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Proteomic Analysis Reveals Differences in Protein Expression in Spheroid versus Monolayer Cultures of Low-Passage Colon Carcinoma Cells

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Spheroid cultures of cancer cells may better reflect characteristics of tumors than traditional monolayer cultures. Furthermore, low-passage cancer cell lines recapitulate the properties of the original tumor cells more closely than commonly used standard cell lines that experience artificial selection processes and mutations over years of passaging. Here we established spheroid cultures of the low-passage colon cancer cell line COGA-5 and stable COGA-12 aggregates with local areas of compaction. The proteomes of both three-dimensional cultures were analyzed versus their corresponding two-dimensional cultures. 2-D gel electrophoresis followed by peptide mass fingerprinting identified three differently expressed proteins in COGA-5 spheroids (acidic calponin, hydroxyprostaglandin dehydrogenase, and lamin A/C) and two in COGA-12 partly compact aggregates (two isoelectric variants of the acidic ribosomal protein P0) compared to the respective monolayer cultures. The lamin A/C spot showed a lower molecular weight in the 2-D gel (30 kDa) than expected for full-length lamin. Further Western blot analysis and immunocytochemistry identified the lamin protein as a caspase-6-cleavage product in apoptotic cells of the spheroid. Similar caspase-dependent lamin cleavage was observed in monolayer cultures after serum withdrawal and further increased under hypoxic conditions, suggesting cleaved lamin as an indicator for apoptotic stress. In conclusion, proteome analysis of multicellular spheroids versus monolayers cultures identifies differential protein expression relevant to tumor cell proliferation, survival, and chemoresistance and thus may reveal novel targets for cancer therapy.

Keywords: acidic calponin • acidic ribosomal protein P0 • colon cancer • hydroxyprostaglandin dehydrogenase • lamin A/C • mass spectrometry • spheroid culture • two-dimensional gel electrophoresis

Introduction

The lack of suitable cancer model systems, which sustain the properties of the original tumor in vitro, is a general flaw in cancer research. For example, obtaining primary cultures of colon carcinoma cells from patients is very laborious, can hardly be standardized, and the success rate is far below 10%. In addition, most cancer cell lines accumulate genetic alterations or undergo artificial selection processes during several in vitro passages, thereby loosing their original phenotype. A promising prospect has been provided by recently developed low-passage colon cancer cell lines that closely resemble the phenotypes of the corresponding original tumors.[1] Extensive investigation of these low-passage cell lines demonstrated, for example, a pronounced diversity of the individual tumor cells regarding oncogenic mutations.

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However, traditional monolayer cultures of these low-passage cell lines still hardly recapitulate characteristics that result from the three-dimensional growth in tumors in vivo. Multicellular spheroid cultures are intermediates between monolayer cultures and tumors [2,3] since they resemble the in vivo situation more closely with regard to cell proliferation, differentiation, and cell environment, i.e., cell—cell contacts and different growth areas. A major feature of multicellular spheroids is that they consist of proliferating cell populations as well as areas with post-mitotic, yet viable, cells and also compact structures, often in the spheroid core, which may contain necrotic or apoptotic cells.[4] This structure reflects the assembly of a tumor better than monolayer cultures and may therefore provide an advanced model system for in vivo tumors. For example, murine EMT6 mamma carcinoma cells exhibiting chemoresistance after treatment of the tumor-bearing mice recapitulated this resistant phenotype in multicellular spheroids but not in monolayer cultures.[5]

Similarly, spheroids of the low-passage colon carcinoma cells may better reflect the original patient phenotype than monolayer cultures and, therefore, provide adequate model systems for analysis of colon cancers and development of novel

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therapeutic strategies. Such spheroid cultures likely exert differences in the protein expression pattern compared to traditional monolayer cultures, which may affect cellular signaling pathways, cytoskeleton structure, proliferation, or sensitivity to therapeutic approaches. In the present work, we therefore established three-dimensional multicellular spheroids of the unique human colon cancer cell lines and compared the proteome of monolayers and spheroids using 2-D gel electrophoresis followed by analysis of differentially regulated proteins with mass spectrometry.

Materials and Methods

Cell Culture. Human low-passage cell lines COGA-1, COGA-2, COGA-3, COGA-5, COGA-5L, COGA-10, and COGA-12 were obtained from colorectal cancer patients and resemble the phenotype of the original tumors as previously described.[1] Cell lines COGA-1, COGA-5, and COGA-10 exhibited epithelial-like COGA-5L and COGA-12 piled-up, and COGA-2 and COGA-3 rounded-up morphology. Only 5−10 passages after harvesting the primary tumor cells from the patients were required for the generation of such low-passage cell lines. The cell lines were grown in RPMI 1640 medium containing 10% serum (Invitrogen, Karlsruhe, Germany) at 37 °C in 5% CO_2 humidified atmosphere.

Multicellular Spheroid Culture. Multicellular spheroids were generated using the liquid overlay technique as described previously.[6] Briefly, 24 well culture plates (Nalge Nunc International, Naperville, IL) were coated with 300 μL of 1% SeaPlague agarose (Biozym, Hess, Germany) in serum-free growth medium. In each well 10^5 cells from a single-cell suspension were added to total volume of 1 mL growth medium containing 10% serum, and multicellular spheroids were allowed to form over 24−96 h. To inhibit caspases-6 induced lamin A cleavage in spheroids, the caspases-6-specific inhibitor Z-VEID-fmk (50 μM) was added to the media previous to spheroid formation. Spheroids were grown 24 h before cell lysis followed by Western blot analysis. Controls were grown in media containing appropriate amounts of DMSO.

