Cell Biology International

Cell Biol. Int. (2010) 34, 267–274 (Printed in Great Britain)

Research Article

Cell death and lumen formation in spheroids of MCF-7 cells

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Abstract

3D (three-dimensional) cell culture permits a more integrated analysis of the relationship between cells, inserting them into a structure more closely resembling the cellular microenvironment *in vivo*. The development of *in vitro* parameters to approximate *in vivo* 3D cellular environments makes a less reductionist interpretation of cell biology possible. For breast cells, *in vitro* 3D culture has proven to be an important tool for the analysis of luminal morphogenesis. A greater understanding of this process is necessary because alterations in the lumen arrangement are associated with carcinogenesis. Following lumen formation in 3D cell culture using laser scanning confocal microscopy, we observed alterations in the arrangement of cytoskeletal components (F-actin and microtubules) and increasing levels of cell death associated with lumen formation. The formation of a polarized monolayer facing the lumen was characterized through 3D reconstructions and the use of TEM (transmission electron microscopy), and this process was found to occur through the gradual clearing of cells from the medullary region of the spheroids. This process was associated with different types of cell death, such as apoptosis, autophagy and entosis. The present study showed that changes in the extracellular matrix associated with long periods of time in 3D cell culture lead to the formation of a lumen in MCF-7 cell spheroids and that features of differentiation such as lumen and budding formation occur after long periods in 3D culture, even in the absence of exogenous extracellular compounds.

Keywords: cell death; cytoskeleton; lumen formation; mammary adenocarcinoma; three-dimensional cell culture

1. Introduction

When we consider cancer to be a product of the interaction between different cell types with their microenvironment (Bissell and Radisky, 2001), the development of *in vitro* experimental models that more faithfully reproduce the 3D (three-dimensional) nature of physiological cellular interactions becomes necessary to study the multiple factors associated with tumour initiation and progression. The ability to create both cell-to-cell and cell-to-matrix interactions (Pampaloni et al., 2007) makes 3D cell culture an attractive model to replicate the peculiarities of the tumour microenvironment *in vivo* (Feder-Mengus et al., 2008). Normal breast cells grown in a monolayer normally lose their polarity, while in 3D cell culture, the glandular architecture is re-established and maintained, thus maintaining cell polarity. This situation is important for the regulation of cell growth, survival and maintenance of their differentiated state. Contrary to what is found in cells grown in a monolayer, 3D cell cultures associated with extracellular matrix elements show a cellular organization similar to that found *in vivo*. With cells grown in 3D culture models, the multicellular tumour spheroids (spheroids) have a well-organized spherical symmetry, with different cell populations arranged concentrically (Mueller-Klieser, 2000; Friedrich et al. 2007). Topics studied in this model of breast carcinoma include analysis of the alterations of the activation of HER2 in the 3D environment (Pickl and Ries, 2009), the effect of marine compounds on the cytoskeleton (Freitas et al., 2008), the role of the stroma in carcinogenesis (Weigelt and Bissell, 2008), drug screening (Friedrich et al. 2009), angiogenic factors/polarity (Chen et al., 2009) and morphogenesis (Yamada and Cukierman, 2007; Mailleux et al., 2008). In the present study, we monitored luminal morphogenesis by taking advantage of the integrative vision provided by this model. Analyses of the baso-apical polarity, tensegrity, cell death and interaction of spheroids with the extracellular matrix (Hebner et al., 2008; Ingber, 2008) provide a less reductionist model of tumour complexity, contributing important information about the roles of these processes in the development of breast cancer.

The objective of the present study was to evaluate cellular differentiation during luminal morphogenesis of cells derived from breast adenocarcinoma (MCF-7) when grown in a 3D cell culture.

2. Materials and methods

2.1. Cell culture

The cancer cell line MCF-7 (Soule et al., 1973) was obtained from the American Type Culture Collection and cultured in DMEM (Dulbecco's modified Eagle's medium) Ham's F12 (Gibco-BRL) supplemented with 10% FBS (fetal bovine serum) (Gibco-BRL). Cells were maintained as a monolayer at 37°C with 5% CO_2.

2.2. Spheroid formation

After treatment with 0.05% trypsin (Sigma–Aldrich), 10^6 cells were seeded in non-adherent 90-mm-diameter Petri dishes (VWR) with

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Abbreviations: 3D, three-dimensional; BrdU, 5-bromo-2-deoxyuridine; PBSA, PBS without Ca^{2+} and Mg^{2+}; PI, propidium iodidie; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

10 ml of cell culture medium. The method used to induce spheroid formation was the anchorage-independent growth protocol described by Pickl and Ries (2009), with modifications. Neither scaffolds nor exogenous cell matrix components were added to the culture. The cell aggregates developed spontaneously, and spheroids were maintained in culture up to 155 days. The ages of the cultures in each kind of experiment are indicated in the Results section. The cell culture was maintained under the same conditions as the monolayer, and the culture medium was changed every 4 days.

