Retention of Intrinsic Stem Cell Hierarchies in Carcinoma-Derived Cell Lines

Matthew Locke, Matthew Heywood, Stuart Fawell, and Ian C. Mackenzie

Abstract

Recent work indicates that the growth and behavior of cancers are ultimately determined by a small subpopulation of malignant stem cells and that information about the properties of these cells is urgently needed to enable their targeting for therapeutic elimination. A key feature of normal stem cells is their asymmetrical division, the mechanism that allows stem cell self-renewal while producing hierarchies of amplifying and differentiating cells that form the bulk of the tissue. Most cancer deaths result from epithelial malignancies, but the extent to which the hierarchical proliferative stem and amplifying cell patterns of normal epithelia are actually retained in epithelial malignancies has been unclear. Here we show that even cell lines generated from carcinomas consistently produce in vitro colony patterns unexpectedly similar to those produced by the stem and amplifying cells of normal epithelia. From the differing types of colony morphologies formed, it is possible to predict both the growth potential of their constituent cells and their patterns of macromolecular expression. Maintenance of a subpopulation of stem cells during passage of cell lines indicates that the key stem cell property of asymmetrical division persists but is shifted towards enhanced stem cell self-renewal. The presence of malignant epithelial stem cells in vivo has been shown by serial transplantation of primary cancer cells and the present observations indicate that stem cell patterns are robust and persist even in cell lines. An understanding of this behavior should facilitate studies directed towards the molecular or pharmacologic manipulation of malignant stem cell survival.

Introduction

Normal tissue renewal depends ultimately on a subpopulation of cells (termed stem cells) that are characterized by an extensive proliferative potential (1). Stem cells are precisely distributed in vivo in relation to units of epithelial structure (2) and epithelial stem cells typically divide both to renew themselves and to generate transit amplifying cells that undergo a series of amplifying divisions as they differentiate (2, 3). Stem cell homeostasis results from a balanced pattern of asymmetrical division that generates one cell that remains a stem cell and one that becomes committed to differentiation (3–5). The concept that cancers contain a similar subpopulation of malignant stem cells was proposed several years ago (6) and the extent to which the hierarchical proliferative stem and amplifying cell patterns of normal epithelia are actually retained in epithelial malignancies has been unclear. Here we show that even cell lines generated from carcinomas consistently produce in vitro colony patterns unexpectedly similar to those produced by the stem and amplifying cells of normal epithelia. From the differing types of colony morphologies formed, it is possible to predict both the growth potential of their constituent cells and their patterns of macromolecular expression. Maintenance of a subpopulation of stem cells during passage of cell lines indicates that the key stem cell property of asymmetrical division persists but is shifted towards enhanced stem cell self-renewal. The presence of malignant epithelial stem cells in vivo has been shown by serial transplantation of primary cancer cells and the present observations indicate that stem cell patterns are robust and persist even in cell lines. An understanding of this behavior should facilitate studies directed towards the molecular or pharmacologic manipulation of malignant stem cell survival.

with at least the stem cell property of indefinite proliferation. However, the nature of stem cell patterns in malignancy remained a matter of controversy (7, 8) until it was shown that human leukemias retain a hierarchical proliferative pattern and that only a small "tumor-initiating" subpopulation, prospectively identifiable by its CD34+, CD38− phenotype, is able to reinitiate tumor growth after transplantation to immune-deficient mice (4, 9). A similar stem cell pattern has since been shown for breast cancers in which the tumor-initiating cells can be prospectively identified by their CD44+, ESA+, CD24+/low, Lineage− phenotype (10).

Morphologic heterogeneity is a typical feature of malignant cell lines and has usually been attributed to genetic instability and clonal evolution (11). The presence of such cellular heterogeneity, together with uncertainty about the effects of cell adaptation to in vitro conditions (12), has cast doubt on the value of using malignant cell lines rather than freshly isolated tumor cells for malignant stem cell studies (13). However, analysis of stem cell properties in vivo is extremely difficult and elucidation of control mechanisms for key properties, such as asymmetrical division (8), would be greatly simplified if they could be studied in in vitro systems (14). Several findings now suggest that malignant cell lines may in fact retain stem cell patterns of behavior. For example, malignant cell lines derived from nonepithelial tumors show behavioral heterogeneity and contain subpopulations of cells with putative stem cell characteristics such as dye exclusion (15–18). For malignant epithelial lines, the isolation of behaviorally different subpopulations, by density sedimentation (19) and by dye exclusion (16, 17), similarly points to the presence of cells with a range of differing proliferative capabilities. Clonogenic assays of malignant epithelial cells in "organotypic" cultures also suggest the presence of cells with differing clonogenic potentials (20).

