Inhibition of mammalian target of rapamycin induces phenotypic reversion in three-dimensional cultures of malignant breast epithelial cells

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Received 27 February 2010; revised 11 March 2010; accepted 17 March 2010.

ABSTRACT

Inhibition of mammalian target of rapamycin (m-TOR) is a potential method for cancer treatment. Effects of rapamycin (RAP) on the reversion of malignant breast epithelial cells were investigated on three-dimensional (3D) basement membrane extract (BME) cultures. Through continuous exposure to 20 nM of RAP, cell colony size was significantly reduced in 3D BME cultures of malignant breast epithelial cells, while normal cell colony size appeared unaffected. In unfixed 3D BME cultures of normal and RAP-treated malignant breast epithelial cells, the presence of luminal cell death was confirmed by ethidium bromide and propidium iodide labeling. Increased structural organization was observed by immunofluorescence staining of F-actin and β-catenin in RAP-treated malignant breast epithelial cells. In monolayer cultures of normal and malignant breast epithelial cells, continuous exposure to 20 nM of RAP increased caspase 3/7 activity and decreased proliferation. Reverse transcriptase polymerase chain reaction (RT-PCR) array analysis indicated a fold increase in the expression of a number of proteins related to polarity, cell-cell adhesion, and cell-matrix adhesion in the presence of RAP. Our data showed that phenotypic reversion of malignancy can be achieved through RAP exposure on 3D BME cultures. This 3D BME culture system will provide correct microenvironments for observing the effects of other mTOR inhibitors on phenotypic reversion of malignant breast epithelial cells.

Keywords: Rapamycin; Three-Dimensional Culture; Breast Cancer Reversion; Basement Membrane Extract; mTOR Inhibitors

1. INTRODUCTION

The use of traditional two-dimensional (2D) cultures for breast cancer studies provides abundant fundamental information on the regulation of breast cancer, but its results often fail to properly translate into clinical applications [1]. The need to effectively address this problem has led to a demand for culture systems which more accurately represent the tissue environment found in vivo. In recent years, three-dimensional (3D) culture systems have been developed which use extra-cellular matrix (ECM) proteins [2,3]. 3D cultures provide epithelial cells with spatial freedom to allow the formation of 3D structures. Furthermore, the presence of reconstituted basement membrane extract (BME) promotes polarization of breast epithelial cells into acinar structures with luminal cell death through communication with membrane-bound integrins [3-7]. BME products are derived from the Engelbreth-Holm Swarm (EHS) tumor and are rich in laminin-1, with other ECM components including procollagen IV, heparin sulfate proteoglycan, and entactin [8]. However, most breast cancer cell lines fail to exhibit a normal phenotype in BME cultures unless accompanied with one or more signal molecules [9]. Several signal molecules have previously been shown to affect phenotypic reversion through limited cell proliferation, increased multi-cellular organization, basolateral polarization, and programmed luminal cell death. These reversion factors include inhibitors of β1-integrin, epidermal growth factor receptor (EGFR), and the MAPK & PI3K kinases [10-12]. Such reversion studies have provided valuable insight for potential methods of cancer treatment.

Rapamycin (RAP), the primary inhibitor for mammalian target of rapamycin (mTOR), has been used for its immunosuppressive properties under the drug name Sirolimus [13]. However, the mTOR signaling pathway has been shown to be a component of several signaling pathways related to breast cancer, including Akt [14], PTEN [15], and PI3K [16]. The web of signaling pathways related to mTOR is complex, and its malignant effects include increased cell growth, proliferation, and
resistance to apoptosis [17]. Presently, RAP and its analogues are under investigation as potential cancer treatments [15]. In breast epithelial cells transformed to exhibit hyperactivity of the Akt kinase, RAP exposure inhibits morphological disruptions caused by Akt overactivity [18]. However, the effects of mTOR inhibition on breast cancer cell lines cultured in 3D BME are not well characterized.

To provide further insight into the value of mTOR inhibition as a potential cancer treatment, we observed and analyzed the phenotypic reversion of malignant breast epithelial cells in 3D BME following exposure to RAP. Two malignant breast epithelial cells (BT-483 and MDA-MB-468) were exposed to RAP and compared to normal breast epithelial cells (184-B5). Changes in multi-cellular morphology, cell proliferation, polarity, and gene expression were investigated in the presence of RAP on 3D BME cultures.

