 1.5% methicellulose (Sigma) at 6 × 10⁵ cells/10 cm poly-HEMA-coated dishes. Keratinocytes were processed for multicellular aggregation by briefly treating monolayers with trypsin-EDTA to prepare single cells, which were then plated on poly-HA-coated dishes (6 × 10⁵ cells/dish) in the presence of serum-free keratinocyte medium supplemented with 1 mM Ca²⁺ for 24 h. Single-cell suspension cultures of keratinocytes were prepared as above, but semisolid medium supplemented with 1 mM Ca²⁺ was used.

Cell Growth Assays—To measure [³H]thymidine incorporation into DNA, 6 × 10⁵ cells were plated on dishes as described above; 1 μCi [³H]thymidine was added to each of the dishes at selected time periods, and the cells were labeled for 24-h periods. The dishes were washed three times with Hank’s balanced salt solution, and cells were collected by centrifugation. The cell pellet was extracted with 2 ml of ice-cold 10% trichloroacetic acid, the washed pellet was dissolved in 0.1 ml formic acid, and radioactivity was determined in a scintillation counter. Data are expressed as the mean of six dishes.

Multicellular aggregates were assessed for growth by determining the total number of cells in the MCA cultures, using the method described by Freyer and Sutherland (15). Cells (6 × 10⁵) were plated in tissue culture dishes as monolayer, suspended single-cell, or MCA cultures. At selected times, cells were collected and processed for counting using a hemocytometer. Results are expressed as the mean of three dishes.

DNA Fragmentation—To assay for intranucleosomal DNA cleavage, low molecular weight genomic DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, and then phenol-chloroform extracted three times and precipitated with ethanol. Samples were then analyzed in a 1.5% agarose gel with 0.25 μg/ml ethidium bromide, visualized by UV fluorescence, and photographed (9). Cells were grown as monolayers, suspended single cells, or MCAs for 72 h. Cells were then harvested from poly-HEMA dishes by pipetting or from tissue culture dishes by scraping into the medium in which they had been incubated. In monolayer cultures, floating cells were collected and combined with the attached cells before DNA extraction.

TUNEL Detection—The suspended single cells and MCAs from 72-h cultures were collected, fixed in freshly prepared paraformaldehyde buffered with 0.1 M sodium phosphate (pH 7.4), and embedded in agar (1%). The agar blocks were dehydrated with graded ethanols and embedded in paraffin according to standard procedures. The technique for in situ visualization of DNA fragmentation was carried out according to the method described by Mori et al. (16), which was a modification of the one developed by Gavrieli et al. (17) using a commercially available kit (MBL Laboratories, Watertown, MA). After staining, samples were processed for examination with a Nikon fluorescence microscope using filters for fluorescein isothiocyanate and rhodamine.

Studies with Divalent Cations and Blocking Antibody—For analysis of divalent cations, HSC-3 monolayers, suspended single cells and MCAs were seeded at 6 × 10⁵ cells/plate in the presence Ca²⁺- and Mg²⁺-free insulin, transferrin, and selenium/Dulbecco’s modified Eagle’s medium, which was supplemented with high (1 mM) or low (data not shown) concentrations of CaCl₂ and MgCl₂ at the indicated concentrations. To study the effect of blocking antibody to E-cadherin, HSC-3 cells were seeded at 6 × 10⁵ cells/plate in the presence of mouse anti-human E-cadherin mAb (clone SHE78-7, Zymed Laboratories) at 1 μg/ml and incubated for up to 72 h (18, 19). Mouse IgG added to monolayer, single cell, and MCA cultures at 1 μg/ml was used as control. [³H]Thymidine incorporation and DNA ladder assays were performed with the cell cultures as described above.

Immunoprecipitation and Immunoblotting—We estimated levels of cadherins, Bel-2, and activation level of focal adhesion kinase (FAK) by immunoblotting. Protein lysates were processed for immunoprecipitation with mouse anti-human Bel-2 antibody (sacite, Dako) and antibody against FAK (2A7, Upstate Biotechnology, Inc.). Immune complexes were recovered with protein A-agarose (13).