Despite a role of "niches" in determining various aspects of epithelial stem cell behavior (21), some control of stem cell division patterns is intrinsic to the epithelium itself and persists after cells are isolated and grown in vitro (5). However, when considering the morphologic heterogeneity displayed by malignant epithelial cells, little attention seems to have been given to the fact that even normal keratinocytes are heterogeneous. When plated at low density, normal keratinocytes generate a range of different in vitro colony forms, classified morphologically as holoclones, meroclones, and paraclones, that are derived from, and contain, cells corresponding to stem and early and late amplifying cells, respectively (22). Holoclones take the form of compact round colonies, paraclones form loose irregular colonies, and meroclones have intermediate features. The observation that malignant cell lines often produce a similar range of colony forms prompted us to undertake a more detailed study of their patterns of colony formation. In so doing, we have been able to show that malignant cells have clonogenic properties closely paralleling those of normal epithelial cells and that the cells of malignant holoclones are, like normal holoclones, smaller than the cells of meroclones or...
paraclines, more highly clonogenic, and more rapidly adherent to a range of substrates (23, 24). The different colony morphologies displayed within malignant cell lines were found to be associated with different patterns of macromolecular synthesis.

**Materials and Methods**

**Cell lines and growth conditions.** Most of the cell lines examined were derived from head and neck squamous cell carcinomas (HNSCC). These were the lines CA1 and UK1 (20); UM-5PT (derived by T. Carey, University of Michigan); the CAL27, SCC4, SCC9, SCC15, SCC25, SCC66, and SCC71 lines (obtained from the American Type Culture Collection); the H314, H400, and H357 lines (25); and the C1 and VB6 subclones that were derived from the H357 line (26). Before experimental use, the cell lines were cloned, and were then passaged several times. Unless otherwise stated, cell culture supplies were bought from Invitrogen Life Sciences.1

Colony formation and staining patterns of the oral cell lines were examined after growth both in a highly supplemented epithelial growth medium termend FAD (27) and in a basal medium consisting of DMEM supplemented with 10% fetal bovine serum. Normal oral keratinocytes were isolated and grown in FAD medium with 3T3 feeder cells as previously described (27), but all of the malignant lines studied grew without the need for 3T3 feeder cells. Two nonoral cell lines were also studied: the DU145 prostate cell line that was grown in the DMEM plus 10% fetal bovine serum and the MCF7 breast tumor line that was grown in the same medium supplemented with 50 μg/ml insulin.

**Colony heterogeneity.** For studies of colony morphology and staining patterns, actively growing subconfluent cells were dissociated in trypsin/EDTA, plated in T75 flasks at densities ranging from 50 to 200 cells per cm², and examined by phase contrast microscopy as colonies developed. To test the assumption that colonies derived from low-density plating typically have a clonal origin, some tumor populations were transduced with a retroviral vector (MGIN) expressing the enhanced green fluorescent protein gene (28), mixed with nonexpressing cells of the same line, and plated at low density for examination of the developing colonies by fluorescence microscopy. To determine whether the full range of colony morphologies present in an individual malignant cell line can be generated from a single colony type, the C1, CA1, SCC71, CAL27, and VB6 cell lines were plated at low densities in 90-mm Petri dishes for identification of colonies classified as holoclones (i.e., the putative stem cell lines). These colonies were isolated with cloning rings, their cells dissociated in trypsin/EDTA and then replated in T75 flasks for analysis of their developing patterns of colony morphology monitored both initially and following further passage.

**Cell proliferation.** To label cells in the S phase of the cell cycle, 5-bromo-2-deoxyuridine (BrdUrd, Sigma) was added to culture medium to a final concentration of 50 μg/ml. After 2 hours of exposure, cells were fixed 70% ethanol for 10 minutes, washed in PBS, treated with 4 mol/L hydrochloric acid for 10 minutes, and then washed sequentially in PBS at pH 8.5 and 7.4. Before application of an anti-BrdUrd primary antibody (DAKO, Clone Bu20a) at a dilution of 1:100, endogenous peroxidase was blocked by treatment for 5 minutes with 3% hydrogen peroxide in PBS containing 0.2% Triton 100-X. After overnight incubation at 4 °C, cells were washed and rabbit anti-mouse secondary antibody conjugated to horse-radish peroxidase applied at a dilution of 1:200 for 2 hours before visualization of binding using a 3,3’-diaminobenzidine substrate kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA).