2-D Electrophoresis and Mass Spectrometry. Monolayer cells or pooled multicellular spheroids were washed with 0.5× PBS and lysed in 460 μL 2-D-lysis-buffer (9 M Urea, 5 mM EDTA, 4% CHAPS, 1% DTE), homogenized (QIAshredder Homogenizer, Qiagen, Hilden, Germany), and centrifuged at 17 600× *g* for 30 min. Isoelectric focusing (IEF) of supernatant protein samples (200 μg protein for analytical gels or the 10-fold amount for preparative gels) was carried out by using commercial 24 cm IPG polyacrylamide strips with a linear pH gradient from pH 4 to 7 (in sample rehydration) and an Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences, Uppsala, Sweden; focusing time: 90 000 Vhs). Second dimension gel electrophoresis was carried out until the bromophenol blue front left the gel by using an Ettan DALTtwelve System (Amersham Biosciences, Uppsala, Sweden). Visualization of peptides was performed by silver staining using formaldehyde for better compatibility with mass spectrometry.[7] Automatic spot detection, spot matching, and normalization were performed with Proteomweaver 2.2 software (Definiens, Munich, Germany). Differential protein expression was identified if the average normalized signals altered at least 2-fold between samples and the alteration was at least detected in three separate gels of at least two independent 2-D electrophoresis runs.

Protein spots of interest were destained with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate and subjected to trypsin digestion (Promega, Mannheim, Germany). Peptides were desalted by using C18 ZipTip pipet tips (Millipore, Bedford, USA), mixed with alpha-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Leipzig, Germany), and spotted onto a MALDI-TOF MS target. MALDI-TOF mass spectrometry was carried out using an autoflex II spectrometer (Bruker Daltonics, Leipzig, Germany) in the reflector mode. The mass spectrometer was calibrated using a peptide calibration standard (Bruker Daltonics, Leipzig, Germany). Mass spectra were processed and analyzed using the flexAnalysis 2.2 software (Bruker Daltonics, Leipzig,Germany). Internal recalibration of the mass spectra was performed using peaks derived from autoproteolysis of trypsin. Peptide mass fingerprinting was performed by using the BioTools 2.2 software (Bruker Daltonics, Leipzig, Germany). Briefly, proteins were identified by database search on MASCOT server (http://www.matrixscience.com) using the MSDB database. Searches were performed with mandatory carbamidomethylation of cysteines and variable oxidation of methionine residues. The most frequently occurring contaminant peptides derived from keratin and trypsin autodigestion peptides were deleted before database searching in order to avoid random matches. Analysis criteria allowed a peptide mass tolerance of 50 ppm and one missing cleavage.

Western Blot Analysis. After SDS-PAGE and transfer to a PVDF membrane (Macharey Nagel, Düren, Germany) the blot was probed with either a rabbit monoclonal antibody detecting full length lamin A/C and cleaved lamin A (1:1000, cell signaling technology, Danvers, U.S.A.), with an antibody detecting only cleaved lamin A (1:1000, cell signaling technology, Danvers, U.S.A.), with a goat polyclonal antibody detecting acidic calponin (1:500, Santa Cruz Biotechnology, Heidelberg, Germany) or a mouse monoclonal antibody detecting keratin 18 (1:500, cell signaling technology, Danvers, U.S.A.) in Tris buffered saline containing 0,5% Tween (TBST) and 5% nonfeat milk powder at 4 °C over night. After washing with TBST membranes were exposed to goat anti-rabbit HRP-conjugated secondary antibody (1:2000; Vector Laboratories, Burlingame, U.S.A.) followed by a chemiluminescence detection (ECL; Amersham Biosciences, Arlington Hights, IL). Equal protein loading was controlled by reprobing the membrane with a mouse monoclonal anti-α-Tubulin antibody (1:5000; Santa Cruz Biotechnology, Heidelberg, Germany) and a secondary anti-mouse HRP-conjugated antibody (1:5000; Vector Laboratories, Burlingame, U.S.A.).

Oxygen and Serum Deprivation. For oxygen-deprivation, cells were incubated in an oxygen-free N_2/CO_2 (95%/5%) atmosphere for 24 h. For serum-deprivation, cells were incubated in RPMI containing different percentages of fetal calf serum (0%, 2%) for 24 h. To block lamin cleavage by inhibition of caspases, cells were preincubated 30 min prior to hypoxia with the inhibitors z-VAD-fmk (10 or 50 μM) or the specific caspase 6 inhibitor z-VEID-fmk (50 μM). Control cultures were grown in RPMI containing 10% fetal calf serum.

Chemotherapy. COGA cells were treated with either Camptothecin (Sigma-Aldrich, Taufkirchen, Germany), (25 μM) or 5-fluorouracil (Sigma-Aldrich, Taufkirchen, Germany), (20 μM) for 3 days before cell lysates were subject to Western blot analysis. Controls were treated with the appropriate amounts of DMSO.

