BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 66–70 (1997)
ARTICLE NO. RC977238

The Expression of the Molecular Chaperone Calnexin Is Decreased in Cancer Cells Grown as Colonies Compared to Monolayer

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Received August 5, 1997

Differential display was used to identify genes which were differentially expressed when HT-29 human colon adenocarcinoma cells were grown as monolayers attached to plastic or as colonies in soft agarose. One of the gel bands differentially displayed corresponded to a 171 bp fragment that showed 99% identity with a sequence of mRNA for human calnexin. The decrease in calnexin gene expression by HT-29 cells growing as colonies in soft agarose was confirmed by reverse transcriptase-PCR (RT-PCR) using calnexin-specific primers. We also used RT-PCR to show that the expression of calnexin was decreased in HT-29 cells and MCF-7 human breast adenocarcinoma cells growing in suspension in poly(hydroxyethyl methacrylate)-coated wells compared to cells growing as monolayers. The results suggest that there is a signal for the up-regulation of calnexin expression when cells contact a substrate which allows cell adhesion.

Cells in tissues are anchorage-dependent. They grow attached to each other and to the extracellular matrix. This attachment, along with contact and spreading, is required for growth and survival (1). When normal cells are grown in culture, they maintain their anchorage-dependence and lose viability and quickly become apoptotic if cultured on substrates that prevent adhesion and spreading, e.g. poly(hydroxyethyl methacrylate) (poly(HEMA)) (1,2) or soft agarose (3,4,5).

A characteristic of malignantly transformed cells is that they have decreased or absent anchorage-dependence, and they are able to grow with or without being attached to a substrate (1,2). The physiological significance of anchorage-dependence for cell survival may be a means of tumour suppression by preventing invasion and metastasis (5). It has been suggested that progressive loss of shape-dependent growth regulation during malignant transformation may lead to cell survival in the absence of extracellular matrix adhesion (6).

Adhesion-induced changes in cell physiology are regulated by adhesion-dependent signalling events in which the integrin cell surface receptors may play an important role. The survival of many cell types requires integrin-mediated adhesion to the extracellular matrix proteins (7). Integrins regulate intracellular signalling pathways such as the Ras-Raf-mitogen-activated kinase pathway, by activating signalling molecules such as focal adhesion kinase, protein kinase C and phosphatidylinositol 3-kinase (4,5,7,8,9). Gene expression is regulated by integrins through activation of tyrosine kinase pathways, possibly involving the mitogen-activated protein kinase pathway (7). Adhesion-dependent signalling is altered when cells become tumorigenic such that the cells remain viable without being attached to a substrate. Thus, differences in cellular attachment are expected to result in alterations in intracellular signalling and ultimately, changes in gene expression.

We used differential display (10) and reverse transcriptase-PCR (RT-PCR) to identify differences in gene expression between HT-29 human colon adenocarcinoma cells and MCF-7 human breast adenocarcinoma cells grown as colonies in soft agarose or in surface suspension in poly(HEMA)-coated wells, and the same cells grown as monolayers on plastic. We report that the level of mRNA for the chaperone protein, calnexin, is higher in HT-29 and MCF-7 cells growing attached to plastic compared to cells growing in an anchorage-independent manner.

MATERIALS AND METHODS

Cell Culture

HT-29 human colonic adenocarcinoma cells and MCF-7 human breast adenocarcinoma cells obtained from the American Tissue Type

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Abbreviations: RT-PCR, reverse transcriptase-PCR; poly(HEMA), poly(hydroxyethyl methacrylate); ER, endoplasmic reticulum.

0006-291X/97 $25.00

Collection (Rockville, MD) were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin in a humidified atmosphere with 5% CO_2 at 37°C, in 6-well Falcon flat bottom polystyrene tissue culture plates (Becton Dickinson, and Co., Lincoln Park, NJ), as monolayers attached to the plastic, or as colonies in 0.3% agarose or in poly(HEMA) (Aldrich Chemical Co., Inc., Milwaukee, WI) coated wells.