**RNA extraction and quantitative PCR.** A pilot examination of holocline and paracline RNA extracts, using Affymetrix HG-U133A Arrays, provided preliminary gene expression data for holoclines and paraclines of the C1 and CA1 cell lines and 10 differentially regulated genes (listed in Table 1) were selected for further examination. Material for analysis by quantitative PCR was generated from large holocline and paracline colonies of seven cell lines (listed on Table 1) that were isolated with cloning rings, replated into T75 flasks, and grown for 2 days to provide expanded cell populations. Total RNA was extracted using an RNeasy (Ambion, Austin, TX) kit, first-strand synthesis from 1 μg of total RNA was achieved using a Bio-Rad iScript CDNA synthesis kit, both according to the manufacturer’s instructions, and cDNA was quantitated using a Genespec 1 spectrophotometer (Naka Instruments, Kyoto, Japan). Thermal cycling conditions and primer specificity were initially determined by PCR and the subsequent quantitative PCR was done using equal quantities of holocline or paracline cDNA and a DyNaMo SYBR Green qPCR kit (Finnzymes, Espoo, Finland).

All assays were repeated at least in triplicate, and the specificity of amplification for all primers (Table 1) was determined from thermal cycler generated melting curves.

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2 http://www.sciencemag.org.
cells derived from holoclones of seven oral cell lines (CA1, C1, VB6, cells.

full morphologic range of cellular phenotypes characteristic of each

those of normal keratinocyte holoclones, are able to regenerate the

were restored indicating that the cells of malignant holoclones, like

and colony proportions characteristic of the original parental lines

were generated even at the initial passage (Fig. 1

produced mainly holoclones, but some meroclones and paraclones

but a similar series of colony morphologies was produced in both

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HNSCC-derived malignant cell lines initially examined showed

The patterns of cell and colony morphologies developed by the 15

Cancer Res 2005; 65: (19). October 1, 2005 8946 www.aacrjournals.org

Results

Colony morphologies. After plating at low densities, malignant
cells formed small colonies within 2 to 3 days (Fig. 1A). Examination
of the colonies formed by mixtures of GFP+ and GFP – cells showed
that the great majority of colonies consisted only of one or other cell
type and had therefore most probably developed clonally (Fig. 1B).
The patterns of cell and colony morphologies developed by the 15
HNSCC-derived malignant cell lines initially examined showed
small individual differences but, superimposed on these, each line
showed a spectrum of colony morphologies ranging from round
colonies of small closely packed cells to irregularly shaped colonies
consisting of larger loosely packed cells. These morphologies were
identifiable at the earliest stages of colony formation and were
maintained as colonies increased in size (Fig. 1B). Cell lines grew
more rapidly in the supplemented FAD medium than in the DMEM,
but a similar series of colony morphologies was produced in both
media. Cells isolated from holoclones and replated in T75 flasks
showed formation of aborted microcolonies with the appearance
of late paraclones (Fig. 2C).

Paraclonal differences between their constituent cells (Fig. 2B).
Microscopic examination of the plated wells indicated that, as for
oral lines, failure of paraclone cells to form colonies was not due to
failure of plating or lack of initial proliferative activity; most wells
showed formation of abortive microcolonies with the appearance
of late paraclones (Fig. 2C). These differed markedly from the
colonies formed by holoclone cells that consisted mainly of small
packed cells that could be removed and replated to generate new
sublines. The early presence of mixed colonies in some of the wells
plated with a single holoclone cell provided further evidence of
the ability of holoclone cells to give rise rapidly to all clonal
morphologies.