2.3. Electron microscopy

The spheroids were fixed for 2 h with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for SEM (scanning electron microscopy) and TEM (transmission electron microscopy). Fixed samples were washed in 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed in 1% osmium tetroxide in the same buffer. The spheroids were dehydrated in a graded ethanol series and propylene oxide. The resin infiltration was performed with a 1:1 mixture of propylene oxide and epon for 5 h, followed by 100% epon for another 5 h. Next, the material was embedded, followed by 48 h of polymerization. Thin sections were produced using an ultramicrotome, and these were stained with Toluidine Blue. The spheroids were then thin-sectioned at 70–90 nm and stained with 4% uranyl acetate and 10% lead citrate solution. The material was analysed with a Jeol 1010 transmission electron microscope (80 kV). For SEM, the spheroids were washed in buffer and dehydrated with a graded ethanol series, followed by drying with hexamethyldisilazane. The material was coated by gold sputtering for 140 s and viewed with a Jeol 6100 scanning electron microscope (30 kV).

2.4. Immunofluorescence and 3D analyses

The cells were fixed with a 3.7% formaldehyde solution, permeated with 0.1% Triton X-100 for 20 min and treated with RNAase (10 mg/ml) for 30 min. The spheroids were then incubated overnight with monoclonal antibodies against α- and β-tubulin (1:100) or anti-laminin (1:100) inside a wet chamber. After three washes with PBSA (PBS without Ca^{2+} and Mg^{2+}), preparations were incubated for 1 h with anti-mouse CY5 (Molecular Probes) (1:100). The spheroids were stained with phalloidin-FITC (Sigma–Aldrich) (7.5 μM) for 40 min. The preparations were washed in PBSA at every step of each reaction. The nuclei were counter-stained using 5 μl of PI (propidium iodide) (10 μl/ml). The cytological preparations were mounted between the slide and coverslip with anti-fading solution (Vectashield, Vector) and observed under a laser scanning confocal microscope (Zeiss LSM 510). Fluorescent images were obtained using argon (at 458, 488 and 514 nm), Helium-Neon1 (at 543 nm) and Helium-Neon2 (at 633 nm) lasers. Optical sections were obtained for the appropriate sectioning ranges of the *z*-axis between 0.3 and 0.7 μm. Different modules from the LSM 510 3D software (Carl Zeiss) were used in the confocal analyses, including slice projections, orthogonal sections and animations.

2.5. BrdU (5-bromo-2-deoxyuridine) incorporation

Spheroids were grown in normal culture medium and, for S-phase identification, BrdU (5-bromo-2-deoxyuridine; 50 μM) was added to cultures for 3 h. After fixation with a solution of ethanol and acetic acid (3:1, v/v) for 30 min, the cells were permeated with 0.1% Triton X-100 for 20 min. The DNA of the spheroids was denatured by incubation in 2 M HCl for 30 min. The cells were then rinsed three times with PBSA and incubated with a primary mouse anti-BrdU antibody (1:100) for 2 h. The spheroids were washed with PBSA and incubated with a secondary antibody, anti-mouse CY5 (1:100), for 1 h.

2.6. Cell death detection

Apoptosis was assessed with the M30 CytoDEATH fluorescein-conjugated antibody (mouse monoclonal, clone M30, Roche Applied Science) diluted 1:250 and incubated for 2 h at room temperature (approx. 25°C) (Freitas et al., 2008). This antibody recognizes caspase-cleaved cytokeratin 18. The M30 antibody was used to monitor caspase-3 activation (Leers et al., 1999). Autophagy and entosis were identified according to the morphological criteria established by Levine and Yuan (2005) and Overholtzer et al. (2007). The criteria described by Kroemer et al. (2009) were used for the classification of the types of cell death.

3. Results

From the first days of 3D cell culture, the MCF-7 cells aggregated and formed spheroids (Figure 1A). Inverted phase-contrast microscopy showed that the spheroid growth and the development of a dark area in the central region, termed the cell death region (Figure 1B), occurred simultaneously.

On approximately the 20th day of culture, there were many mitotic cells showing a greater degree of cortical cell proliferation. This observation was also confirmed through the examination of BrdU incorporation (Figure 6), showing the presence of S-phase cells in these spheroids. Regionalized cortical buds that formed (Figure 1B) had a tendency to lengthen in the spheroids over the course of the culture and formed a luminal arrangement very similar to that seen in breast glands (Figure 1C, 75 days). Interestingly, we were able to maintain the 3D cultures for up to 155 days, by which time they had reached a diameter of approx. 500 μm. It was difficult to determine the final size of the spheroids as they continued to elongate throughout the culture period. Mitotic cells were also observed in these structures; however, it seems that the cell population achieved a balanced size.