Immunoblotting was performed as described previously (13). The immunoprecipitates or total cellular lysates were fractionated by SDS-polyacrylamide gel electrophoresis, were transferred to Immobilon membranes, and probed with an anti-E-cadherin monoclonal antibody (from Dr. Caroline Damsky, University of California, San Francisco), anti-human P-cadherin monoclonal antibody (Transduction Laboratories), anti-human N-cadherin polyclonal antibody (Zymed Laboratories), anti-human Bel-2 antibody (Dako) or anti-phosphotyrosine antibody (PY20, Upstate Biotechnology, Inc.). Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech).

RESULTS

MCAs, but Not Single Cells, Proliferate in Suspension Culture—The HSC-3 squamous epithelial cells proliferate rapidly in conventional monolayer culture even in the absence of serum (Fig. 1A). However, when HSC-3 cells were deprived of their anchorage by suspension in methylcellulose as single cells in poly-HEMA-coated dishes, they failed to proliferate and were unable to incorporate [³H]thymidine. In contrast to single cells in suspension, HSC-3 cells that were permitted to form MCAs in suspension were able to proliferate, as assessed by incorporation of [³H]thymidine and corresponding increase in total cell number (Fig. 1, B and C). Similar results were obtained with the MTT reduction assay (data not shown). Although the proliferation rate of MCAs was significantly less efficient than that displayed in monolayer culture, the cell aggregates were clearly able to survive and replicate. FACS analysis of propidium iodide-stained MCAs showed a reduced fraction in G2/M as compared with monolayers and a higher fraction in G0; in contrast, suspended single cells had reduced numbers in the S and G2/M fractions as compared with the multicellular aggregates with a larger proportion of cells in G0/G1.

Suspected Single Cells, but Not MCAs, Undergo Programmed Cell Death—By morphological criteria, the suspended single HSC-3 cells appeared abnormal and displayed signs of spontaneous cell death. To assess whether this process represented programmed cell death (PCD), cultures of suspended single cells or MCAs and control monolayer cultures were analyzed for nucleosomal fragmentation and in situ DNA end-labeling. For detection of DNA laddering, genomic DNA was isolated and analyzed for degradation by display in agarose gels (Fig. 2A). Whereas only high molecular weight DNA was detected in both monolayer (Fig. 2A, lanes 2 and 5) and MCA cultures (lanes 4 and 7), DNA isolated from 3- and 6-day single-cell suspension cultures revealed extensive DNA laddering typical of apoptotic intranucleosomal cleavage (Fig. 2A, lanes 3 and 6). Usually, the degraded DNA fragments banded at 200–300 bp and above. In other experiments, the appearance of DNA fragmentation after suspension of single cells was monitored; at 12 h postseeding, DNA fragmentation was not evident, but by 24 h, there was significant PCD (data not shown). Moreover, analysis of annexin levels by fluorescence-activated cell sorting showed extensive expression only in the suspended single cells but not in monolayers or MCA cultures (data not shown).

We also analyzed DNA isolated from monolayer and suspension cultures of human foreskin keratinocytes to assess whether survival upon MCA formation can occur in normal epithelial cells. Human foreskin epithelial cells were grown as monolayers, suspended single cells, and MCAs. DNA was an-
alyzed for nucleosomal fragmentation after 24 h of culture as described above for HSC-3 cells. Monolayer cultures of keratinocytes showed no detectable DNA fragmentation (Fig. 2A, lane 2), whereas both suspended single cells and MCA demonstrated extensive DNA laddering (Fig. 2B, lanes 3 and 4, respectively).

Next, we analyzed DNA fragmentation to identify apoptotic cells by processing preparations of suspended HSC-3 single cells and multicellular aggregates for the TUNEL labeling method. Suspended single cells (Fig. 2C, panel a) and MCAs (Fig. 2C, panel c) were stained for DNA with propidium iodide to identify nuclei. Fragmented DNA was labeled with dUTP-fluorescein. In the 3-day cultures of suspended single cells, a majority (~90%) displayed nuclear fragmentation, indicating a marked induction of apoptosis (Fig. 2C, panel b). However, in the MCAs, only a minor and variable population of cells (~4%) were apoptotic (Fig. 2C, panel d). In most cases, the small fraction of positive cells in MCAs appeared at the outer boundary of the MCA or in single cells, suggesting that these cells had failed to properly integrate into the aggregates.