Cell adhesion and cell proliferation. Malignant cells also
showed a clear relationship among their rates of adherence, their
size, and their subsequent growth patterns. For each of five HNSCC

cell lines examined (CA1, C1, 5PT, VB6, and UK1), the mean size of

Table 1. Assessment by q-PCR of differences in gene expression between holoclones and paraclones for each of seven
cancer cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>C1</th>
<th>CA1</th>
<th>VB6</th>
<th>SCC71</th>
<th>CAL27</th>
<th>MCF7</th>
<th>DU145</th>
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<tr>
<td>Notch 3</td>
<td>Forward TGGTGACGAGTGCTCTATCG; Reverse AATGTCACCTCCTCGCAATAGG</td>
<td>16.7</td>
<td>42.1</td>
<td>2.1</td>
<td>42</td>
<td>1.3</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>Forward CCTGGCGTAGAAAATGGA; Reverse AGGCCTCAGGATCTTCCA</td>
<td>5.2</td>
<td>4.9</td>
<td>4.7</td>
<td>5.2</td>
<td>0.1</td>
<td>7.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Hur7</td>
<td>Forward TGACTCTGCCGACCTGTTG; Reverse TAAACCTTCGTCCTCCATTGGC</td>
<td>2.8</td>
<td>4.3</td>
<td>3</td>
<td>17.1</td>
<td>5.2</td>
<td>12.1</td>
<td>3.6</td>
</tr>
<tr>
<td>CEBP</td>
<td>Forward GAAAGCTGGTTGCGGTTCA; Reverse TCATACCTCAGGCCCTCCCTTG</td>
<td>2.4</td>
<td>4.6</td>
<td>4</td>
<td>8.1</td>
<td>4</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>Pirin</td>
<td>Forward CCAAGGTTTTACACTCGACA; Reverse GCCCTTCAACCCATTTTT</td>
<td>14.7</td>
<td>5.2</td>
<td>132</td>
<td>3.2</td>
<td>8</td>
<td>20</td>
<td>0.3</td>
</tr>
<tr>
<td>RDHL</td>
<td>Forward TGCAGTTAATCGTGCCCTGTC; Reverse CACCGGCAATAAGTGAAGTCTT</td>
<td>3.6</td>
<td>29.8</td>
<td>4</td>
<td>4.9</td>
<td>4.6</td>
<td>7.7</td>
<td>2.1</td>
</tr>
<tr>
<td>PSCA</td>
<td>Forward TACCTCAGGACGGACCGCT; Reverse GCTTGAGCTGCTGGAGTACT</td>
<td>2.1</td>
<td>2.1</td>
<td>4</td>
<td>7.9</td>
<td>104</td>
<td>13.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Erb-B3</td>
<td>Forward GTCTGTTGACACCTGCAACT; Reverse GGCTGACAGGAAGACXCTT</td>
<td>18.9</td>
<td>13.6</td>
<td>3.1</td>
<td>80</td>
<td>9.5</td>
<td>2.5</td>
<td>2</td>
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<tr>
<td>HPGD</td>
<td>Forward CGTGAACGGCAAAATGGCCTGGTGT; Reverse ACACCTGTCAAGATTTCAACCT</td>
<td>58</td>
<td>2.3</td>
<td>8.7</td>
<td>4.6</td>
<td>10.2</td>
<td>11.9</td>
<td>6.5</td>
</tr>
<tr>
<td>E-CAD</td>
<td>Forward CCATCCACCCACGTCACAG; Reverse TACATCTCAGACTGGCCCGAAG</td>
<td>14.7</td>
<td>5.5</td>
<td>211</td>
<td>3.5</td>
<td>5.2</td>
<td>44.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

NOTE: >1, fold higher expression in holoclones; <1, lower expression in holoclones. Full gene names (accession nos.) are Notch 3 (gb:NM_000435.1); IGFBP3, insulin-like growth factor binding protein 3 (gb:BF340228); Hur7, UV-B-repressed hurpin 7 (gb:BE148534); CEBP, CCAAT enhancer-binding protein a (gb:NM_004364.1); Pirin, UV-B-repressed hurpin 7 (gb:BE148534); PSCA, prostate stem cell antigen (gb:NM_000587.2); Erb-B3, v-erb-b2 avian erythroblastic leukemia viral oncogene homologue 3 (gb:NM_001982.1); HPGD, hydroxyprostaglandin dehydrogenase (gb:NM_000860.1); and E-CAD, E-cadherin (gb:BF125158).
the more rapidly adherent cells was consistently smaller than that of the less rapidly adherent cells (Fig. 3A). Counts of the types of colony morphologies formed by early, intermediate, and late-adhering cells indicated that the smaller early adherent cells formed a greater proportion of holoclone colonies (ANOVA, P = 0.003), indicative of their greater clonogenicity. Staining for BrdUrd incorporation into large colonies indicated that there was little cell proliferation in paraclones that consisted only of greatly flattened cells. However, all other types of colonies, from holoclone to early paraclones, showed a large proportion of cells that had incorporated BrdUrd (Fig. 2D), and these colonies also showed similar rates of increase in size with further time in culture.

