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Advances in Brief

Global Effects of Anchorage on Gene Expression during Mammary Carcinoma Cell Growth Reveal Role of Tumor Necrosis Factor-related Apoptosis-inducing Ligand in Anoikis[1]

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Abstract

Anchorage-independent growth is a hallmark of tumor cells. We compared gene expression profiles of anchored and nonanchored human mammary carcinoma cells to study this phenomenon. In this study, we show that anchorage had striking effects on cell growth and morphology but altered transcript levels from a limited number of genes. Only about 1% of mRNA transcripts detected in these cells was altered by anchorage. These include genes related to amino acid and polyamine metabolism, apoptosis, ion channels, cytoskeletal and stress proteins, transcription factors, and growth factors. Some of these may be crucial for the survival of transformed cells. For example, clusterin and the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) were suppressed by anchorage, which could help prevent programmed cell death of these tumor cells. In addition to suppressing TRAIL expression, anchorage also decreased the susceptibility of these tumor cells to TRAIL-induced apoptosis as determined by poly(ADP-ribose) phosphorylase cleavage, annexin-V binding ($P < 0.01$), and cell cycle analysis ($P < 0.0001$). These data may help explain mechanisms by which anchorage prevents apoptosis of cells that would otherwise experience anoikis. Thus, genes found to be altered by this analysis could serve as potential targets for anticancer therapy. These findings suggest that TRAIL may be used as a means to target circulating epithelial tumor cells before their attachment and colonization at new sites.

Introduction

Positional cues normally dictate how cells act in the body. Cancer cells tend to ignore these cues, grow uncontrollably, and cause harm to the entire system. Whereas normal cells generally require integrin signaling to survive and grow, transformed cells do not. Accordingly, the ability for anchorage-independent growth is a hallmark of tumorigenicity (1, 2), including that of breast carcinoma cells (3, 4). However, the nature of this phenomenon is not well understood (1, 2). In addition to integrin signaling, anchorage to a substrate influences cell shape. Indeed, morphological constraints imposed by this action are required for adherence to affect cell growth. The structural and mechanical complexities of these events make the problem difficult to address (2). We compared gene expression profiles of anchored and nonanchored human mammary carcinoma (MCF-7) cells to help clarify this situation.

About half of the several thousand genes examined in these cells were found to be active, with about 0.5% augmented and 0.5% curtailed by anchorage. These encode proteins with important functions relating to carcinogenesis. Half of the transcripts increased by anchorage are involved with amino acid synthesis, whereas anchorage suppressed genes causing apoptosis, including TRAIL.[3] In addition, anchorage protected these tumor cells against the cytotoxic effects of TRAIL.

Materials and Methods

Cell Culture and Apoptosis Assays. MCF-7 cells (700,000 or 350,000) were seeded in 4 ml of DMEM + 10% fetal bovine serum on 6-cm polyHEMA-coated or noncoated tissue culture dishes to produce nonanchored or anchored cells, respectively. Cell numbers were obtained by Coulter counter at time points indicated in Fig. 1. Cells were harvested for RNA extraction on day 3.

Apoptosis induced by TRAIL was evaluated by PARP cleavage, annexin-V binding, and cell cycle analysis. PARP cleavage was detected by Western blot analysis with an anti-PARP polyclonal antibody (Boehringer Mannheim), whereas actin was detected by an anti-actin antibody (I-19; Santa Cruz Biotechnology) as a control, as described (5, 6). Cell numbers in subG$_1$ were determined by fluorescence-activated cell sorting analysis as described (6). Annexin-V binding was analyzed with an annexin-V-EGFP apoptosis detection kit (BioVision, Palo Alto, CA).

Analysis of Gene Expression. HuGeneFL GeneChip arrays were probed with RNA and analyzed with Genechip Expression Analysis software according to protocols developed by the manufacturer (Affymetrix, Inc.). These arrays contain approximately 6800 probe sets to represent about 5600 different genes. Signals produced by RNA from nonanchored cells were used as baselines for comparison of data obtained by RNA from anchored cells. Bordering probe cell pixels were excluded from the analysis, and all of the probe sets found to be altered by anchorage were verified by visual inspection. Only alterations of genes that were consistently found to be differentially expressed by anchored and nonanchored cells in all of the four analyses were considered significant.