The external surface of the spheroids was analysed using SEM (Figures 1D and 1D1), and the MCF-7 cells appeared highly juxtaposed with cell plasma membrane projections on the external spheroid surface. This pattern was maintained throughout the 3D cell culture period, and components of the cell matrix were not observed on the surface of the examined spheroids (Figure 1D1).

Hypothesizing that alterations in the morphology of the spheroids were accompanied by cytoskeletal rearrangement, we

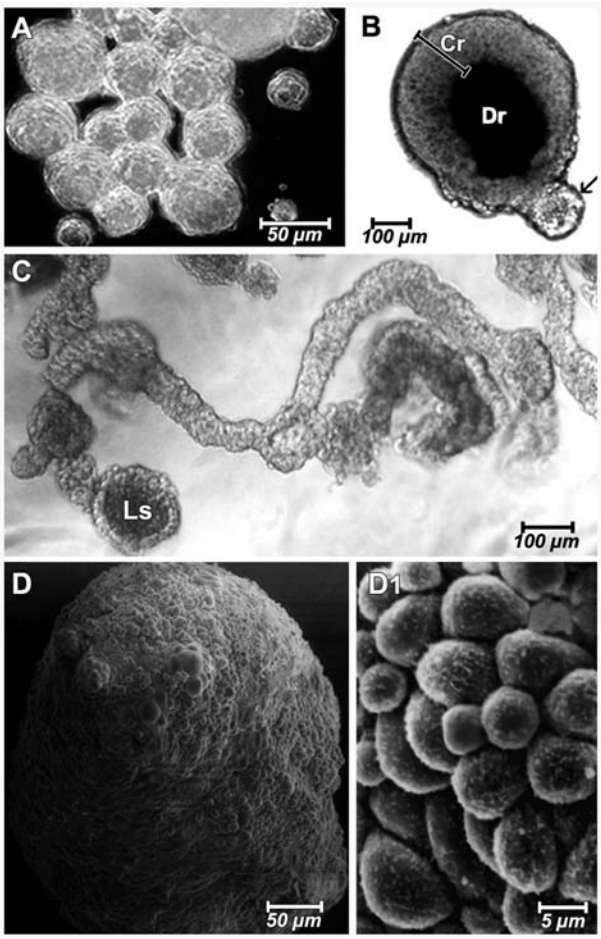

Figure 1 External and internal morphology alterations of spheroids in MCF-7 cells

The spherical morphology (**A**, 3 days) changed with cell budding (arrow) and increased size of the cell death region (Dr) (**B**, 20 days). At longer culture times, spheroids showed an elongated arrangement with lumen (Ls) (**C**, 75 days). Externally, SEM showed cell membrane projections of MCF-7 cells with little extracellular matrix (**D** and **D1**, 30 days). Cr, cortical region. Figures (**A**), (**B**) and (**C**) are images from inverted phase-contrast microscopy.

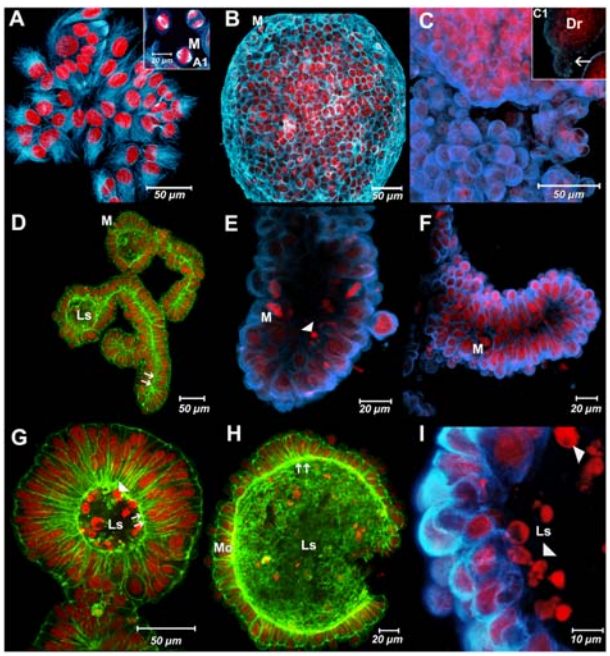

Figure 2 Cytoskeleton and lumen formation in MCF-7 spheroids

Laser scanning confocal microscope images are shown: monolayer of MCF-7 cells (**A**) until the formation of the cysts with luminal space (**H**). Microfilaments of actin (green) (**D**, **F**, **G** and **H**) are concentrated (double arrow) on the inner surface of the lumen (**D**, **G** and **H**). The microtubules (α- and β-tubulin anti-CY5 in blue) are uniformly arranged at the monolayer stage (**A**), 10 days of culture (**B**), 35 days of culture (**C** and **C1**) 75 days of culture (**E** and **I**) and 155 days of culture (**F**). Inside the luminal space (LS) there was nuclear fragmentation (head of arrow in **G**, **E** and **I**). M, mitosis; Mo, adluminal monolayer; Dr, cell death region. (**C1**) Arrow shows detail of (**C**).