Cell-Cell Adhesion-induced Escape from PCD Is Ca²⁺-dependent—Epithelial cells require Ca²⁺ to form homophilic cell-cell adhesions (6). We therefore tested whether high concentrations of divalent cation were necessary for the survival and growth of suspension cultures of HSC-3 cells. Monolayer and MCA cultures were initiated in culture medium containing the following combinations of Ca²⁺ and Mg²⁺ at high (1 mM) or low (0.09 mM) concentrations: 1) high Ca²⁺ and low Mg²⁺, 2) low Ca²⁺ and high Mg²⁺, and 3) low Ca²⁺ and low Mg²⁺. In monolayer cultures, HSC-3 cells proliferated well in standard medium containing 1 mM of each cation (Fig. 1A) or in medium containing either high Ca²⁺ or high Mg²⁺ but failed to proliferate when both divalent cations were supplied at low concentration (Fig. 3A). In contrast, when cells were cultured as MCAs, proliferation was evident only when Ca²⁺ was present at high concentration (Figs. 1B and 3B). If low Ca²⁺ was used for MCA cultures, no growth occurred, and there was actually a significant decrease in cell number regardless of the Mg²⁺.

**Fig. 1. Growth of HSC-3 monolayers (ML), suspended single cells (SC), and multicellular aggregates (MCA).** HSC-3 cells (6 × 10⁵) were plated on tissue culture dishes (for MLs) or on poly-HEMA-coated dishes (for MCAs) or on poly-HEMA-coated dishes in the presence of 1.5% methylcellulose (for SCs). A, growth of ML and SC cultures as assessed by [³H]thymidine incorporation. To measure DNA synthesis, cultures were labeled with [³H]thymidine for 24 h after their respective growth periods and then processed as described under “Experimental Procedures.” Values are means of six dishes; bars show S.D. B, growth of HSC-3 cells grown in suspension as MCAs and SCs as assessed by [³H]thymidine incorporation. Values are means of six dishes; bars show S.D. C, growth of HSC-3 cells in MCA or SC culture as assessed by cell number. HSC-3 cells (6 × 10⁵) were cultured as described in A for SCs and MCAs. At the indicated time intervals, cells were collected, and total cell number was determined from each dish. The values are the mean of triplicate dishes for each time point; bars show S.D.
HSC-3 multicellular aggregates under the different growth conditions were evaluated for DNA fragmentation. Culturing of MCA in high Ca\textsuperscript{2+} and high Mg\textsuperscript{2+} did not produce apoptosis (Fig. 2A). DNA laddering was detected only in MCA cultures grown without high Ca\textsuperscript{2+}, and substituting 1 mM Mg\textsuperscript{2+} for Ca\textsuperscript{2+} failed to rescue the cells from suspension-induced apoptosis (Fig. 3C, lane 5). Monolayer cultures showed no evidence of PCD when either high Ca\textsuperscript{2+} or high Mg\textsuperscript{2+} was provided; only when medium contained low levels of both divalent cations was apoptosis evident in the monolayer cultures (not shown). Because integrins are known to function effectively in the presence of Mg\textsuperscript{2+} and are usually inhibited in high Ca\textsuperscript{2+} (20), these results suggest that the rescue from apoptosis following the formation of survival-promoting cell-cell adhesions in MCAs is not mediated by integrins but via Ca\textsuperscript{2+}-dependent cell contacts. It is well known that cadherins are known to form epithelial cell-cell adhesions through a Ca\textsuperscript{2+}-dependent mechanism (6, 21).

Anti-cadherin Antibody Suppresses MCA Growth and Induces PCD—We analyzed the stepwise formation of HSC-3 cell aggregates, which begins with single cells that subsequently form cell chains by 8 h, collect into loose, irregular clumps of cells by 12 h, condense into compacted MCAs with smooth margins between 15 and 24 h, and remain viable with evidence of growth at 72 h (Fig. 4, A–F). Because squamous epithelial cells are known to use E-cadherin as the primary cell-cell adhesion molecule, we tested the effect of a function-perturbing anti-E-cadherin mAb on multicellular aggregate formation. When the cells were cultured in the presence of mAb SHE78-7, the formation of the large, compact aggregates was severely inhibited at 24 h (Fig. 4G) and 72 h (Fig. 4H). Although some limited cell aggregates formed in the presence of the antibody, they were of smaller size, with many visible single cells, and failed to compact, remaining loosely adherent. These effects of mAb SHE78-7 indicated that the assembly and compaction of multicellular aggregates involves functional E-cadherin.