Results and Discussion

Human (MCF-7) mammary carcinoma cells plated on polyHEMA coated or noncoated tissue culture dishes grew as nonanchored and anchored cells, respectively. As shown in Fig. 1, anchored cells were flat with a cobblestone appearance typical of epithelia, whereas nonanchored cells were round, disorganized, presented evidence of anoikis, and lagged for about 24 h after seeding. GeneChip techniques have been demonstrated to be extremely accurate and reliable in the

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[3] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADP-ribose) phosphorylase; polyHEMA, poly(2-hydroxyethyl methacrylate).

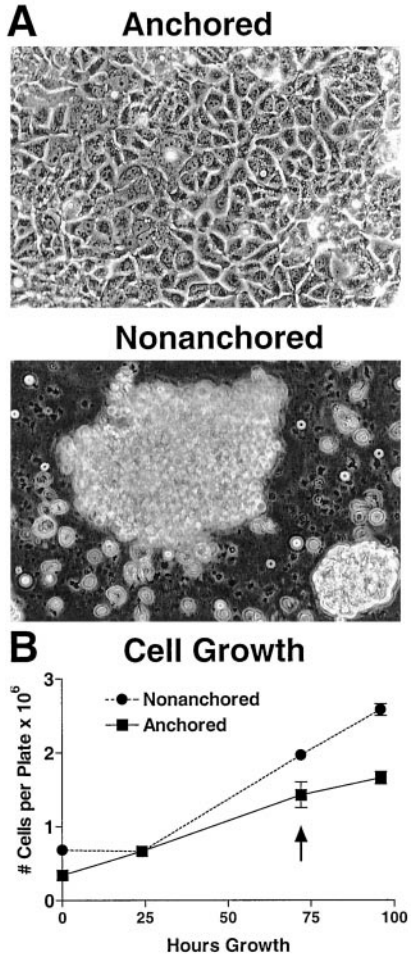

Fig. 1. Morphology and growth of MCF-7 cells. Cells (700,000 or 350,000) were seeded on 6-cm polyHEMA-coated or noncoated tissue culture dishes to produce nonanchored or anchored cells, respectively. Cells were counted at time points indicated in *Panel B*, photographed, and harvested for RNA extraction on day 3 (*arrow*). Morphology of anchored and nonanchored cells is shown in *Panel A* as indicated.

comparison of gene profiles from many cell types (7–10). We used this methodology to find genes affected by anchorage. RNA was extracted from cells 3 days after seeding, which was before they reached contact growth inhibition or cell saturation density. This RNA was used to screen arrays containing approximately 5600 different human genes. About 50% of these genes were expressed by both anchored and nonanchored cells. Only 27 (about 1%) of the approximately 2800 genes that were found to be expressed by these cells were significantly affected by anchorage; 14 were increased, and 13 were decreased. These are shown with reference to function in Table 1. Putative effects of these genetic modulations with respect to each other and cell behavior are outlined schematically in Fig. 2.

Half of the 14 genes increased by anchorage were involved in amino acid metabolism. However, asparagine and argininosuccinate synthetase may also affect breast tumor cell growth and regulate NO production at anatomical sites (11, 12). An arginine-rich protein that is often mutated in tumor cells (13) was also induced by anchorage. In contrast, anchorage decreased levels of mRNA encoding spermidine/spermine acetyltransferase, which is consistent with its putative role in polyamine metabolism in tumorigenesis (14).

As shown in Table 1, only four genes expressed by nonanchored cells were suppressed by anchorage to the extent that transcripts were not detectable in anchored cells. One of these, TRAIL, can selectively kill transformed cells, including breast tumor cells, *in vitro* and *in vivo* (15, 16). In addition, anchorage decreased levels of clusterin (TRPM-2) mRNA, which can also induce apoptosis of breast cancer cells (17, 18). Therefore, as discussed below, suppression of these genes may help account for protective effects of anchorage on cell viability.

Along with TRAIL, expression of the transcription factor hEGR1 was also completely suppressed by anchorage. Hence, it is tempting to speculate that this early growth response factor may induce TRAIL production in nonanchored cells. The epithelial-specific transcription factor (ESE-1b) was also decreased by anchorage, although not completely suppressed as in the case of hEGR1.