Immunostaining of Lamin A/C in Multicellular Spheroids. After 4 days in culture, multicellular spheroids were transferred

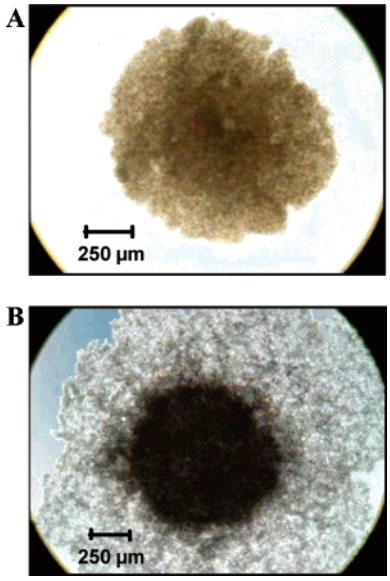

Figure 1. Three-dimensional cultures of the cell lines COGA-5 (A) and COGA-12 (B) using the liquid overlay technique. Cells (1×10^5) were seeded in agarose-covered 24 well plates and cultivated in medium containing 10% growth factors (FCS) for 96 h (COGA-5) or 72 h (COGA-12). COGA-5 cells are forming fully compact spheroids, and COGA-12 cells are forming partly compact aggregates with local areas of compaction.

to tissue freezing medium (Leica Microsystems, Nussloch, Germany) and frozen at −80 °C. Cryosections (5 µm) were obtained with a Leica CM3050S cryostat (Leica Microsystems, Nussloch, Germany). Sections were transferred to microscope slides (SuperFrost, Menzel, Braunschweig, Germany), fixed in 4% paraformaldehyde, and subsequently treated with 0.2% Triton-X for 5 min. After blocking with 3% goat serum in PBS for 1 h, the sections were incubated with rabbit anti-lamin A/C (1:100, cell signaling technology, Danvers, USA) or anti-cleaved lamin A antibody (1:100, cell signaling technology, Danvers, USA) in 3% goat serum in PBS at 4 °C over night. Afterward, the sections were exposed to biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, USA) in 3% goat serum in PBS for 2 h at RT followed by incubation with Oregon Green streptavidin (Invitrogen, Karlsruhe, Germany) for 40 min in PBS. For counterstaining of the nuclei, the slides were incubated with DAPI (4′,6-diamidino-2-phenylindole) at a concentration of 1 µg/mL in PBS for 15 min. Detection of Oregon Green indicating lamin A/C or fragmented lamin A was performed using a fluorescent confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) at 488 nm excitation (530 nm emission), and DAPI fluorescence was detected at 364 nm excitation (385 nm long-pass emission). Image recording was performed with the LSM 5 software, version 3.0 (Carl Zeiss, Jena, Germany).

Immunostaining of Ki67 in Multicellular Spheroids. Immunostaining was performed by the avidin−biotin−peroxidase complex staining method according to standard protocols. Briefly, paraffin sections were dewaxed and rehydrated. Antigen retrieval was performed by heat induction incubating the sections in 0.1 M citrate buffer (pH 6.0) for 60 min in the microwave. Sections were incubated with the primary antibody for 60 min at room temperature in a moist chamber. The proliferation antigen Ki67 was detected with mab MIB-1 (0.8 µg/mL, IgG1, Dako, Germany). After washing in PBS, sections were incubated with a biotinylated antibody diluted (1:4000)