We next tested whether mAb SHE78-7 could block proliferation of HSC-3 monolayer, suspended single-cell, or MCA cultures. In these experiments, cultures were initiated with or without blocking SHE78-7 antibody, and after 72 h the incorporation of [\textsuperscript{3}H]thymidine by the cells was measured. The SHE78-7 mAb had no significant effect on the proliferation of HSC-3 cells in monolayer cultures, but [\textsuperscript{3}H]thymidine incorporation by MCA suspension cultures was inhibited by nearly 60% (Table I). The blocking antibody did not significantly alter the low level of [\textsuperscript{3}H]thymidine incorporation in suspended single cells during the same period. Inclusion of an irrelevant mAb for SHE78-7 produced the same negative result as medium alone.\textsuperscript{3} Analysis of DNA extracted from HSC-3 MCAs cultured in the presence and absence of SHE78-7 showed significant DNA laddering only in cultures treated with the blocking mAb (Fig. 5A, lane 4), whereas no DNA laddering was visible in the HSC-3 MCAs cultured in the absence of the antibody (Fig. 5A, lane 3) or in control monolayer cultures (Fig. 5A, lane 2). These results indicate that hindering the formation of E-cadherin-mediated cell-cell contacts by suspended cells significantly reduces their growth and induces PCD. This suggests that engagement of intercellular E-cadherin contacts in multicellular aggregates may substitute for the loss of cell-matrix signaling.

Elevated Bel-2 Is Linked to HSC-3 Cell Survival—Levels of Bel-2 protein estimated by immunoprecipitation and Western blot analysis revealed that Bel-2 was elevated in HSC-3 monolayer and MCA cultures but was down-regulated in suspension cultures of single cells (Fig. 5B). These results indicate that the inhibition of growth and susceptibility to apoptosis in sus-
pend single cells is associated with the loss of Bcl-2 expression. Next, we tested whether E-cadherin-mediated cell-cell adhesions are involved in the maintenance of Bcl-2 levels by examining the effect of the anti-E-cadherin mAb (Fig. 5C).

Bcl-2 expression was down-regulated by 15–20-fold in HSC-3 multicellular aggregate in the presence of the anti-E-cadherin mAb SHE78-7 when compared with control HSC-3 multicellular aggregates.

FAK Is Not Activated in Suspended HSC-3 Cells—FAK is an important mediator of integrin-induced signaling involved in anchorage-dependent survival and growth (22). The level of activated FAK was assessed by immunoprecipitation and detection of autophosphorylation in monolayer, suspended single cell, and MCA cultures of HSC-3 cells (Fig. 5D). As expected for monolayer cultures, FAK was fully activated as evidenced by its high level of tyrosine phosphorylation. Also as expected for suspended single cells lacking substrate adhesions, no detectable tyrosine phosphorylation of FAK was observed. However, FAK activation was also lacking in MCA cultures, indicating that cell-cell adhesion fails to trigger significant downstream FAK autophosphorylation. By immunoblotting analysis, the relative levels of FAK protein were unchanged in the different culture conditions (not shown).

E-cadherin-negative Cells Fail to Survive and Grow in Suspension Culture—To further test whether E-cadherin engagement rescues cells in suspension from apoptosis, we studied another human oral squamous carcinoma cell line, HOC-313. The parent population of this cell line has low levels of E-cadherin and is unable to form compacted MCAs. However, this cell line contains a mixed population of both high and low expressors of E-cadherin, and we were able to isolate individual receptor-negative and receptor-positive single-cell clones by serial dilution. A total of nine random clones were isolated, and immunoblotting was used to assess E-cadherin expression. Whereas the parental HOC-313 cells expressed low levels of E-cadherin, the D1 and D2 clones had high levels of E-cad-
herein, and the C7 and C8 clones had no detectable signal for the receptor (Fig. 6A). Two clones were selected for further study: the C8 receptor-negative clone and the D1 receptor-positive clone.

The ability of the C8 and D1 clones to form cell-cell aggregates was evaluated in suspension cultures on poly-HEMA-coated plates. At 8 h postplating, the E-cadherin-negative C8 clone formed loose chains of aggregates in suspension (Fig. 6B, panel a) that failed to fully compact by 24 h (Fig. 6B, panel b).