HSP70B was completely suppressed by anchorage, whereas the stress protein heme oxygenase, which may promote cell proliferation and angiogenesis, was decreased by about 2-fold. Heme oxygenase is induced by Ets family members (19) such as ESE-1b, suggesting that suppression of ESE-1b by anchorage may, in turn, suppress heme oxygenase. In contrast, the GRP78 and ORP150 stress proteins were induced by anchorage. These genes may neutralize hypoxia during invasion after anchorage to metastasiz-

Table 1 *Effects of cell anchorage on gene expression*

Functional descriptions of altered genes are given with GenBank accession numbers. The fold increase or decrease resulting from anchorage is shown as mean ± SE ($n = 4$). Only alterations of genes that were consistently found to be differentially expressed by anchored and nonanchored cells in all of the four analyses were considered significant.

GenBank accession no.	Fold δ	Genes
		INCREASED BY ANCHORAGE
		Amino acid metabolism
M36400	3.1 ± 0.8	Cytosolic aspartate aminotransferase
M27396	2.7 ± 0.1[a,b]	Asparagine synthetase
X01630	2.2 ± 0.1[a]	Argininosuccinate synthetase
D28473	2.2 ± 0.2	T-lymphocyte isoleucyl-tRNA synthetase
X92720	2.0 ± 0.1	Phosphoenolpyruvate carboxykinase
U09587	1.9 ± 0.1	Glycyl-tRNA synthetase
U09510	1.9 ± 0.1	Glycyl-tRNA synthetase
D32050	1.9 ± 0.1	Alanyl-tRNA synthetase
		Protein glycosylation
D87989	1.8 ± 0.1	UDP-galactose transporter related isozyme 1
		Growth factors
X51801	1.7 ± 0.0	Osteogenic protein (OP-1)
		Transcription factors
U74612	2.9 ± 0.1[a]	Hepatocyte nuclear factor-3/fork head homolog 11A (HFH-11A)
U20240	2.3 ± 0.3	CCAAT/enhancer binding protein (C/EBP) gamma
		Stress Proteins
M19645	2.3 ± 0.3[a,b]	M_r 78,000 glucose-regulated protein (GRP78)
U65785	2.1 ± 0.1[a]	M_r 150,000 oxygen-regulated protein (ORP150)
		Unknown
M83751	2.4 ± 0.1[a]	Arginine-rich protein (ARP)
		DECREASED BY ANCHORAGE
		Apoptosis
U37518	**(3.7 ± 0.5)**[a,c]	TNF-related apoptosis inducing ligand (TRAIL)
M63379	1.8 ± 0.0[a,b]	Clusterin (TRPM-2)
		Cytoskeleton
X07696	**(3.6 ± 0.2)**[a,c]	Cytokeratin 15
		Polyamine metabolism
U40369	2.1 ± 0.1[a,b]	Spermidine/spermine N1-acetyltransferase (SSAT)
		Ion channels
X76180	3.3 ± 0.2	Lung amiloride sensitive Na+ channel protein
X93036	2.5 ± 0.1[a]	Mammary tumor 8kD protein (MAT8)
		Growth factors
X62320	2.0 ± 0.1[a,b]	Epithelin 1 and 2
		Transcription factors
X52541	**(7.7 ± 0.4)**[c]	Early growth response protein 1 (hEGR1)
U73843	2.3 ± 0.2	Epithelial-specific transcription factor (ESE-1b)
		Stress proteins
X51757	**(5.5 ± 0.1)**[c]	Heat-shock protein 70B (HSP70B)
X06985	2.2 ± 0.3[a]	Heme oxygenase
		Unknown
D87953	2.5 ± 0.2[a]	Tunicamycin-responsive protein (RTP)
U88964	2.0 ± 0.1[a]	Human estrogen-regulated mRNA (HEM45)

[a] Previously reported roles in tumorigenesis in MCF-7 cells.
[b] Previously reported roles in expression in MCF-7 cells.
[c] Bold numbers in parentheses indicate transcripts absent in anchored cells, with the fold decrease being required to achieve background values.