**Figure 1.** Colony morphologies in malignant cell lines. A, range of early colony morphologies formed by the lines CA1, C1, and UK1, respectively. Colony morphology varies from that of holoclones (left) to paraclones (right). Intermediate meroclone forms. The continuous gradient of colony morphologies lacks clear cutoff points for classification, but holoclones are recognized as tight clusters of uniformly small cells producing a smooth outline and paraclones are characterized by loss of tight cell packing, larger cell size, and irregular cell and colony shapes. The cell and colony morphologies vary somewhat from one cell line to another with most differences being apparent for paraclones cells which can be highly flattened (CA1), show multiple filopodia (C1), or be spindle-like (UK1). The range of CA1 colony morphologies was developed at the first plating of cells isolated from a single CA1 holoclone. B, phase-contrast and fluorescent images of colonies formed by low-density plating of a mixture of CA1 cells expressing or not expressing green fluorescent protein. Right, a holoclone, is entirely green fluorescent protein positive, whereas the other colony, a paraclone, is entirely negative indicating the typically clonal origin of colonies formed at low plating densities. C, colonies produced by the VB6 line 12 days after plating indicate that early differences between colony morphologies (A) are maintained as colonies expand. D, holoclone and paraclone morphologies developed by the MCF7 cell line 3 days after plating and by the DU145 cell line 14 days after plating. Bar, 0.1 mm (A), 0.25 mm (B-C), 0.07 mm for MCF7 and 0.3 mm for DU145 (D).

**Figure 2.** Twenty-four-well plates in which each well was individually seeded with a single cell derived from either a holoclone or paraclone. A, holoclone cells of the CA1 line (top left) have given rise to expanding cell populations, but paraclone cells (top right) failed to show such expansion and only one or two small colonies can be seen with the naked eye. Microscopic examination showed that nearly all wells contained small abortive colonies, indicating that cells had plated and undergone a few divisions. B, for the DU145 line 17 of 24 wells seeded with individual holoclone cells (bottom left) show expanding colonies compared to only four wells seeded with paraclone cells. In other similar experiments, it was shown that colonies derived from holoclones were capable of indefinite further passage. C, markedly different microscopic appearance of colonies are formed by holoclone and paraclone cells. Holoclone cells of the DU145 line form large colonies of packed small cells and paraclone cells form colonies with spaced, often spindle-shaped, cells with little growth. The paraclone cell from the MCF7 line shows little growth even 21 days after plating cells. For all cell lines, plating single holoclone cells initially generated colonies with holoclone morphologies but, as shown by the singly plated CA1 holoclone cell (bottom right), meroclones can arise at an early stage, and following further growth, paraclone colonies developed. D, however, two colonies, a holoclone (left) and a paraclone (right), after staining to display cells incorporating BrdUrd. More than half of the holoclone cells are labelled and also quite a high proportion of the early paraclone cells. Bar, 0.3 mm (C) and 0.2 mm (D).

Immunofluorescent staining patterns of oral cell lines. Each of the cell lines examined showed differing patterns of staining for holoclones and paraclones with antibodies against the majority of the markers examined. Cell lines varied in their degree of staining with particular antibodies, but holoclones typically showed stronger staining than paraclones for molecules that have been reported to be expressed more strongly by normal stem cells. For example, in all cell lines, most colonies showed some staining of cell peripheries for b1 integrin, but staining of holoclone cells was much brighter than paraclone cells (Fig. 4A). Similarly, all cell lines showed stronger staining of the cell peripheries of holoclone cells for b-catenin (Fig. 4A) and E-cadherin (Fig. 4B). Maintenance of an epithelial phenotype was indicated for all cell lines by staining for cytokeratin 5 (Fig. 4B), and cytokeratin 14, typically basal cell markers in stratifying squamous epithelia. Staining for cytokeratin 15 was absent from the CAL27 and DU145 cell lines and showed only weak cytoplasmic staining for most colonies of other cell lines. However, much stronger cytoplasmic staining of holoclones for K15 was found in the C1 and CA1 (Fig. 4C). Staining for cytokeratins 6 and 16, markers of early cell differentiation in normal oral mucosa, was present in most cell lines with large flattened cells in
paraclines being strongly stained, a pattern typical of normal oral keratinocytes in culture (Fig. 4D).