Growth of cells in poly(HEMA)-coated wells. Poly(HEMA) was dissolved at 1% (w/v) in 95% (v/v) ethanol and filtered through a 0.2 μm filter. Five hundred microlitres of the solution was pipetted into the wells and the plates were allowed to dry for at least one day at 37°C with the lids in place. The wells were washed twice with a phosphate-depleted buffer (0.14 M NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 0.6 mM Na_2HPO_4, pH 7.4) before the cells were seeded at 4000 cells per well.

Colony formation in soft agarose. A 1 ml basement layer of 0.5% (w/v) agarose was solidified in each of the wells, and the cells were placed on top of this in 1 ml of 0.3% (w/v) agarose at a density of 4000 cells per ml. When this layer of agarose had solidified, 1 ml of complete medium was pipetted over it to prevent dehydration.

Differential Display

Differential display was performed according to Bauer *et al.* (29) with some modifications. Cytoplasmic RNA was extracted from cells with TRIzol reagent (Gibco BRL, Gaithersburg, MD). Total RNA was reverse transcribed with Superscript II RNase H^- reverse transcriptase for 60 minutes at 37°C in four separate reactions, using four different anchored downstream primers, *viz.* $T_{11}MG$, $T_{11}MC$, $T_{11}MT$ and $T_{11}MA$ (where M = any nucleotide except T). PCR amplification of the cDNA was performed in the presence of [α-^{33}P]-dATP, with the matching downstream primer and one of the 26 arbitrary 10-mer upstream primers (Keystone Laboratories Inc., Menlo Park, CA) described in Bauer *et al.* (29) using the following cycling parameters: 94°C for 30 sec, 40°C for 60 sec and 72°C for 60 sec for 40 cycles, followed by 5 minutes at 72°C (Stratagene Robocycler Gradient 40, La Jolla, CA). The PCR amplified fragments were displayed on a nondenaturing 6% (w/v) polyacrylamide gel. X-ray film (Kodak Bio-Max, Eastman Kodak, Rochester, NY) was exposed to the gel in a cassette overnight at −80°C. Fragments of cDNA corresponding to bands that were differentially displayed between sets of cells were recovered from the gel by boiling the gel segment in dH_2O for 10 minutes. The DNA was re-amplified using the same set of primers and the same cycling parameters which had been used to generate the original PCR product. The fragments were then cloned into the pCR II vector using the Original TA Cloning kit (Invitrogen, Carlsbad, CA), and sequenced using the Sequenase Version 2.0 kit (United States Biochemical Co., Cleveland, OH). Sequences were compared to known nucleotide sequences in GenBank using the Basic Local Alignment Tool (BLAST).

Confirmation of Differential Expression: RT-PCR

We used reverse transcription-PCR (RT-PCR) to confirm that the genes represented by the individual candidate bands were being differentially expressed in the different sets of cells. RT-PCR provides comparable results to Northern blotting or *in situ* hybridisation but is more sensitive, thus allowing confirmation of differential expression even when the sample quantity is low or the target message is rare (30). We also used RT-PCR to determine whether calnexin was differentially expressed in MCF-7 cells and HT-29 cells growing in poly-(HEMA) coated wells and attached to plastic. Cytoplasmic RNA was extracted from cells with TRIzol reagent (Gibco BRL, Gaithersburg, MD) and total RNA was reverse transcribed with Superscript II RNase H^- reverse transcriptase for 60 minutes at 37°C using oligo-dT primers (Gibco BRL, Gaithersburg, MD). Calnexin-specific primers were designed such that a DNA product of known length would be selectively amplified if the mRNA transcript for calnexin was present in the RNA sample that had been reverse transcribed. RT-PCR amplification of calnexin mRNA was repeated several times with different RNA isolates and different cDNA syntheses. In negative control samples, dH_2O was substituted for DNA. Glyceraldehyde-3-phosphate dehydrogenase (GDH) was amplified from the same samples as an internal control for RNA quality and quantity, and for the efficiency of the reverse transcription and PCR steps. PCR amplified products were visualised on ethidium bromide-stained 1.2% (w/v) agarose gels.