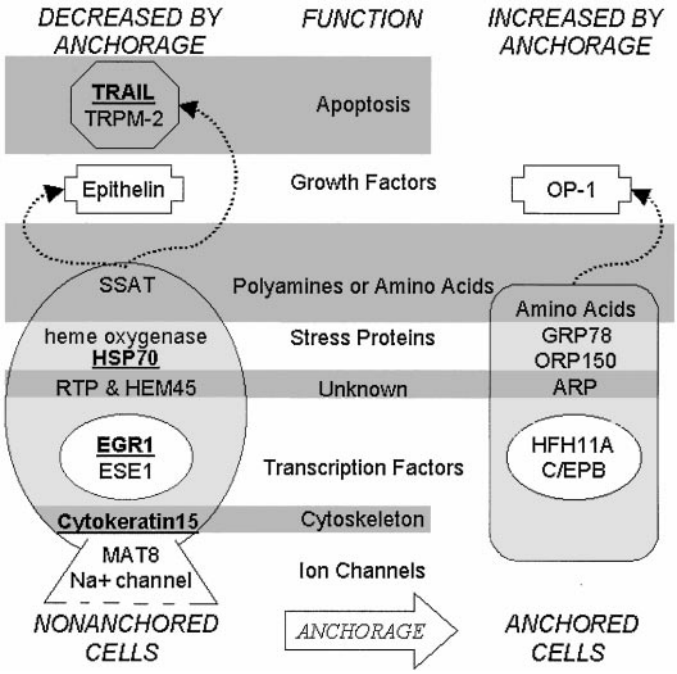

Fig. 2. Schematic diagram of genes altered by anchorage. Transcripts increased or decreased by anchorage are shown in anchored and nonanchored cells, respectively. Transcripts completely suppressed by anchorage are *bold* and *underlined*.

ing sites, which is consistent with reports of their elevated levels in breast tumor cells (20, 21).

Repression of ESE-1b by anchorage was also accompanied by complete suppression of cytokeratin 15, which, along with heme oxygenase, is induced by Ets transcription factors (22). This was the only cytoskeletal gene found to be affected by anchorage and, thus, it may be implicated in the morphological differences seen between anchored and nonanchored cells in Fig. 1*A*. Transcripts encoding epithelin 1 and 2, unique low molecular weight growth factors with contrasting abilities to induce or suppress epithelial cell growth, respectively (23), were also decreased by anchorage.

In contrast to ESE-1b, hEGR1, and epithelin, the transcription factors HFH-11A and C/EBP γ were increased by anchorage along with the growth factor OP-1. This may underlie some effects of anchorage on genes involved with amino acid synthesis. For example, C/EBP regulates transcription of phosphoenolpyruvate carboxykinase (24), which was also increased by anchorage. Interestingly, HFH-11A mRNA levels decline with age, which may contribute to decreased utilization of amino acids during senescence (10). OP-1 is a transforming growth factor-β family member implicated in tumor cell malignancy (25). The UDP-galactose transporter-related isozyme 1 was also increased by anchorage, which could affect extracellular matrix interactions by regulating proteoglycan synthesis (26).

Two of the 13 genes suppressed by anchorage encode ion channel proteins. These include the lung amiloride sensitive sodium channel protein and the mammary tumor M_r 8,000 (MAT8) protein; MAT8 encodes or contributes to chloride channels. The sodium channel is required for dome formation of mammary cells (27), whereas MAT8 is expressed in a variety of breast tumor cells (28). RTP and HEM45 were also suppressed by anchorage; they are both responsive to a variety of stimuli and convey unknown functions, although HEM45 may possess nuclease activity (29).

The effects of anchorage reported in this study presumably resulted from integrin signaling. $\alpha_v\beta_5$, which binds vitronectin, is the major integrin receptor expressed by MCF-7 cells, along with low levels of α3 (4, 30). This is consistent with our findings in which integrin expression was not affected by anchorage. However, we also found transcripts encoding integrin αE in these cells, which has been found by others in breast tumor epithelia (31). Integrin αE is generally found in lymphocytes where it associates with integrin β7 to interact with E-cadherin and promote lymphocyte infiltration into epithelia (32). It is tempting to speculate that such interactions may promote invasion of mammary carcinoma cells into surrounding epithelium. However, β7 was not expressed by these cells, and it is not known if integrin αE combined with β5 here.