The clonal heterogeneity present in the MCF7 breast cell line allowed examination of how holoclone morphology, considered predictive of the stem cell phenotype, correlated with the expression of CD44 and ESA, markers used in the isolation of “tumor-initiating cells” from freshly isolated or in vivo pasaged breast tumor cells (10). Colonies formed by the MCF7 line showed tight restriction of staining for CD44 to the peripheries of cells in holoclones (Fig. 5A). To examine whether the MCF7 pattern of staining for CD44 was characteristic of other cell lines, colonies formed by the CA1, C1, VB6, and DU145 cell lines were stained for CD44 expression and each line showed a similar pattern of restriction of CD44 staining to cells within holoclones (Fig. 5A-C). Holoclones formed by the MCF7 line also stained strongly for ESA, but staining was less tightly restricted and extended weakly to meroclines. The other cell lines similarly showed stronger staining of holoclones with ESA and extension of decreasing staining to meroclines (Fig. 5D and E). Staining for cytokeratin 15 was present in the MCF7 cell line and was restricted to holoclones (data not shown).

**Discussion**

The primary aims of these investigations were to determine whether the morphologic heterogeneity typically found in malignant epithelial cell lines is mainly the result of an underlying stem and amplifying cell pattern and, if so, how differing cellular properties are predicted by such morphologic differences. Given the often controversial nature of stem cell studies, a problem initially encountered was to define what experimental criteria might reasonably be accepted as indicating the functional presence in malignant cell lines of stem cell patterns similar to those of normal keratinocytes. Three criteria were chosen, based on those for normal epithelia: (a) the presence of a subpopulation of cells with the capacity for extensive self-renewal, (b) their generation of an amplification hierarchy, and (c) their production of cells entering a differentiation pathway (5).

Fulfillment of the first criterion could be inferred from the continuous expansive growth of malignant cell lines, both in vivo and in vitro. In addition, as new lines can be repeatedly generated from singly plated holoclone cells, it is apparent that cells with the property of indefinite self-renewal are present and are able to expand their numbers under in vitro conditions. The clonal assays that were done with malignant cell lines were essentially similar to those used previously for in vitro investigation of stem and amplifying cells from normal epithelia (22). All of the cell lines examined showed marked clonal heterogeneity and developed a range of colony morphologies paralleling the holoclone, meroclone, and paracline morphologies produced by normal keratinocytes. The relatively high rates of cell proliferation in meroclone and early paracline colonies suggest that they contain the equivalent of early- and late-amplifying cells with holoclone cells differing from paracline cells in being smaller, more rapidly...
Hoechst staining of the same field (VB6 holoclones for CD44 but lack of staining of larger, surrounding paraclone staining (spaced, nuclei in paraclones. Bar 0.25 mm (A, B)

Figure 5. Immunofluorescent staining of colonies. A, MCF7 line stained for CD44 shows staining of a tight cluster of holoclone cells and Hoechst staining of the same field shows the presence of unstained, large, and spaced paraclone cells (top and right). B, the CA1 line similarly shows staining of holoclone but not paraclone cells for CD44. Staining of the VB6 line for ESA is similarly restricted to the holoclone. C, immunofluorescent (left) and Hoechst staining (right) shows surface staining of the central cells of two small developing VB6 holoclines for CD44 but lack of staining of larger, surrounding paraclone cells. D, strong cell surface staining of a large holoclone of the C1 line. Hoechst staining of the same field (right) clearly illustrates the typical appearance of small packed nuclei in holoclone colonies and the larger, more spaced, nuclei in paraclones. Bar 0.25 mm (A, B, and D) and 0.15 mm (C).