2. MATERIALS AND METHODS

2.1. Cell Culture

Malignant human breast epithelial cells (BT-483 and MDA-MB-468) and normal human breast epithelial cells (184-B5) were purchased from ATCC (VA). For monolayer cultures: BT-483 was cultured using RPMI-1640 (Sigma-Aldrich, MO) with 20% of fetal bovine serum (FBS) (Thermo Fisher Scientific, UT) and 0.01 mg/mL of bovine insulin, MDA-MB-468 was cultured using Leibovitz’s L-15 (Sigma-Aldrich) with 10% of fetal bovine serum (FBS) (Thermo Fisher Scientific, UT) and 0.01 mg/mL of bovine insulin, MDA-MB-468 was cultured using Leibovitz’s L-15 (Sigma-Aldrich) with 10% of FBS in air with no added CO₂, and 184-B5 was cultured using MEGM (Lonza, Switzerland) with 1 ng/mL of cholera toxin. To achieve luminal morphology, cells were cultured using 3D on-top method as previously described [2,3]. 3D culture matrix BME (phenol red free), with reduced growth factors, was obtained from Trevigen (MD). The 3D culture media were modified to contain 2-10% of BME, with 2% of FBS for malignant cells with or without EGF (epidermal growth factor) supplements. All media were supplemented with 1% of Penicillin/Streptomycin (Invitrogen, CA) and 0.1% of Fungizone (HyClone, UT). Cells were seeded at 0.25 × 10⁵ (184-B5), 0.22 × 10⁵ (BT-483), and 0.18 × 10⁵ (MDA-MB-468) cells/cm². Media were changed every two days subsequent to seeding. RAP (Sigma-Aldrich, MO) was reconstituted in DMSO and stored at -20°C at a stock concentration of 1 μM.

2.2. Morphological Observation

Morphology was observed using a TE2000-S Eclipse Microscope (Nikon, Japan) or a TS100 Eclipse Microscope (Nikon, Japan). All images were taken with a Digital Sight DSQi1Me Camera (Nikon, Japan). Fluorescence images were detected with an Intensilight C-HGFI lamp (Nikon, Japan). For the detection of non-viable (apoptotic) cells, non-fixed cultures were incubated for 15-30 minutes with 1 μM of ethidium bromide or 500 nM of propidium iodide and observed for fluorescence. Cell colonies were extracted using PBS-EDTA solution, fixed using 4% of paraformaldehyde, and blocked with 10% of goat serum and 1% of goat F(ab’) IgG (Sigma-Aldrich, MO). Monoclonal anti-actin (1A4) and monoclonal anti-β-catenin (15B8) were obtained from Sigma-Aldrich (St. Louis, MO) for use as primary antibodies. Secondary antibody for FITC-conjugated goat anti-mouse was obtained from Santa Cruz Biotechnology (CA). Image analysis was conducted using NIS-Elements v3.0 (Nikon). Areas of cell colonies (in the x-y plane) in all images were analyzed for the calculation of mean areas under different conditions.

2.3. Caspase 3/7 Assay

Monolayers of BT-483 and 184-B5 cells were prepared in a 96-well plate to analyze the effects of RAP on caspase activity. Caspase activity was measured at 24 and 48 hours following exposure to RAP at 0, 5, and 20 nM. One hundred μL of Caspase-Glo 3/7 Reagent (Promega, WI) was added to each sample, including two blank wells containing 100 μL of growth media, and incubated for 2 hours. Luminescence was then measured using a Synergy 4 Plate Reader (Biotek, VT).

2.4. Cell Viability Assay

CellTiter-Blue Cell Viability Assay reagent was obtained from Promega (WI). MDA-MB-468 and 184-B5 cells were seeded in 96-well plates, beginning on day 2, and were treated continuously with 0 nM and 20 nM of RAP. On day three and day seven, cell viability assays were conducted and measured for fluorescence using a Synergy 4 Plate Reader (Biotek, VT).

2.5. Real-Time RT-PCR Arrays

Real time RT-PCR (reverse transcriptase polymerase chain reaction) Arrays for human extracellular matrix and adhesion molecules were obtained from SABiosciences (MD). MDA-MB-468 cells cultured in 3D BME were treated with 0 nM and 20 nM of RAP for 6 days. Cell colonies were extracted using PBS-EDTA, mRNA was extracted using RNeasy RNA isolation kit (Qiagen, CA), and cDNA synthesis was conducted using the Reverse Transcriptase First Strand Kit (SABiosciences, MD). Real time RT-PCR arrays were run using a Bio-Rad Opticon 2 thermocycler system (MJ Research, Canada) and analyzed using the Opticon 2 software provided. C₅ values were acquired graphically.