The approach taken in this study was limited to one cell line and approximately 5600 genes. Nonetheless, expression of a surprisingly small number of genes was altered at the RNA level by anchorage, which had profound effects on cell growth and morphology. The apoptotic signals TRPM-2 and TRAIL were suppressed by anchorage of MCF-7 cells, possibly as a consequence of suppression of the transcriptional regulators hEGR1 and ESE-1b. This effect was evidently mediated by signaling through integrin $\alpha_v\beta_5$ and mechanical forces imposed by this interaction on cell shape (2). TRAIL induces apoptosis via its R_1 (DR4 or TNFR1SF10A) or R_2 (KILLER/DR5 or TNFR1SF10B) receptors, whereas normal cells may be protected by the decoy receptors TRID (DcR1) or TRUNDD (DcR2; Refs. 33, 34). Suppression of TRAIL by anchorage could prevent programmed cell death or anoikis of mammary tumor cells. In contrast to other necrotic

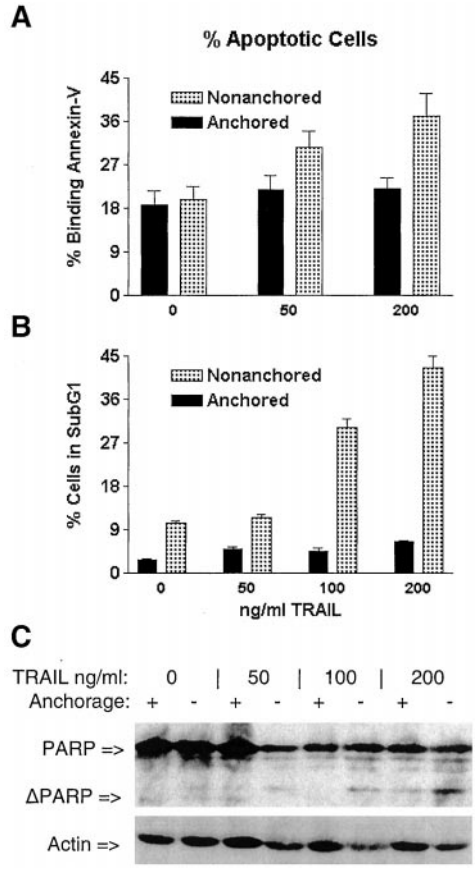

Fig. 3. Anchorage suppresses sensitivity of MCF-7 cells to TRAIL-induced apoptosis. MCF-7 cells, 5×10^5 cells/ml, were seeded on polyHEMA-coated or noncoated culture dishes to produce nonanchored or anchored cells, respectively. After 3 days of growth, cells were treated with TRAIL at 0, 50, 100, or 200 ng/ml for 12 h, as indicated. *Panels A* and *B* contain the percentage of cells in $SubG_1$ or binding Annexin-V, respectively, as determined by flow cytometry (mean $\pm$ SE; $n = 3$). *Panel C* illustrates the affect of TRAIL on caspase-3 activity based on cleavage of PARP from M_r 113,000 (PARP) to M_r 89,000 (ΔPARP). Detection of actin is shown below to demonstrate equal loading of protein for each sample.

agents, TRAIL exhibits very limited side effects and is selectively toxic to tumor cells, including breast carcinoma (15).

We addressed the functional significance of these findings by examining the effects of anchorage on the sensitivity of these cells to TRAIL. As shown in Fig. 3, anchored cells were significantly less sensitive to TRAIL than nonanchored cells as evaluated by three different methods (5, 6). Anchorage significantly reduced the inversion of phosphatidylserine from cells exposed to TRAIL as detected by annexin-V binding ($P < 0.01$ by two-way ANOVA; Fig. 3*A*). In addition, anchorage drastically reduced the numbers of cells exposed to TRAIL in the $SubG_1$ phase of the cell cycle; the interaction between anchorage and TRAIL accounted for 21.08% of the total variance, with anchorage alone accounting for 48.66% ($P < 0.0001$ by ANOVA for the interaction between anchorage and TRAIL, as well as anchorage alone; Fig. 3*B*). Finally, anchorage suppressed the caspase-3 activity in cell lysates exposed to TRAIL as detected by PARP cleavage (Fig. 3*C*). Similar results were observed using anchored and nonanchored 293 cells in the presence *versus* absence of TRAIL (data not shown).

Thus, anchorage not only suppressed TRAIL expression but also made these human mammary tumor cells less sensitive to its effects. These results may help explain mechanisms by which anchorage and integrin signaling prevent apoptosis that would otherwise be associated with nonanchored cells experiencing anoikis. This study demonstrates that applications of gene chip technology in a basic experimental approach may reveal avenues for the development of potential anticancer agents and protocols. For example, these results suggest that TRAIL, and possibly other natural apoptotic agents, may be used to specifically target circulating epithelial cancer cells before they attach and colonize to sites where they may have the potential to form tumors.

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