Next we analyzed samples of DNA isolated at 72 h from monolayer, suspended single-cell, and MCA cultures formed by the C8 and D1 clonal cell lines. DNA was analyzed on agarose gels (Fig. 4). Multicellular aggregate formation requires E-cadherin. HSC-3 cells were plated on poly-HEMA-coated dishes, and cell aggregation over time was followed by phase contrast microscopy. A, single cells immediately after plating. B, by 4 h, cells had started to aggregate. C, chains and small clumps were observed at 8 h. D, larger, irregular aggregates were visible at 12 h, followed by compacted MCA formation by 24 h postplating (E) and larger MCAs by 72 h (F). That this aggregation and compaction is E-cadherin-dependent is demonstrated in G and H. HSC-3 cells were plated on poly-HEMA in the presence of 1 μg/ml anti-E-cadherin mAb SHE78-7 for 24 h (G) and 72 h (H). The presence of anti-E-cadherin reduced the extent of aggregation and inhibited compaction, as evidenced by the presence of small clumps and single cells.

| Culture conditions | SHE78–8 mAb | [3H]Thymidine incorporation | Inhibition |
|--------------------|-------------|----------------------------|-----------|
| Monolayer          | 1           | 25.9 ± 1.8                 | 4.6       |
| Single cell        | 1           | 24.7 ± 2.1                 |           |
| MCA                | 1           | 1.22 ± 1.1                 | 1.6       |

a Values represent the mean cpm ± S.D. × 10^4 of TCA-insoluble radioactivity from triplicate plates.

Fig. 5. Analysis of E-cadherin, Bel-2, and FAK in HSC-3 cell survival. A, effect of anti-E-cadherin mAb on HSC-3 cell survival. HSC-3 cell MCA cultures were incubated in the absence or presence of 1 μg/ml anti-E-cadherin mAb SHE78-7, and after 3 days, the cells were processed as in Fig. 2A. Lane 1, standard 100-bp DNA ladder. Lane 2, control monolayer (ML) culture; lanes 3 and 4, MCA in the absence (lane 3) or presence (lane 4) of anti-E-cadherin antibody. Note extensive fragmentation of DNA from MCA cultures in the presence of anti-E-cadherin mAb but not in control ML or MCA cultures. B, immunoblot analysis of levels of Bel-2 in HSC-3 cell ML, single cell (SC), and MCA cultures. 500 μg of protein from each type of culture was immunoprecipitated with anti-Bcl-2 mAb. Immunoprecipitates were resolved by 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions, transferred to Immobilon membranes, and subjected to immunoblot analysis. Proteins were visualized by incubation with anti-Bcl-2 mAb followed by incubation with streptavidin-horseradish peroxidase, and then detected by ECL as described under “Experimental Procedures.” ML, HSC-3 monolayer; SC 2d and 3d, 2- and 3-day SC cultures, respectively; MCA 2d and 3d, 2- and 3-day MCA cultures, respectively. K562, Bel-2 in K562 cells as a positive control. C, immunoblot analysis of Bel-2 protein in MCA in the absence (MCA) and presence (MCA + mAb) of anti-E-cadherin mAb SHE78-7. MCA, Bel-2 expression in HSC-3 monolayers. Note down-regulation of Bel-2 in MCA in the presence of SHE78-7 mAb. D, tyrosine phosphorylation of FAK in HSC-3 cells. Protein lysates from HSC-3 ML, SC and MCA cultures were immunoprecipitated with FAK, analyzed by 7.5% SDS-polyacrylamide gel electrophoresis, and immunoblotted with Py20. In control monolayers, FAK was tyrosine-phosphorylated (ML) whereas no phosphorylated p125FAK could be detected in SC and MCA cultures.
Intercellular Adhesion Regulates Growth

**Fig. 6.** E-cadherin levels, MCA formation, and apoptosis in HOC-313 cell clones. A, HOC-313 cells were cloned by serial dilution as described under “Experimental Procedures.” E-cadherin expression in the parental and four representative clonal cell lines was analyzed by immunoblotting. B, MCA formation by C8 and D1 cell clones. Phase-contrast microscopy shows the process of aggregation at 8 h (panels a and c) and 24 h (panels b and d). Note that the receptor-positive D1 clones (panels c and d) form compact MCAs, whereas the receptor-negative C8 clones (panels a and b) only generate loose aggregates. C, analysis of DNA fragmentation of D1 and C8 cells cultured as monolayers (ML), single cells (SC), or MCAs for 3 days; samples were processed as in Fig. 2A. Lane 1, 100-bp DNA ladder; lanes 2–4, samples from D1 clone; lanes 5–7, samples from C8 clone. Extensive DNA fragmentation was observed in MCA from the receptor-negative C8 cells (lane 7) but not in MCA from the receptor-positive D1 cells (lane 4).

gels for intranucleosomal cleavage. We found that monolayer cultures from either cell line lacked any evidence of DNA fragmentation (Fig. 6C, lanes 2 and 5). However, both cell types showed extensive PCD when cultured as single-cell suspensions. Importantly, the E-cadherin-positive D1 cells that formed compacted MCAs survived and grew in suspension, whereas the cadherin-negative C8 cells underwent apoptosis, as indicated by extensive DNA laddering (Fig. 6C, lane 7).