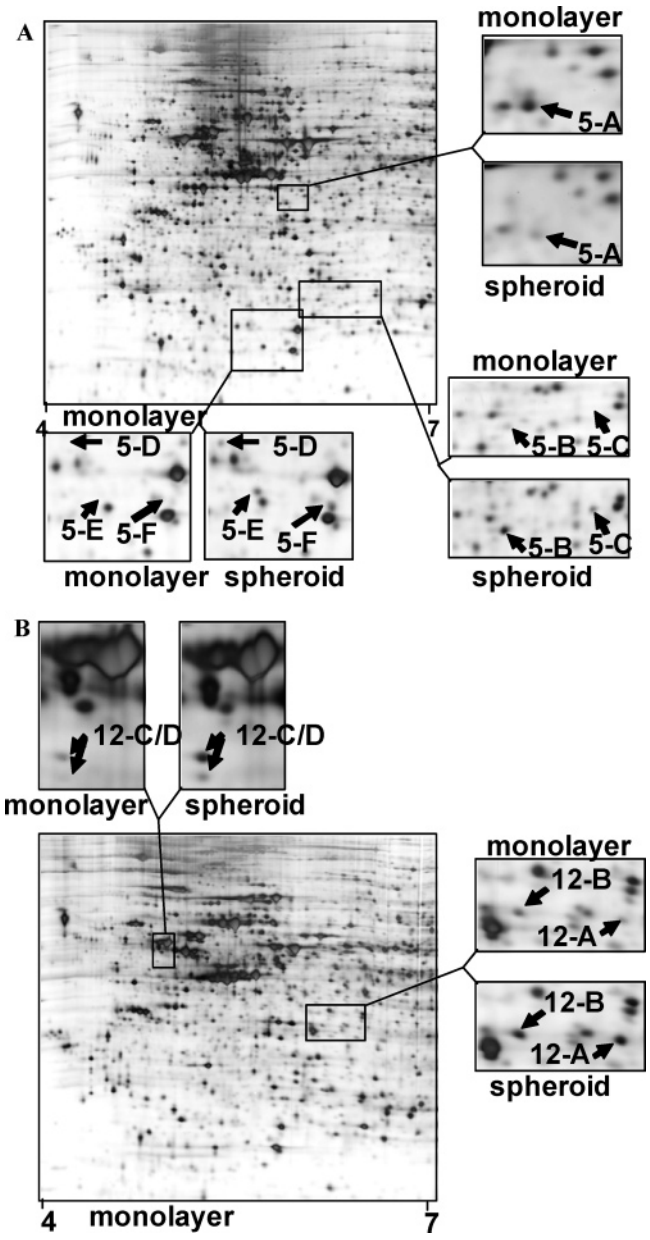

Figure 2. Differences in the expression profiles of multicellular spheroids/aggregates and their corresponding monolayer cultures obtained by 2-D gel electrophoresis. 2-D gel electrophoresis of cell lysates of the cell lines COGA-5 (A) and COGA-12 (B) were performed with IPG dry strips pH 4−7 in the first dimension and 11% SDS-polyacrylamidgels in the second dimension. Representative silver-stained gels are shown. Differences in the expression profiles are marked with an arrow.

in PBS for 30 min. Slides were incubated with peroxidase conjugated streptavidin (MIB-1). Peroxidase reaction was developed in AEC for 8 min at room temperature. The sections were counterstained and mounted.

Detection of Apoptosis in Multicellular Spheroids. Apoptotic cells were detected on paraffin embedded section of multicellular spheroids at different time points 24 h and up to 120 h after spheroids formation using a TUNEL assay (Roche) according to manufacturer instructions. Briefly: After dewaxing and rehydration according to standard protocols, the spheroid sections were treated with proteinase K working solution (Roche) for 10 min. The TUNEL reaction mixture was added

Table 1. List of Proteins Identified by MALDI-TOF MS

spot no.	identified protein	accession no.	theoretical MW (Da)	theoretical p*I*	protein score	sequence coverage %	no. of matched peaks
5-A	Acidic calponin	JC4501	36562	5.69	116	55	18
5-B	15-hydroxyprostaglandin dehydrogenase	A35802	29187	5.56	86	53	13
5-C	Lamin A/C	Q5TCJ4	53219	6.13	74	31	18
12-A	Acidic ribosomal protein P0	R5HUPO	34423	5.71	155	65	19
12-B	Acidic ribosomal protein P0	R5HUPO	34423	5.71	184	65	20

and incubated for 60 min at 37 °C in a humidified atmosphere. After intensive washing with PBS, the sections were mounted with antifade and analyzed using a fluorescence microscope at 488 nm excitation (530 nm emission).

Results

Establishment of Multicellular Spheroids of Low-Passage Colon Cancer Cell Lines. Seven low-passage colon cancer cell lines of three different phenotypic morphologies as described earlier[1] were tested for their ability to form multicellular spheroids. However, only one cell line, COGA-5 (epithelial-like), generated fully compact multicellular spheroids as shown in Figure 1A. Furthermore, the cell lines COGA-5L and COGA-12 (both of piled-up morphology) formed partly compact multicellular aggregates with local areas of compaction (Figure 1B). The other cell lines tested only formed loose, unstable aggregates or did not survive as a three-dimensional culture and died rapidly after detaching from the surface of the cell culture dish. The cell lines COGA-5, representative for fully compact multicellular spheroids, and COGA-12, representative for cells forming partly compact clustered aggregates, were subject for further investigations. Spheroids of the cell line COGA-5 had a constant small diameter of 1200 μm, whereas partly compact aggregates of the cell line COGA-12 exhibited a larger size of approximately 2300 μm. Ninety-six hours after their formation, COGA-12 cells aggregates developed rosette-like structures within the areas of compaction (Supplementary Figure 1B, Supporting Information). Staining with an antibody detecting the proliferation marker Ki67 showed that spheroids/aggregates of both cell lines still contained proliferating cells even after 96 h (Supplementary Figure 1, Supporting Information). For these reasons spheroids were grown for 96 h before analyzed by 2-D gel electrophoresis.

Different Protein Expression Pattern in Monolayer Cultures Compared to Spheroids. Both multicellular spheroids of cell line COGA-5 and partly compact aggregates of cell line COGA-12 were further investigated by 2-D electrophoresis. Proteome analysis revealed different expression of six proteins in COGA-5 spheroids (five up- and one down-regulated) (Figure 2A and Supplementary Figure 2A, Supporting Information) and altered expression of four proteins in COGA-12 aggregates (all up-regulated) (Figure 2B and supplementary Figure 2B, Supporting Information) compared to the respective monolayer cultures. The average normalized signals of peptide spots 5-D, 5-F, 12-A, 12-B, and 12-C were increased approximately 2-fold, the signal of spot 5-B was increased approximately 3.5-fold, and the signal of spot 5-A was decreased about 3-fold in multicellular spheroids/aggregates compared to respective monolayers. Spots 5-C, 5-E, and 12-D were only detected in samples of multicellular spheroids/aggregates. The altered expression of the proteins identified in multicellular spheroids of cell line COGA-5 could be demonstrated in cell lysates of both 48 and 96 h old spheroids. Aggregates of COGA-12 cells were not subject of analysis prior to 96 h after their formation as no rosettes were detected before this time point.