PCR primers (Biosynthesis Inc., Lewisville, TX) used for gene-specific RT-PCR: GDH forward primer, bp 68-90 and reverse primer bp 588-565 (GenBank accession number X01677); and Calnexin forward primer bp 82-102 and reverse primer bp 1534-1514 (GenBank accession number L18887).

RESULTS

MCF-7 and HT-29 cells grown in poly(HEMA)-coated wells had the same appearance as the colonies of the same cells growing in soft agarose: they were rounded and formed multicellular, roughly spherical clusters. Fukazawa *et al.* (2) have previously reported a close correlation between viability and morphology of cells in poly(HEMA)-coated plates and colony formation in soft agarose.

Differential Display

Eighty combinations of primer sets, consisting of the four anchored downstream primers and twenty arbitrary 10-mer upstream primers were used for differential display of total RNA from HT-29 cells growing as monolayers and as colonies in soft agarose. Approximately two-thirds of the total differential display library was examined and fourteen cDNA fragments were found to be differentially displayed. Three bands showed an increase and eleven bands were decreased or absent in HT-29 cells growing as colonies. Two of the cDNA fragments corresponded to known gene sequences, one shared identity with the human homologue of a yeast ribosomal protein, four showed homology to segments of *Caenorhabditis elegans* cosmids, while the other seven did not correspond to known sequences in GenBank. PCR with the anchored downstream primer $T_{11}MG$ and the upstream primer 5′ TGG ATT GGT C 3′ amplified a band that was detectable only in HT-29 cDNA generated from cells grown in monolayer. This band was recovered from the gel, re-amplified with the same primer set, cloned and sequenced. This revealed an 171 bp fragment (Figure 1), of which bp 3-128 demonstrated 99% identity ($P = 7.6 \times 10^{-46}$: GenBank search 4 August, 1997) with fragment 659-778 of human calnexin mRNA. The mismatched base pair occurred in the upstream primer region and was presumably caused by primer mismatch and subsequent amplification.

Confirmation of Differential Expression and RT-PCR

RT-PCR with calnexin-specific primers confirmed that calnexin was highly expressed in HT-29 cells

5' TGG ATT GGT CCA GAT AAA TGT GGA GAG GAC TAT AAA CTG CAC TTC
ATC TTC CGA CAC AAA AAC CCC AAA ACG GGT ATC TAT GAA GAA AAA
CAT GCT AAG AGG CCA GAT GCA GAT CTG AAG ACC TAT TTA CTG ATA
AGA AAC ACA TCT TAC ACA CTA TCT GAT CAG ATA TAG 3'

FIG. 1. Sequence of the cloned 171 bp fragment differentially expressed in HT-29 cells growing as monolayers and as colonies, which demonstrated identity to human calnexin mRNA by comparison with sequences in GenBank. The underlined bases indicate the upstream primer sequence.

grown as monolayers but not in cells grown as colonies (Figure 2). For the amplification of the GDH product, 0.5 μl or 1 μl of cDNA was added to the PCR (Figure 2 lanes 3 and 5, and lanes 4 and 6 respectively) and for the amplification of the calnexin product, 2 μl or 3 μl of cDNA was added to the PCR (Figure 2, lanes 9 and 11, and lanes 10 and 12 respectively). The level of GDH product was approximately the same in samples from cells grown as monolayers or as colonies, indicating that the amount of starting material, cDNA and hence RNA, was approximately the same in the samples tested.