looked for possible modifications of the actin filament and microtubule structures going from the monolayer (Figure 2A) to the formation of spheroids with a large luminal space (Figures 2G–2I). Immunofluorescence staining with anti-α-tubulin and anti-β-tubulin antibodies showed a typical arrangement of these components, mainly in the process of cell division (Figure 2A1). During the key stages of lumen formation, such as the formation of spheroids (Figure 2B), cortical budding (Figures 2C and 2C1), elongation of the spheroids (Figures 2E and 2F) and formation of the adluminal monolayer (Figure 2I), the general microtubule arrangement did not change. The actin microfilaments became more concentrated in the apical region of MCF-7 cells in contact with the lumen. This characteristic occurred from the beginning of the formation of the luminal space (spheroid elongation) and was more prominent after the formation of the adluminal monolayer (Figures 2D, 2G and 2H). Observations using a laser scanning confocal microscope also made it possible to observe changes in nuclear morphology, mainly in the central region of the spheroids. An increasing number of picnotic nuclei in the cell death region in the spheroids after 35 days in culture (Figure 2C) was slowly substituted with a luminal space containing nuclear fragments in its interior, indicating apoptosis (Figures 2D–2E and 2G–2I). This observation was also confirmed with reactivity to the M30 antibody (detecting cleaved cytokeratin 18) (Figure 6).

The process of lumen formation was analysed using TEM, focusing on cell differentiation in the cortical region and the progressive process of cell death in the medullary region.

In the initial stages of the 3D cell culture, the presence of mitosis and euchromatic nuclei indicated an increase in cellular activity (Figure 3A). Despite the strong aggregation of cells, the formation of cell junction components was rarely observed (Figures 3A and 3A1 at 10 days). The increase in diameter of the medullary region was associated with a greater number of autolysosomes, different nuclear condensation stages and apoptotic cells (Figures 3B and 3B1 at 30 days). On approximately the 60th day of cell culture, the adluminal monolayer was formed by columnar cells, superimposed and polarized. In this differentiation phase of the spheroids, the cellular junctions were already well-established (Figure 3C), and these junctions (especially the desmosomes) were associated with bundles of tonofilaments. The luminal space formed was delimited by single layer of cells, and the apical region of these cells was facing this lumen (Figures 3C1 and 3C3). When observed at high magnification (Figures 3C2a and 3C4), the apical cell region was found to have