2.6. Statistical Analysis

With the SYSTAT 12 software, statistical analyses on all quantitative data were carried out using ANOVA (ana-
lyses of variance) to verify statistical significance ($p < 0.05$).

3. RESULTS

3.1. Effects of EGF on Phenotype of Normal Breast Epithelial Cells

Correct signaling from growth factors (EGF) and ECM proteins (BME) contribute to the development and maintenance of a hollow luminal structure within normal human breast epithelial acini, which is schematically described in Figure 1. Cells seeded on BME proliferated to form acini. Several events contributing to the formation of a luminal structure preceded the suppression of proliferation within the normal breast epithelial acini. Two populations of cells within each acinus became evident from day 6 to 8: 1) An outer layer of cells that was in direct contact with the matrix and 2) an inner subset of cells that underwent luminal death (apoptosis) (Figure 1(c)) and that lacked contact with the matrix. Cells in contact with BME were apparently protected from a global death signal. Normal (184-B5) breast epithelial cells were initially cultured in a MEGM-based medium containing epidermal growth factor (EGF) additive. Resulting cultures contained large, morphologically disrupted structures with no apparent limitations to growth. Culturing the cells in EGF-reduced media resulted in the formation of significantly smaller structures with uniformity in shape (Figure 2(a)). The mean area for cell colonies in EGF-reduced media at day 7 was significantly lower than those in EGF-enhanced media at day 7 (Figure 2(b)).

Figure 1. Schematic of biological events during normal human breast epithelial cells’ acinar morphogenesis. Normal human breast epithelial cells were cultured using the 3D on-top method. Cell colonies were immunostained against. (a) actin (green); (b) β-catenin (green); (c) ethidium bromide (red). Nuclei were counterstained with DAPI (blue) in (b). Experimental procedures were adapted from [2,3].

Figure 2. (a) Effects of EGF-reduction on morphology of normal human breast epithelial cells (184-B5) in 3D BME cultures with and without EGF. Top: EGF-reduced media induced a normal phenotype, including smaller, round morphology with maximum size at day 5 of culture. Bottom: EGF-enhanced media led to increased proliferation and disrupted morphology. (Scale bars = 25 µm). (b) Effects of EGF on average cell colony size: Average cell colony areas were measured from normal human breast epithelial cells (184-B5) in 3D BME cultures with and without EGF on day 7 of culture. Colonies in EGF-reduced media showed a significantly lower average area than the colonies in EGF-enhanced media. (Error bars ± st. dev, * significantly lower than control, $p < 0.05$).

3.2. Effects of RAP Exposure on Malignant Cell Morphology

Morphologies of normal and malignant breast epithelial cells (BT-483 and MDA-MB-468) were compared in 3D BME cultures. Malignant breast epithelial cells did not form organized structures, had no uniformity in shape, and continued to proliferate. Malignant cell colonies exposed to 20 nM of RAP, starting on day 2, formed significantly smaller colonies with a maximum size reached at 6-8 days (Figure 3(a)). The mean area for RAP-treated malignant cell colonies was significantly reduced than the untreated malignant cell colonies on day 7 (Figure 3(b)).

An inner subset of cells undergoing luminal cell death (apoptosis) was observed with ethidium bromide and propidium iodide staining. Apoptotic colonies were commonly found at the center of colonies in normal
(184-B5) breast epithelial cell cultures on day 8, which indicated luminal formation. This was also observed in RAP-treated malignant (BT-483 & MDA-MB-468) cell colonies, providing evidence that the treated malignant breast epithelial cells were reverting to normal phenotypes (Figure 4(a)).

Structural organization was also observed using anti-actin and anti-β-catenin immunofluorescence staining (Figure 4(b)). RAP-treated malignant colonies exhibited cells arranged around the center, showing similarities to the structure of normal breast epithelial cells in 3D BME cultures. Exposure to up to 20 nM of RAP had no apparent effect on the morphology of either EGF-reduced or EGF-enhanced cultures of normal (184-B5) cells (Data not shown).