The staining of colonies produced by malignant cell lines indicate that holoclone morphology predicts higher levels of expression of stem cell–related molecules such as β1 integrin, E-cadherin, and β-catenin (24, 30). The holoclones of some cell lines also express cytokeratin 15, a marker of stem cell zones in hair follicles and in oral mucosal epithelium (2, 5). Similarly, staining for CD44, the major distinguishing marker for tumor-initiating cells in breast cancers (10), was also much stronger in holoclones, as was staining for ESA. Malignant holoclone cells thus share some patterns of marker expression with both normal epithelial stem cells and with tumor-initiating cells. The functional contribution to "stemness" of most of the genes examined at the RNA level is uncertain, but some studies associate somatic stem cell differentiation with altered expression of molecules such as E-cadherin, CCAAT enhancer–binding proteins, and dehydrogenases (30–33). Progress characterizing molecules of significance to stem cell behavior has been limited (4, 13, 34), but the expression differences detected between holoclones and meroclones seem sufficiently marked and consistent at least to provide putative new markers for malignant stem cell identification. Greater expression of molecules such as pirin, hurpin, and Erb-B3 has previously been associated with epithelial malignancy (35–37) but has not been related particularly to stem cells. Their higher expression in malignant holoclones raises the possibility that some markers may be selectively expressed by stem cells in malignant tissues.

Differences between the stem cells of a cell line and the stem cells that were present in its tumor of origin are likely to result both from cellular changes acquired during the generation of the cell line and from the tissue culture environment itself. Thus, although cells acting as malignant stem cells in vitro can be prospectively identified by morphologic and expression characteristics, it remains uncertain how closely they correspond to "tumor-initiating cells" identified by in vivo transplantation of cells freshly isolated from tumors (10). The in vitro colonies generated by malignant cell lines, like those of normal keratinocytes, form a continuous gradient extending from "ideal" holoclone morphology, through meroclones, to early and late paraclones, but where on this morphologic gradient the property of in vitro self-renewal is lost remains to be determined. Observations made during the present work suggest that cell lines differ in the fraction of holoclone colonies they contain, whether these are identified morphologically or by staining for markers such as CD44. Furthermore, the percentage of singly plated holoclone cells found to generate new holoclone colonies varied quite widely between cell lines (range, 47-100%; data not shown). In a previous study (20), we found that the fraction of total cells with in vitro clonogenic abilities also varies between cell lines, and it will be interesting to compare different cell lines for the proportions of cells capable of initiating new cell lines in vitro and the proportion able to act as tumor-initiating cells after in vivo transplantation.

Demonstration of stem cell patterns in hematologic malignancies (9), and more recently in malignancies of epithelial origin (10), has indicated that elimination of the stem cell subpopulation within tumors is a necessary component of effective therapy (4, 13, 38). Although the great majority of cancer deaths are caused by malignancies of epithelial origin (39), less is known about the behavior of epithelial stem cells, whether normal or malignant, than about their hematopoietic equivalents. However, it is known that normal epithelial stem cells differ from amplifying cells in their growth patterns, apoptotic sensitivities, and motilities (40, 41). They also have higher expression of multidrug resistance transporters (42), but how the retention of this and other differential properties in epithelial tumors (15–17) influences the outcomes of therapeutic procedures presently available remains to be determined.

Another point of interest is that normal homeostatic renewal of epithelia depends on a balanced pattern of asymmetrical stem cell division (4, 5) and thus differs from the growth typical of tumors in which stem cell expansion results from a persistent shift towards enhanced self-renewal (4, 43, 44). The changes that underlie altered patterns of stem cell self-renewal, in either normal or malignant tissues, remain uncertain (31, 32, 43, 45), but normal stem cell self-renewal is at least partially responsive to environmental conditions and is, for example, reduced by cell isolation but increased during growth, in wound healing, and under certain in vitro conditions (8, 30). Possibly, therefore, the self-renewal patterns of malignant stem cells could be manipulated to produce tumor atrophy by directing malignant stem cells away from symmetrical division, a modification of the concept of "directed differentiation" previously raised (46). Analyses of molecular mechanisms associated with asymmetrical division should be facilitated by the persistence of asymmetrical division patterns in malignant cell lines. The in vitro differences identified between
holoclone and paracline cells provide a range of potential molecular and metabolic properties for selective therapeutic actions and the readily quantifiable stem and amplifying populations present in malignant cell lines provide a relatively simple in vitro system for the initial screening of therapeutic agents for their actions on malignant stem cells.

References


Acknowledgments

Received 3/20/2005; revised 5/26/2005; accepted 8/3/2005.

Grant support: Grant number 75/G 14661, Biotechnology and Biological Sciences Research Council, United Kingdom.

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