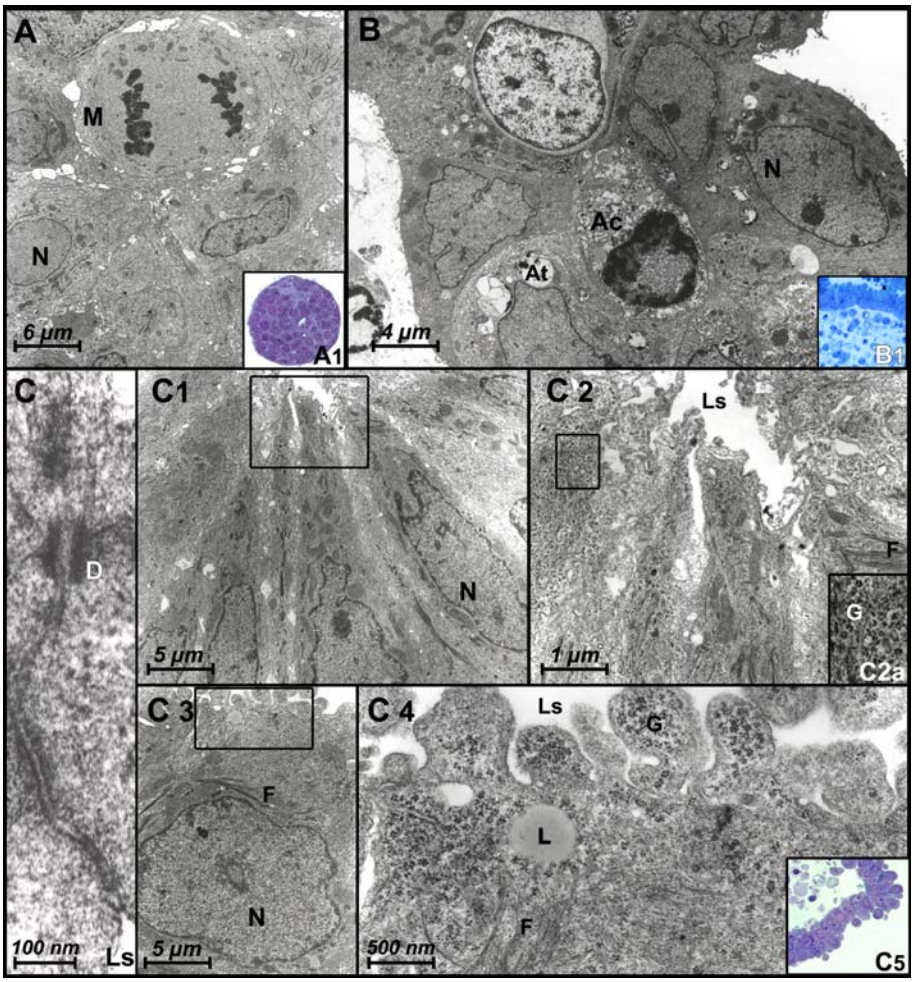

Figure 3 **TEM analysis of cell morphology and differentiation in MCF-7 cells**

Extended 3D culture resulted in a decrease in the number of MCF-7 cells in the luminal space and polarization of cells located in the cortical region of spheroid (compare 5 days, 30 days and 75 days in **A**, **B** and **C1**). Cells adopted a columnar-like form and developed some polarized desmosomes (**D** and **C**). Electron-dense material in the apical region (**G**) near the luminal space (Ls) (detail in **C2**, **C2a** and **C4**). Panels **A1**, **B1** and **C5** show thin slices stained with Toluidine Blue, which allows an overview of the material analysed. N, nucleus; L, lipid; Ac, apoptotic cells; At, autolysosomes; F, intermediate filaments; M, mitosis.

vesicles with granular, electron-dense material, as well as associated lipids and intermediary filaments (Figure 3C4). Different cell death processes occurred mainly in the medullary region (Figures 4A–4E). As many cell death processes other than necrosis were observed, we suggest that the 'cell death region' is a more appropriate term than the 'necrotic nucleus'. In the spheroids, the gradual cellular clearance in this region was observed to occur as the result of different processes such as apoptosis (Figure 4B), death by autophagy (Figure 4D) and entosis (Figure 4E). In spheroids that had been cultured for longer times (more than 60 days), the lumen created as a result of this cellular clearance maintained some cells in the interior during the cell death processes (Figure 4D1). The apoptotic cells presented a greater electron density compared with adjacent cells (Figures 4A and 4B), showing gradual nuclear compaction and posterior cell fragmentation through plasma membrane blebs. At the region of cell death, many cells presented a very active vesicular–lysosomal complex (Figure 4A), and it was also possible to observe many autophagic vesicles and autolysosomes (Figure 4C). Cells also died through autophagy, presenting nuclear compaction, a large number of autolysosomes and later rupture of the plasma membrane of cell (Figure 4D). Some cells in the process of autophagy were interiorized through entosis (Figures 4E and 4E1). The process of entosis was observed both in the cell death region (Figures 4E and 4E1) and the cortical region (Figures 4F and 4F1). During the entosis process, the phagocytic cells contained euchromatic nuclei and a well-developed and wrinkled endoplasmic reticulum (see cell C1 in Figure 4F). Cells in the process of necrosis were also processed through entosis (Figure 4F).

4. Discussion

The intrinsic complexity of the luminal morphogenesis in breast tissue derives from an intimate interaction between the epithelial