Mass Spectrometry-Based Identification of Differentially Expressed Proteins. MALDI-TOF mass spectrometry and subsequent peptide mass fingerprinting identified three of the differently expressed peptides in COGA-5 multicellular spheroids as acidic calponin, 15-hydroxyprostaglandin dehydrogenase, and lamin A/C (Table 1). The altered expression of acidic calponin was verified by Western blot analysis (Figure 3A). In COGA-12 partly compact aggregates, it was possible to identify two differently regulated peptides, which are two isoelectric variants of the same protein, namely acidic ribosomal protein P0 (Table 1). According to the spot position in the 2-D gel, the protein identified as lamin A/C exhibits a much lower molecular weight (approximately 30 kDa) than expected for the intact lamins A or C. This spot probably represented a fragment of lamin A/C, and thus, this protein was further investigated in detail. 15-Hydroxyprostaglandin dehydrogenase, acidic calponin, and the acidic ribosomal protein P0 will be addressed in detail in separate studies.

Lamin A/C Fragmentation in Multicellular Spheroids. Western blot analysis revealed the presence of a lamin A/C fragment (approximately 30 kDa) in COGA-5 spheroids in addition to the intact lamins A and C (Figure 3B). Immunoblot analysis further revealed that the lamin A/C fragment was also detectable in partly compact COGA-12 aggregates (Figure 3B). Notably, expression of lamin A/C and lamin A cleavage was less pronounced in COGA-12 aggregates compared to COGA-5 spheroids. The fragmentation of lamin A was already present after 24 h in COGA-5 spheroids but increased in more compact spheroids, e.g., 96 h after spheroid formation (Figure 3C). TUNEL staining at different time points 24 h and up to 120 h after formation of COGA-5 spheroids revealed that the number of apoptotic cells also increased with time similar to the rising levels of fragmented lamin A in the COGA-5 spheroids (Figure 3D).

Lamin A/C Fragmentation in Monolayer Cultures After Serum Withdrawal. Since COGA-5 spheroids were more compact and expressed more lamin A/C fragment compared to COGA-12 aggregates, we addressed the question of whether the presence of truncated lamin A/C in spheroids could be associated with the lack of oxygen and/or trophic support, conditions known in compact areas of the spheroids.[4] Mimicking these conditions in monolayers Western blot analysis revealed that the lamin A/C fragment was detectable in monolayer cells after serum withdrawal (Figure 4A). Hypoxia further enhanced the level of the protein fragment in cultures deprived of serum, whereas hypoxia alone was not sufficient to induce a lamin A fragmentation in cells cultured with medium containing 10% serum. The fragment was also detected in cells cultivated in medium with reduced serum concentration (2%) when simultaneously exposed to hypoxia (Figure 4A).

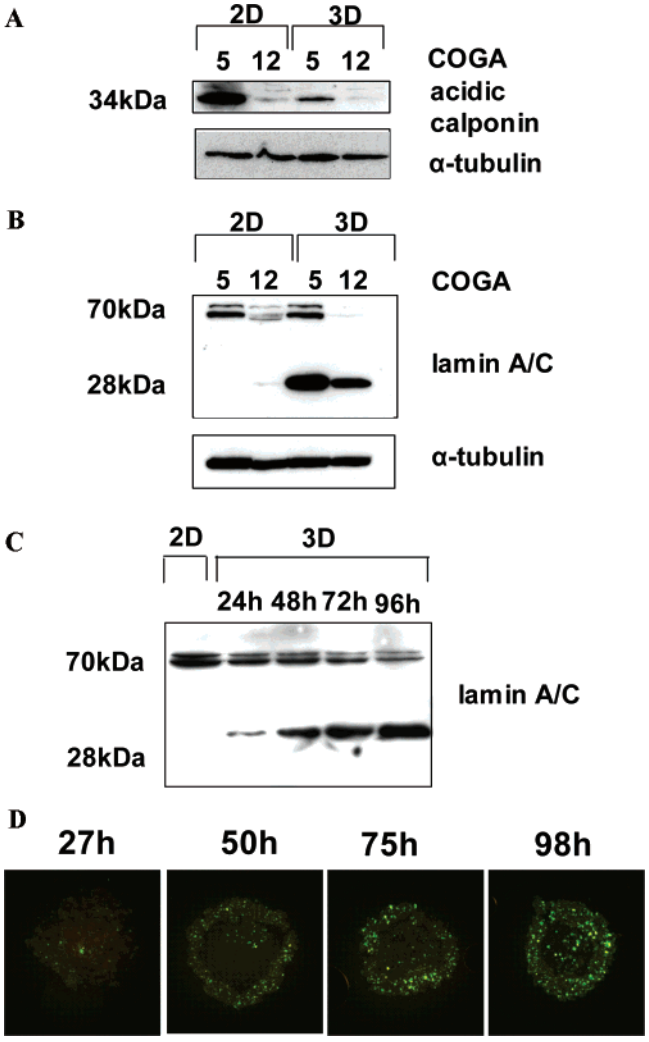

Figure 3. Proteins differentially regulated in three-dimensional cultures of low-passage colon cancer cells. Expression of acidic calponin (A) and a lamin A/C fragment (B) in multicellular spheroids of COGA-5 cells and partly compact aggregates of COGA-12 compared to corresponding monolayer cultures analyzed by Western blotting using an anti-acidic calponin or anti-lamin A/C antibody, respectively. (C) Expression of the lamin A/C fragment in COGA-5 spheroids analyzed by Western blotting and (D) TUNEL staining in paraffin embedded sections of COGA-5 spheroids at indicated time points after spheroid formation.