Fragments of calnexin cDNA amplified by PCR using calnexin-specific primers were barely detectable in the samples using cDNA from cells grown in poly(HEMA)-coated wells, but were readily detectable in samples using cDNA from HT-29 cells and MCF-7 cells grown as monolayers attached to plastic (Figure 3). The same graded increments in the amount of cDNA added to the PCR as described above showed that the apparent difference in the level of calnexin product was not due to quantitative differences in the mRNA. Again, the level of GDH product was approximately the same in

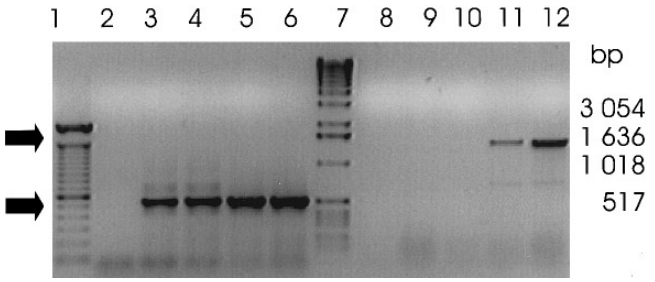

FIG. 2. Ethidium bromide-stained 1.2% agarose gel of RT-PCR products confirming differential expression of calnexin gene by HT-29 cells growing in soft agarose (lanes 9 and 10) and on plastic (lanes 11 and 12). The products in lanes 9 and 11 were amplified from 2 μl cDNA; the products in lanes 10 and 12 were amplified from 3 μl cDNA. Lanes 3 and 4 are GDH PCR products from 0.5 μl and 1 μl respectively of HT-29 cDNA from cells growing in soft agarose, lanes 5 and 6 GDH PCR products from 0.5 μl and 1 μl respectively of HT-29 cDNA from cells growing attached to plastic. Lanes 2 and 8 are negative controls for GDH and calnexin amplification respectively. Lane 1, 100bp molecular weight standard and lane 7, 1kb molecular weight standard (Gibco BRL, Gaithersburg, MD). Arrows indicate the positions of the GDH (520 bp) and calnexin (1.4 kb) PCR products.

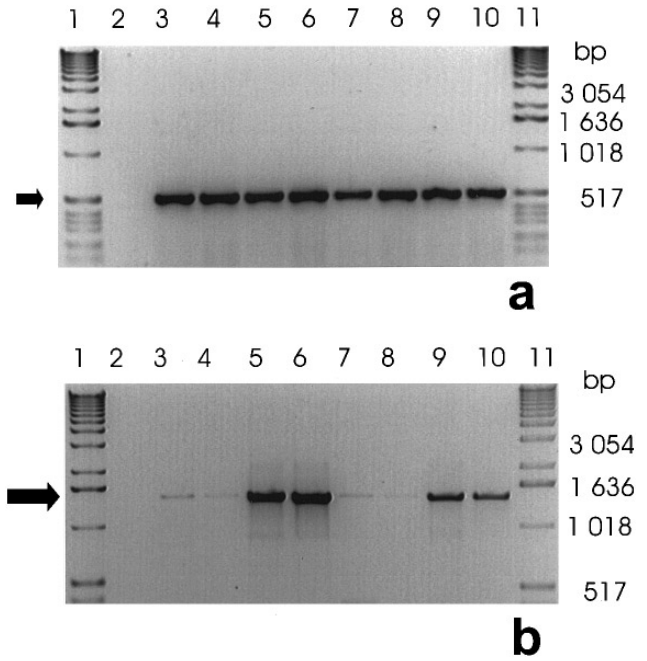

FIG. 3. GDH (a) and calnexin (b) PCR amplification products from MCF-7 and HT-29 cells, visualised by ethidium bromide-staining on a 1.2% (w/v) agarose gel. Lanes 1 and 11 in both (a) and (b) show 1 kb ladder molecular weight standards (Gibco BRL, Gaithersburg, MD), and lane 2 in both (a) and (b) show the negative controls for GDH and calnexin respectively. In both (a) and (b) lanes 3 and 4: samples amplified from MCF-7 cells grown in poly(HEMA)-coated wells; lanes 5 and 6: MCF-7 cells grown attached to plastic; lanes 7 and 8: samples from HT-29 cells grown in poly(HEMA)-coated wells; and lanes 9 and 10: HT-29 cells grown attached to plastic. Products in (a) lanes 3, 5, 7 and 9 were amplified from 0.5 μl of cDNA, and in lanes 4, 6, 8 and 10 were amplified from 1 μl of cDNA. In (b) 2 μl of cDNA were added to the PCR of the samples in lanes 3, 5, 7 and 9, and 3 μl of cDNA were added to the PCR of the samples in lanes 4, 6, 8 and 10. Arrows indicate (a) the position of the GDH PCR product (520 bp), and (b) the position of the calnexin PCR product (1.4 kb).

the tested samples. Up to 3 μl of cDNA was added to the PCR which corresponds approximately to the quantity reverse transcribed from 0.3 μg of total RNA.