**DISCUSSION**

Most squamous epithelial cells, including their transformed counterparts, are strictly anchorage-dependent; they fail to survive in single-cell suspension culture and undergo terminal differentiation and programmed cell death (9, 23, 24). Our analysis of squamous cell carcinoma cell lines showed that forced anchorage deprivation triggered apoptosis. Suspen- sion-induced cell death was confirmed by several independent criteria, including morphology and DNA fragmentation assessed by gel fractionation and TUNEL. However, if the cells formed cell-cell aggregates, they not only survived in suspension but also proliferated. Moreover, if cells were suspended in low calcium medium to prevent cell-cell adhesion, they failed to proliferate and rapidly entered the death pathway. Importantly, we found that although HSC-3 cells in MCA cultures were able to proliferate, they did so at a reduced rate compared with monolayer cultures. This suggests that cell-cell adhesion is only partially able to compensate for loss of integrin engagement or, alternatively, that intercellular adhesion, while stimulating proliferation may also produce some degree of growth inhibition. A number of other oral SCC cell lines showed a similar growth response in MCA cultures. Taken together, these observations provide evidence that intercellular adhesion could suppress apoptosis and promote growth.

An obvious candidate for mediating this intercellular adhesion is the family of classical cadherins. These transmembrane homophilic adhesion receptors are Ca\(^{2+}\)-dependent and are expressed at high levels in all epithelial cells. We used specific inhibitory antibodies to E-cadherin to show that this receptor was involved in promoting cell-cell contact-dependent survival and proliferation in anchorage-deprived cells. For HSC-3 cells, E-cadherin appears to be the major receptor involved in mediating intercellular adhesion and anchorage-independent growth. Squamous epithelial cells may also express some amounts of P-cadherin (25) and less frequently may express N-cadherin and desmosomal cadherins. The potential role of these receptors in growth regulation needs to be examined (26).

Previous work provides some evidence that supports the role of cadherins in regulating PCD and growth. Recently Zhu and Watt (27) reported that a dominant-negative E-cadherin mutant cDNA lacking the extracellular domain partially suppressed growth of monolayer cells when transfected into normal keratinocytes. Results from other studies demonstrate a role for N-cadherin in inhibition of granulosa cell apoptosis (11, 28). However, other investigators have suggested that cadherin expression can inhibit cell growth. For example, in studies with transgenic mice, Allen et al. (29) showed that disruption of desmosomal formation by a defective cadherin gene enhanced epidermal cell proliferation. Cell growth was also inhibited in a catenin-deficient lung carcinoma cell line when transfected with functional α-catenin (19). The opposing results observed in these various studies following manipulation of cadherins may reflect the divergent functions performed by these adhesion receptors and the complexities of cell growth regulation.

Although our results indicate that E-cadherin is involved in mediating anchorage-independent survival and growth of epithelial cells, the exact mechanism is unknown. Recent studies have provided evidence for the linkage of cadherins to intracellular signaling pathways (30–34). β-Catenin binds both cadherin and other catenins and is involved in signal transduction, gene expression, and developmental patterning (35). The polymerization of actin into a cytoskeleton during compaction clearly indicates generation of focal signals. Catenins bind the actin-bundling protein fascin, and β-catenin is associated with both cell-cell contacts and the leading edge of the cell with adenosomatous polyposis coli (36, 37). Furthermore, it has been shown that the epidermal growth factor receptor family of tyrosine kinase receptors can physically couple with catenin/cadherin complexes (38, 39). In addition, there is evidence of cross-talk between cadherins and integrin receptors (29, 40, 41). Recently, the adapter protein Shc, which is involved in growth factor activation by Ras, and the tyrosine kinase substrate p120Cas have been shown to associate with E-cadherin (42–44). Finally, cell-cell contact regulating proliferation has been linked to alterations in cyclin-dependent kinases (45).
We suspect that the crucial event during cadherin-mediated anchorage-independent growth is the process of compaction that proceeds during the final stages of intercellular adhesion. Compaction of cell aggregates is analogous to the process observed during blastocyst formation. The phenomenon consists of a mechanical stretching and compression between adjacent cells and requires active contraction of the actin cytoskeleton tethered to the cadherin intercellular contacts (30). That compaction is required for anchorage-deprived cells is suggested by the observation that under conditions when compaction does not occur (with anti-E-cadherin antibody or with E-cadherin-negative cells), the cells are still able to form rudimentary, loose cell aggregates, but their growth is inhibited, and they are still forced into the PCD pathway.