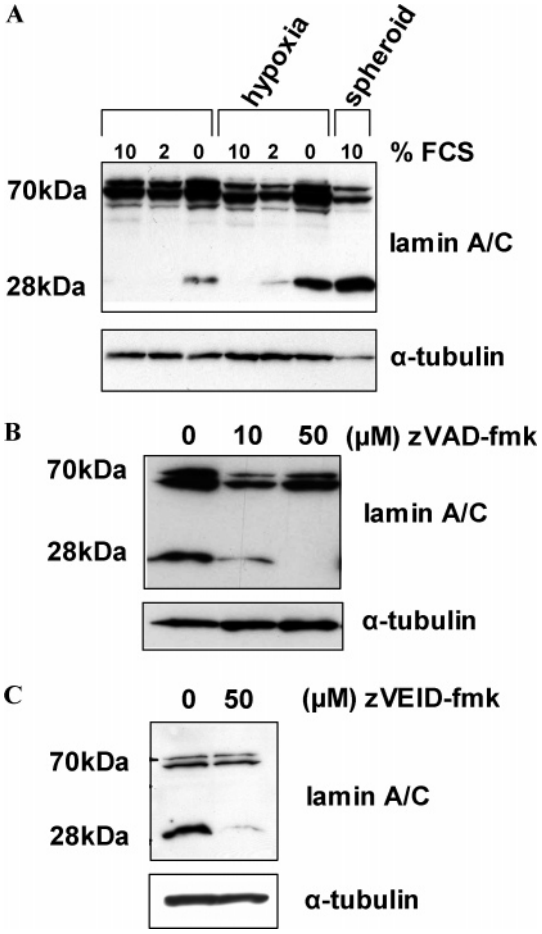

Figure 4. Expression of the lamin A/C fragment in monolayer cultures after depletion of oxygen and serum analyzed by Western blotting using an anti-lamin A/C antibody. (A) COGA-5 cells were incubated for 24 h at hypoxic and normoxic conditions and indicated serum concentrations. Results obtained in spheroid culture are given for comparison. (B) COGA-5 cells were incubated for 24 h at normoxic conditions in absence of growth factors and were treated with different concentrations of a pan-caspase-inhibitor. (C) Caspase 6 inhibition in COGA-5 multicellular spheroids. Spheroids of COGA-5 cells were incubated with the specific caspase 6 inhibitor z-VEID-fmk. Lamin A/C expression was analyzed 24 h after spheroid formation by Western blotting using an anti-lamin A/C antibody.

Caspase Mediated Lamin A/C Fragmentation. It has been suggested that lamin A/C is cleaved in a caspase-dependent manner during apoptosis.[8] To confirm the involvement of activated caspases in the observed lamin A/C fragmentation in monolayer cultures, we applied the pan caspase inhibitor zVAD-fmk (10 and 50 μM) during serum deprivation. Caspase inhibition abolished the appearance of the lamin A/C fragment after serum deprivation in a dose-dependent manner (Figure 4B). Further, the caspases 6-specific inhibitor zVEID-fmk (50 μM) significantly attenuated the cleavage of lamin A/C (Figure 4C) in spheroid cultures of COGA-5 cells, confirming that the observed lamin cleavage was mediated by caspase-6.

Western blot analysis using an antibody specific against caspase-cleaved lamin A fragment confirmed the previously observed lamin A/C fragment in monolayer cells challenged by serum withdrawal as a product of caspase cleavage (Figure 5A). Such stress-induced caspase cleavage product was observed in cells deprived of serum in normoxia as well as in hypoxic conditions. In COGA-5 spheroids, immunocytochemistry revealed high levels of caspase-cleaved lamin A in individual cells that were distributed throughout spheroid without restriction to inner areas (Figure 5B). In most cells positive for fragmented lamin A/C, the nuclei appeared pyknotic or fragmented, indicating that enhanced lamin cleavage occurred in cells undergoing apoptotic cell death.

Since we could observe caspases-6 dependent lamin A cleavage in multicellular spheroids, we further investigated if other caspases-6 substrates, e.g., cytokeratin 18, [9] were also cleaved during the apoptotic process in COGA-5 spheroids or COGA-12 aggregates. Western blot analysis revealed cytokeratin 18 cleavage in both three-dimensional cultures (Figure 5C). Notably, induction of COGA cell apoptosis by 5-fluorouracil or camptothecin was also accompanied by cleavage of lamin A and cytokeratin 18, suggesting activation of similar caspase-dependent cell death pathways in COGA cells after spheroid