DISCUSSION

Calnexin, also referred to as IP90 (11) or p88 (12), is a calcium-binding (13,14), endoplasmic reticulum (ER)-resident transmembrane protein which acts as a molecular chaperone for glycosylated and oligomeric proteins (11,15). Newly synthesised, incompletely folded, glycosylated proteins associate with calnexin and the closely related soluble protein, calreticulin (14,16) until they are correctly processed and folded. Mutant proteins, partially assembled oligomers in cell lines in which a component of the complex is not expressed, or proteins misfolded due to the incorporation of azetidine-2-carboxylic acid, display prolonged association with calnexin and their rates of secretion are slowed

(11,15,17,18). Calnexin has an ubiquitous tissue expression and associates with many different proteins (11), and it has been suggested that calnexin plays a major role in the quality control mechanisms for secretory glycoproteins (15). The synthesis of mammalian calnexin, unlike molecular chaperones of the Hsp families, is not significantly affected by stresses such as heat shock, ionophores, or amino acid analogues (18). The event that induces calnexin appears to be the initial contact of the cell with a surface to which it can attach, possibly *via* integrin signalling (4,7,8).

Calnexin-like proteins are found in yeast, plants and mammals, [(18) and references therein] suggesting an important role for the protein. Calreticulin and calnexin share extensive molecular homology (11,14,19), and there is considerable overlap between the substrate glycoproteins of calnexin and calreticulin, and they can associate with the same protein simultaneously or sequentially (16). Some proteins which associate with calnexin are: MHC class I molecules (20), T-cell receptor complex (11), the cystic fibrosis transmembrane conductance regulator (18) and integrin chains β_1 and α_6 (21). It has been shown that calnexin is not absolutely required for the surface expression of major histocompatibility class I molecules nor for the viability of a human leukaemic cell line which lacked both calnexin protein and mRNA (22). However, in the yeast *Schizosaccharomyces pombe* which lacks a calreticulin homologue, loss of calnexin is lethal (16,23). In cells transfected with truncated variants of calnexin, proteins which would normally be retained by calnexin in the ER are redistributed to the cell surface (24). Thus, it would be expected that the decrease in calnexin during anchorage-independent growth would also result in a re-distribution of proteins to the cell surface.

An increase in the expression of variant glycoprotein adhesion molecules has been associated with transformation and tumour progression, for example the tumour-specific antigens CD44E (25), CD44v (26) and MUC18 (27). However, when cancer cells are grown as a monolayer on plastic they lose some of their tumour-specific antigens (28). This may be because calnexin which is expressed by cells attached to a support retains incorrectly folded or mutant glycoproteins in the ER and prevents them from being expressed at the cell surface. Our studies suggest that the increase in variant adhesion molecule cell surface glycoproteins that occurs in some cancers and can be indicative of tumour progression, may be due to the down regulation of calnexin as the cells become anchorage-independent. The differential expression of calnexin by cells growing as monolayers and in three-dimensional culture may also explain the loss of tumour-specific antigens when cancer cells are grown in monolayer and their re-expression when the cells are grown in three-dimensional cultures. While our studies cannot distinguish whether the decrease in calnexin expression occurs as a result of colony formation, or colony formation is allowed to proceed as a result of the decrease in calnexin expression, an increase in cell surface glycoproteins as a result of lower calnexin levels, which may be seen as an effort by the cell to adapt to its new environment by producing novel attachment factors that may be better suited to the available substrate.

ACKNOWLEDGMENTS

This work was supported by NIH Grants CA52995 and CA61015. We are grateful to Dr Mark Kunkel for his computer expertise, for useful discussion, and for critically reading the manuscript.

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