Another possible mechanism by which cadherin-dependent interactions could modulate cell growth and survival is by promoting the juxtaposition of specific growth factor receptors and their ligands as cells form intercellular junctions. Although cadherins are the initiators of cell-cell contact and stabilize junctional assemblies, this process of cell adhesion may facilitate potential receptor-ligand interactions that could trigger intracellular signaling pathways involved in survival and proliferation. An example of this phenomenon is the observation that transmembrane TGF-β precursors on one cell are able to activate epidermal growth factor receptors expressed on an adjacent cell during intercellular adhesion and that these interactions have been shown to regulate cell growth (46). However, there are few, if any, examples showing that juxacrine signaling is important in vivo. Obviously, additional studies are required to determine which of these potential signaling pathways are involved in mediating cell-cell junctional promotion of cell survival and growth.

Integrins do not appear to be involved in the anchorage-independent growth and survival described in the present study. However, it is well established that integrins function as signal-transducing receptors capable of modulating cell growth and gene expression (47, 48). For HSC-3 cells, induction of apoptosis following loss of anchorage clearly implicates integrins in promoting cell survival/growth in substrate-adherent cells. Interestingly, in a previous study colon carcinoma organoids were reported to avoid apoptosis via cell-cell aggregation-mediated by the ov integrin (49), but the interpretation of these results has been questioned (2). The observation that aggregates of HSC-3 cells in suspension cultures were resistant to PCD and proliferated in medium containing high Ca²⁺ (cadherin-permissive) but not in high Mg²⁺ (integrin-permissive) argues that cadherin and not integrin is the important receptor for survival in suspension. Furthermore, engagement and cross-linking of integrins by ligand is sufficient to induce the tyrosine phosphorylation of FAK (50), and the lack of FAK autophosphorylation in MCA cultures is evidence for the absence of integrin-mediated downstream signaling in anchorage-deprived HSC-3 cells. It is unlikely that in suspension culture any significant extracellular matrix is present: experiments with aggregates in serum-free growth conditions failed to detect the presence of matrix molecules (laminins, collagen, or fibronectin), and a combination of blocking anti-integrin mAbs at concentrations that inhibit adhesion of HSC-3 cells to ECM molecules (51) had no effect on either HSC-3 cell-aggregation or anchorage-independent growth.²

The current observations may have relevance to tumor progression. In the case of well and moderately differentiated human squamous cell carcinoma, studies have found that, in general, modest but variable expression of E-cadherin is preserved as lesions advance through premalignant to invasive and metastatic stages (52, 53). Importantly, squamous cell carcinoma cells deep within solid tumor islands tend to exhibit high levels of mitotic activity in cells that are distal from the neighboring basement membrane at the advancing edge of the tumor, and these cells continue to express E-cadherin. Loss of cadherin may occur in more progressed and dedifferentiated tumors in which invasion into the surrounding ECM via integrins is facilitated. Furthermore, squamous cell carcinoma cells have been reported to display altered basement membrane deposition resulting from accelerated degradation or from defects in matrix assembly. Thus, it would appear that these malignant cells have escaped the normal pattern of regulated growth control found in basal keratinocytes and, in the absence of anchorage to the basement membrane, may utilize cell-cell adhesive interactions for cell survival and proliferation. We found that normal keratinocytes that proliferate as adherent cells fail to survive in suspension even when permitted to form cell aggregates. Thus, in the case of normal epithelial cells, formation of cell-cell contacts via cadherin was not sufficient to prevent PCD, suggesting that E-cadherin signaling is distinct from that in carcinoma cells. This suggests that transformation may be a necessary requirement for cell-cell contact-induced survival and growth.

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