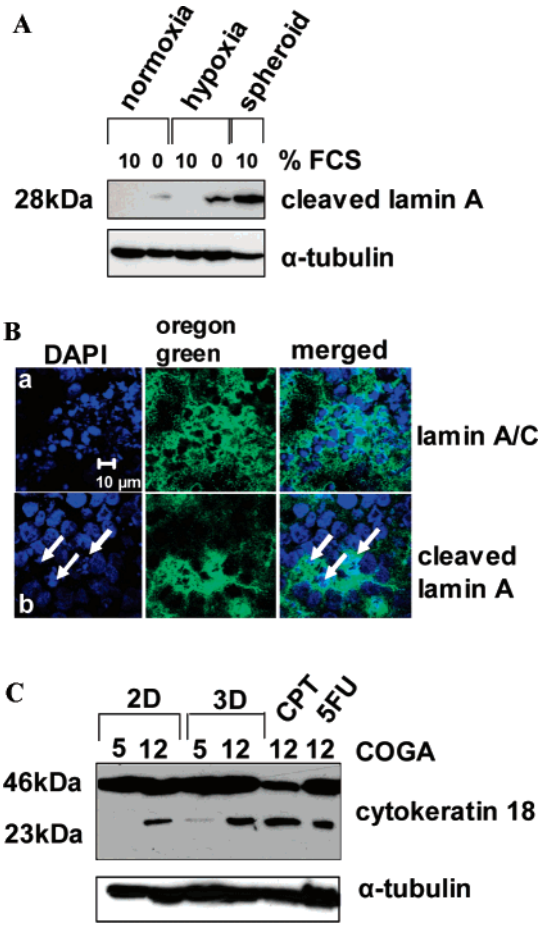

Figure 5. Caspase cleavage products. (A) Caspase 6 cleavage product of lamin A in serum-deprived monolayer cultures. COGA-5 cells were incubated for 24 h at hypoxic and normoxic conditions and different amounts of growth factors. Lamin A/C fragment expression was analyzed by Western blotting using an antibody specific against the caspase cleavage product of lamin A. Results obtained in spheroid culture are given for comparison. (B) Distribution of lamin A/C and its caspase cleavage product within spheroids. Cryosections of COGA-5 spheroids were incubated with antibodies against full length lamin (a) and cleaved lamin (b) followed by a biotinylated antibody which was visualized by oregon green-streptavidin (green). The sections were analyzed by confocal microscopy. Control sections were only incubated with a biotinylated secondary antibody, counterstaining was performed with DAPI (blue). Arrows mark pyknotic nuclei from apoptotic, cleaved lamin positive cells. Photomicrographs representative for the whole spheroid are shown. (C) Expression of cytokeratin 18 in 96 h hold three-dimensional cultures of COGA 5 and COGA 12 compared to corresponding monolayer cultures and after 3 days treatment with chemotherapeutics such as camptothecin (25 μM) and 5-fluorouracil (20 μM).

formation, by deprivation of trophic support and hypoxia, or after exposure to chemotherapeutics.

Discussion

In the present work, the low-passage human colon cancer cells COGA-5[1] were cultured as multicellular spheroids to provide a model system that reflects the three-dimensional structure of the respective tumor in vivo. The COGA-5 cell line was the only one out of seven tested low-passage colon cancer cell lines that generated fully compact multicellular spheroids. Further, the COGA-12 cell line formed partly compact multicellular aggregates with local areas of compaction. The COGA-12 aggregates were additionally investigated in this work as representatives for a three-dimensional cell culture model exhibiting a lower degree of compaction compared to the fully compact multicellular spheroids of cell line COGA-5. Proteome analysis revealed several alterations in the protein expression pattern of the COGA-5 spheroids compared to the corresponding monolayer cultures. Five proteins were found to be up-regulated and one protein was down-regulated in spheroids compared to the monolayer culture. In COGA-12 partly compact aggregates, four proteins were found up-regulated compared to the respective monolayer. In the proteome comparisons between monolayers and the respective spheroids, only 2-fold or higher alterations in protein expression were taken into account. This stringent analysis provided a highly reproducible detection of regulated proteins. However, this restriction significantly reduced the number of detected spots. Furthermore, the current analysis was restricted mainly to cytosolic proteins, whereas detection of hydrophobic membrane proteins or basic proteins was limited.

The spots 12-A and 12-B, both up-regulated in COGA-12 aggregates, were identified as two isoelectric variants of acidic ribosomal protein P0. P0 is highly conserved in vertebrates and forms the lateral stalk of the 60S ribosomal subunit together with two hetero- or homo-dimers of acidic ribosomal proteins P1 and P2; P0 functions as ribosomal core protein for the anchorage of P1 and P2.[12] The function of P0 up-regulation under anoxia, however, remains to be clarified. Hence, P0 might be up-regulated in COGA-12 aggregates as a consequence of the lower oxygen supply in compact areas that are restricted from oxygen and serum supply. In addition, it was previously demonstrated that mRNA levels of P0 were increased in hepatocellular and colon carcinoma in accordance with increased biological aggressiveness, i.e., enhanced tumor outgrowth and cancer progression.[13,14] Overall, these data link altered P0 expression to cellular stress as well as cancer cell proliferation, which both are features in three-dimensional cultures.

The protein down-regulated in COGA-5 multicellular spheroids was identified as the acidic isoform of calponin, as also confirmed by immunoblot analysis. Calponins are actin-associated proteins and besides acidic calponin two other isoforms are known, namely basic and neutral calponins.[15,16] Basic calponin is the best studied isoform and is exclusively expressed in smooth muscle cells where it plays a role in the regulation of contraction.[17] Less is known about acidic calponin, which is expressed in a wide variety of tissues, including the colon.[18] Acidic calponin is not involved in contraction [19] but may play a role in cytoskeletal organization, since it binds to actin.[15,20] The down-regulation of calponin in multicellular spheroids therefore implies differences in cytoskeletal structures compared to monolayer cultures. In fact, changes in the tumor architecture of multicellular spheroids compared to the corresponding monolayers have been reported in a variety of tumor types including renal,[21] gastric,[6] and colorectal carcinomas.[2,22] Altered cell and nucleus shapes attributable to changes in cytoskeletal elements and nuclear structures are hallmarks of cancer cell morphology that may contribute to altered properties compared to normal cells.[23] It is likely that cells in multicellular spheroids represent such alterations of in vivo tumor cells better than monolayer cells. Another protein that was up-regulated in multicellular spheroids of COGA-5 cells