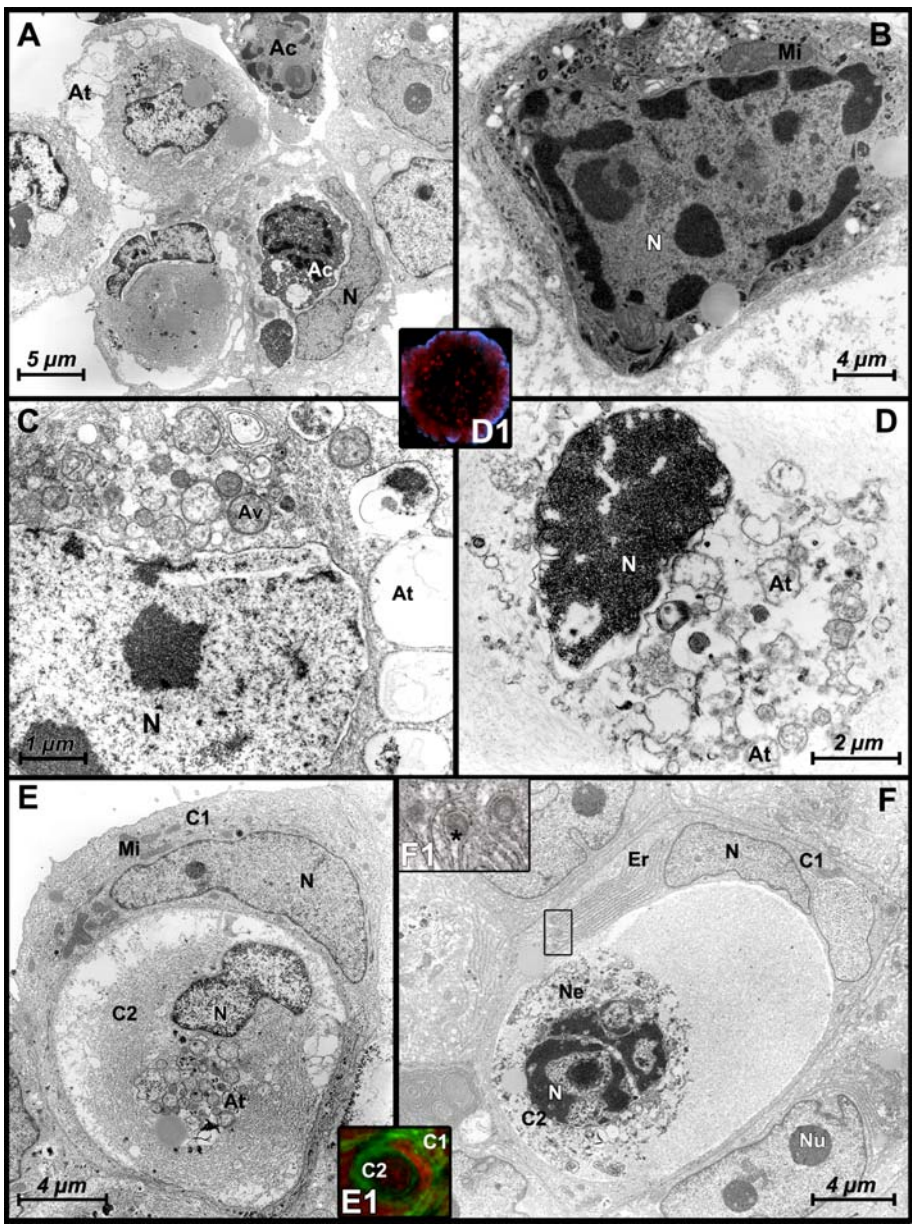

Figure 4 **Cell death in MCF-7 cell spheroids**

Overview of the cell death region, showing some cells in the process of apoptosis (Ac in **A**). Apoptotic cell (**B**). Cell with autophagic vacuoles (Av) and autolysosomes (At) (**C**). Autophagic cell death (**D**). Cells in the process of entosis in cell death region—cell (C2) with a large number of autolysosomes (At) is internalized by another cell (C1) (**E**). Entosis in the cortical region showing a cell in the process of necrosis (Ne-C2) being internalized (C1) (**F**). Detail of (**F**) showing vesicles (*) next to the rough endoplasmic reticulum (**F1**). (**D1**) Microtubules/blue and nuclei/red and (**E1**) F-actin shown in green and nuclei in red show similar regions visualized by laser scanning confocal microscopy. Er, rough endoplasmic reticulum; N, nucleus; Nu, nucleolus; Mi, mitochondria. (**A**, **B** and **E**) Spheroids after 25 days of culture. (**C**, **D** and **F**) Spheroids after 155 days of culture.

cells and their microenvironment. The reproduction and analysis of this process *in vitro* are a challenge because different regulatory pathways participate in lumen formation. The re-establishment of non-tumoral characteristics in the MCF-7 lineage in 3D cell culture suggested the possibility of reversal of some pathways, which resulted in morphological alterations. The absence of a lumen is a characteristic of spheroids originating from breast adenocarcinomas. This peculiarity was used by Kenny et al. (2007), who described the morphology of spheroids originating from different breast cell lineages and divided them into four groups: round, mass, grape-like and stellate. The spheroids of the MCF-7 lineage were classified as a mass of cells, with disorganized nuclei, strong cell-to-cell bonds and an absence of lumen formation. Similar characteristics to the ones described by those authors were observed in the initial phases of the present study. However, the gradual cell clearance in the cell death region in these spheroids culminated in a polarized cortical monolayer (see Supplementary movie at http://www.cellbiolint.org/cbi/034/cbi0340267add.htm),