was identified as 15-hydroxyprostaglandin dehydrogenase (15-PGDH). 15-PGDH mediates prostaglandin degradation.[25] For example, prostaglandins promote growth factor signaling and antagonize apoptosis.[25] Therefore, 15-PGDH is considered to expose tumor suppressor activity, since it may antagonize prostaglandin-mediated tumor progression. In multicellular spheroids, 15-PGDH may contribute to proliferation inhibition, which is an established feature of multicellular spheroids where proliferation is restricted to local areas.[4] In addition, the up-regulation of 15-PGDH as a potential tumor suppressor fits well with the observed lamin cleavage as a result of enhanced apoptosis rate in the multicellular spheroids.

Proteome analysis revealed a 28 kDa fragment of the protein lamin A/C in COGA-5 spheroids, and low levels of this lamin A/C fragment were also detected in COGA-12 partly compact aggregates but not in the respective monolayer cultures. Lamins are type V intermediate filament proteins that form the nuclear lamina, a filamentous network that connects the inner nuclear envelope membrane with chromatin.[26] Lamins form stable structures in the nuclear lamina and the nucleoplasm, thereby determining nuclear shape and size, stability, and the position of nuclear pore complexes.[27−29] Furthermore, it has been suggested that lamins are involved in DNA replication and transcription.[26,30]In caspase-dependent apotosis, lamin A/C is cleaved by caspase-6, which is a requirement for the initiation of the nuclear events of apoptosis, e.g., condensation of chromosomal DNA and formation of apoptotic bodies.[31,32] Inhibition of caspase-6, and thus inhibition of lamin cleavage, enables chromatin to fully condense leading to the suggestion that lamin cleavage frees chromatin from the lamina.[32] However, additional experiments performed in other studies in neuronal cells showed that caspase-6-dependent lamin A cleavage is not a general phenomenon in apoptotic cell death. For example, in a model of glutamate-induced cell death in neuronal HT-22 cells, we could not detect cleaved lamin A in the apoptotic cells (Supplement Figure 3, Supporting Information). In this model system, the cells undergo caspase-independent apoptosis that is mediated by mitochondrial release of apoptosis inducing factor AIF.[33] Thus, the absence of lamin A cleavage serves as additional evidence for the lack of caspase-6 activation after glutamate-induced apoptosis.

The detected truncation of lamin A/C in the spheroids may be a result of apoptotic stress induced by the lack of oxygen and growth factors. Our results clearly show that truncation of lamin A/C was induced in monolayer cultures by withdrawal of trophic support. Serum deprivation alone was sufficient for lamin A/C fragmentation and enhanced by simultaneous hypoxia, whereas hypoxia alone was not sufficient to produce the protein fragment. Using an antibody that only recognizes caspase-cleaved lamin A and by pharmacological inhibition of caspases, we could further verify that the detected lamin A fragment was indeed a product of caspase cleavage.

Recent studies demonstrated that lamin A/C is a preferred substrate of caspase-6 in apoptotic cells.[8,34,35] Consistent with these previous reports, in our studies the pan caspase inhibitor zVADfmk reduced lamin A/C cleavage in a dose-dependent manner. Furthermore, addition of a caspase-6-specific inhibitor to spheroid cultures of COGA-5 cells confirmed that caspase-6 is responsible for the cleavage of lamin A/C in spheroid cultures of the COGA cells. Thus, the results indicate that apoptosis is a feature of the investigated spheroids of low-passage colon cancer cell lines. Similar findings were made with spheroids from other cancer cell lines.[9] Such apoptotic signaling involves the activation of caspases and cleavage of lamin A/C and may be triggered by reduced trophic support rather than hypoxia. In compact areas of multicellular spheroids, hypoxia may further amplify the apoptotic stress similar to the additive effect of hypoxia on lamin A/C cleavage in serum-deprived monolayer cultures. Interestingly, immunochemical analyses suggest a distribution of caspase-cleaved lamin A throughout the COGA-5 spheroid, not only in compact inner areas but also in cells in the outer layers. This finding correlates with results from the TUNEL assay indicating apoptotic cells in different areas in compact COGA-5 spheroids. This leads to the assumption that apoptosis may take place not only in inner spheroid areas due to reduced trophic support and hypoxia. Another possibility is that apoptotic cells are transferred to outer regions of the spheroid instead of resting encapsulated within the spheroid.

In summary, proteome analysis of the three-dimensional colon cancer cell cultures compared to the respective monolayer cultures revealed a panel of alterations that may affect a wide variety of cellular functions related to protein synthesis, proliferation, regulation of the cytoskeleton, and apoptosis, among others. Some of these alterations were previously demonstrated in tumors in vivo. Therefore, the proteome analysis of multicellular spheroids could identify relevant protein regulation and signaling pathways related to tumor cell proliferation, survival, and chemoresistance and thus may reveal novel targets for effective cancer therapy.

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Supporting Information Available: Supplemental Figures 1−5. This material is available free of charge via the Internet at http://pubs.acs.org.

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