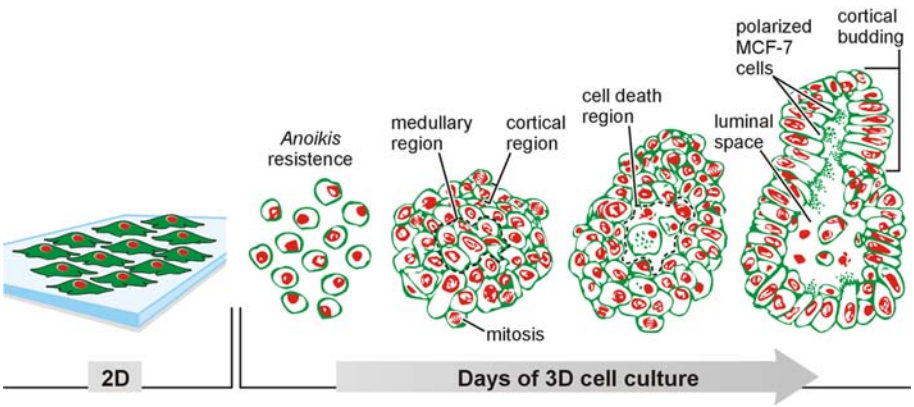

Figure 5 Illustration of lumen formation in MCF-7 cell spheroids

which would put the lineage in the round classification. Kirshner et al. (2003) were able to establish cellular polarization in spheroids of MCF-7 cells, modulating cell-to-cell adhesion molecules and showing the role of CEACAM1-4S (carcinoembryonic antigen-related cell adhesion molecule 1) and the role of apoptosis in lumen formation.

The necrotic core in the central region of the multicellular tumour spheroids has been compared with avascular metastases. The necrotic process that has long been associated with hypoxia (Acker et al., 1984; Bjerkvig, 1992) did not present itself as a critical factor in the formation of the lumen, as shown by the remarkable presence of apoptosis and cell death by autophagy. Through ultrastructural analysis, cells in the necrotic stage were identified in all the spheroids, including within the lumen. The single layer of cells surrounding this central core reduces the chances that these necrotic cells are experiencing hypoxia. Degenhardt et al. (2006) discuss the necrosis signalling pathways, suggesting that this process could possibly be a result of the combined impairment of apoptosis and autophagy.

The dynamic balance between cell proliferation and cell death is responsible for the formation of the lumen in MCF-7 spheroids. This begins with the selection of cells resistant to the anoikis process (Simpson et al., 2008) in which cells are grouped form spheroids (Figure 5). The growth of spheroids is the result of this aggregation and cell proliferation (Figure 6).

Cell death by apoptosis was present in all regions of the spheroid, but was more frequent in the cell death region (central region). Debnath et al. (2002) described the role of apoptosis in maintaining the glandular architecture in spheroids of the breast lineage MCF-10A, showing that lumen formation was associated with selective apoptosis in the central region. When apoptosis was blocked, the lumen formation continued, suggesting that another regulatory pathway participated in lumen formation. Mills et al. (2004), using a similar model, suggested that the cavities in MCF-10A spheroids would be a consequence of cellular elimination by autophagy and apoptosis, two distinct pathways that complemented each another. This complementary effect was also observed in the present study; however, there was participation of another non-apoptotic cell-death pathway in the clearing of cells for lumen formation: entosis. Entosis involves the invasion of

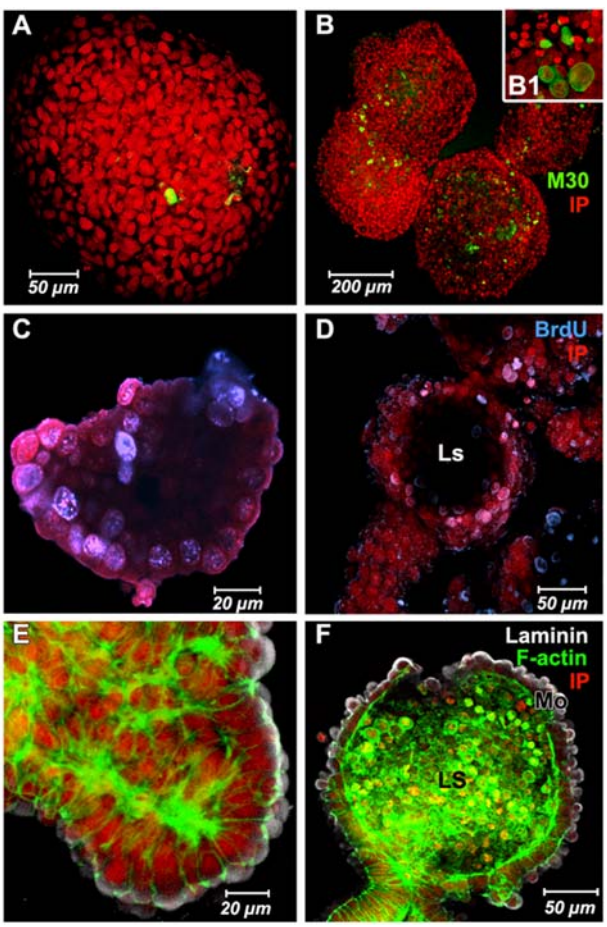

Figure 6 Immunolabelling for laminin, proliferation and cell death in the MCF-7 spheroids

Images were obtained by laser scanning confocal microscopy. (**A** and **B**) Lower level of M30 (anti-M30/FITC, green) in spheroids after 10 days of culture (**A**) compared with 115 days of culture (**B**). The incorporation of BrdU (anti-BrdU/CY5, blue) showed greater cell proliferation in spheroids after 7 days of culture (**C**) than after 60 days of culture (**D**). In spheroids with a developed luminal space (LS) (75 days) the external monolayer (Mo) expressed laminin (anti-laminin/CY5, white). The nuclear morphology was analysed using PI. (**E** and **F**) Labelled F-actin microfilaments stained with phalloidin–FITC (green). (**B1**) shows detail of the central region of (**B**).

one live cell by another cell, followed by the degradation of the internalized cell by lysosomal enzymes (Overholtzer et al., 2007). The occurrence of entosis, as has been described in tumours and cell lineages (including MCF-7 in suspension), is associated with cell populations deprived of attachment to the extracellular matrix.

When grown in a 3D environment, the MCF-7 cells present apoptotic and autophagic cells during lumen formation, but cells in the process of entosis were also found in the cortical region of the spheroid. This fact suggests that cell-to-cell contacts can regulate this process, permitting the occurrence of entosis in different regions of the spheroid, not only within the lumen. These results show that the lumen formation occurs in a regulated manner in the cell death region, not as a result of necrosis. Resistance to anoikis, apoptosis, entosis and autophagic cell death that occur in the medullary region resulted in the establishment of a cell monolayer. This region presented a greater concentration of microfilaments in the apical region (a characteristic of microvilli, according to Hogan and Kolodziej, 2002) and delivered secretion products to the region facing the lumen (Figure 5).

The regulation of the tension mediated by the cytoskeleton components dynamically interacts with factors external to the cell. Therefore, an environment that allows for cell growth in different planes *per se* can already be considered a stimulus that permits consequent morphological alterations. When analysing the formation of MCF-7 cell spheroids with a lumen (cystic arrangement), the presence of regionalized cortical buds was observed. The development of this characteristic associated with the formation of lumen in these buds shows that this method of culture, with increased culture time and the absence of exogenous extracellular compounds, can be used for the study of tubulogenesis. The formation of these structures was similar to the micromorphogenesis described by others (Huang and Ingber, 1999; Ingber, 2008), who related this to mechanical stress due to alterations of the extracellular matrix. When the endogenous and exogenous tension force increases are mediated by a prolonged hardness of the matrix, this can activate related mechanoregulatory pathways [ERK (extracellular-signal-regulated kinase) and Rho] and stabilize focal contacts (Paszek et al., 2005). Wozniak et al. (2003) suggest that, for breast cells in environments where the matrix is more flexible, ROCK (Rho kinase) down-regulates the activity of Rho, which leads to a sequence of events that result in the differentiation in tubules.

It has been shown that epithelial breast cell morphogenesis seems to be driven by laminin when 3D cultures are grown in reconstituted basement membranes derived from Engelbreth–Holm–Swarm tumour supplemented culture medium (Gudjonsson et al., 2002). This study shows that features of differentiation, such as lumen and bud formation, occur after long periods in 3D culture in the absence of exogenous extracellular compounds. Thus, if laminin is important in the induction of this differentiation process, this culture system would depend on its own synthesis of this protein.

Author contribution

Jônatas do Amaral established the spheroid culture conditions, carried out the electron microscopy and immunofluorescence experiment and drafted the manuscript. Marcel Urabayashi partipated in morphological analysis and conducted some immunofluorescence reactions. Gláucia Machado-Santelli designed the study, co-ordinated the experiments, participated in confocal imaging and analysis and wrote the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Roberto Cabado Modia Jr (for aid with the artwork and 3D reconstructions) and Edson Rocha de Oliveira and Gaspar Ferreira de Lima (for electron microscopy support).

Funding

This work was supported by FAPESP (Fundacão de Amparoà Pesquisa do Estado de São Paulo) [process number 06/01026-0]; CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico); Capes (Coordenacão de Aperfeiçoamento de Pessoal de Nivel Superior); and by the Secretaria da Educação do Estado de São Paulo.

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Received 1 July 2009/26 August 2009; accepted 26 October 2009

Published as Immediate Publication 26 October 2009, doi 10.1042/CBI20090024