Figure 4. (a) Selective staining of non-viable cells. Top: Staining of day 8 3D cultures of normal (184-B5) human breast epithelial cells with ethidium bromide or propidium iodide resulted in detection of cell death at the center of cell colonies. Bottom: Similar occurrences of centrally-localized apoptosis were detected in both malignant cell lines when treated continuously with 20 nM of RAP; (b) Immunofluorescence staining of fixed cell colonies from 3D culture. Malignant (BT-483) cell colonies from day 7 of culture, after continuous treatment of 20 nM RAP from day 2, exhibited multi-cellular organization similar to day 7 cultures of normal (184-B5) human breast epithelial cells. Top: Anti-Actin (green) counterstained with DAPI (blue). Bottom: Anti-β-Catenin (green) counterstained with DAPI (blue). (Scale bars = 25 µm)

3.3. Effects of RAP Exposure on Caspase Activity and Proliferation

Confluent monolayers of normal (184-B5) and malignant (BT-483) breast epithelial cells were exposed to RAP at 0, 5, and 20 nM. Caspase activity was measured after 24 and 48 hours of exposure (Figure 5). For normal breast epithelial cells, the only condition which showed significant deviation from the control was 20 nM of RAP after 48 hours of exposure (Figure 5(a)). For malignant breast epithelial cells, 20 nM of RAP exposure showed a significantly higher effect on caspase activity than 5 nM, but no significant difference was observed with respect to exposure time (Figure 5(b)). An increase in caspase activity indicates the presence of programmed cell death, which is essential for the formation of the luminal space in 3D BME cultures.
3.4. Effects of RAP Exposure on Gene Expression of Extracellular Matrix and Adhesion Molecules

RT-PCR arrays were prepared from malignant breast epithelial cells (MDA-MB-468) in 3D BME cultures containing 0 and 20 nM of RAP on day 6 of culture. Quality controls included with the arrays verified successful RT-PCR and the absence of genomic DNA contamination. C\textsubscript{i} values were cut off at > 35. Genes were grouped into six major classifications, and reported with expression fold change (DDCT method) of untreated controls compared to RAP treated cells (Table 1). In RAP-treated samples, 25 genes related to ECM and adhesion molecules showed a significant (at least 5-fold) increase in expression, while no genes showed a significant decrease in expression (Table 1).

4. DISCUSSION

In this study, the effects of RAP exposure on the phenotypic reversion of malignant breast epithelial cells were investigated in 3D BME culture. Microscopic observation of malignant breast epithelial cells treated with RAP showed greater similarities in morphology to normal cells than in untreated malignant breast epithelial cells in 3D BME cultures. Comparison of the average horizontal area confirmed that RAP-treated malignant cell colonies are significantly smaller in size. Increased structural organization was observed by immunofluorescence staining of anti-Actin and anti-β-Catenin. On day 8 of 3D BME culture, RAP-treated malignant breast epithelial...
Table 1. RAP’s effects on gene expression of human extracellular matrix and adhesion molecules. Fold change was calculated using the DDCT method, with GADPH as the normalizing gene.

<table>
<thead>
<tr>
<th>Gene Category</th>
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<th>Description</th>
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cells stained with ethidium bromide and propidium iodide showed similar structural organization with luminal cell death to that observed in normal breast epithelial cells, indicating re-polarization of the epithelial cell layers. Supporting data from monolayer cultures indicated that levels of caspase activity were increased in the presence of RAP, and that proliferation over time was decreased. RT-PCR array analysis of RAP-treated malignant breast epithelial cells in 3D BME culture indicated an increase in the expression of several ECM and adhesion molecules important to polarity and structural organization. This collection of data supports our hypothesis that RAP exposure can revert malignant breast epithelial cells to a normal phenotype.

In order to understand why breast cancer develops, as well as to predict the outcome of pharmacological treatments, we need to model the structure and function of organs or tissues in culture so that our experimental manipulations occur under physiological contexts. 3D cultures provide a model to analyze the spatial and temporal aspects of key biological processes (e.g., proliferation and apoptosis) and signal transduction events during morphogenesis. Many of these studies are impractical or impossible to perform in native breast tissue. Cultured normal human breast epithelial cell monolayers in the absence of BME fail to assemble organized structures, and arrest growth when they reach confluence (Figure 7(a)). We recently found that signaling from epidermal growth factor (EGF) and ECM proteins contributes to the development and maintenance of a hollow luminal

Figure 7. Comparison of normal and malignant human breast epithelial cells grown in 2D monolayer and 3D culture matrix. Normal human breast epithelial cells grown in the 3D BME culture form a luminal structure with normal phenotype (images not to scale). (a) Normal human breast epithelial cells in monolayer without BME (2D); (b) Normal human breast epithelial cells in 3D BME with normal EGF; (c) Normal human breast epithelial cells in 3D BME with reduced EGF; (d) Malignant human breast epithelial cells in 3D BME with reduced EGF. Phenotypic reversion of malignancy can be achieved through RAP exposure on EGF-reduced 3D BME cultures.
structure within normal breast epithelial acini. In our 3D culture system, non-malignant breast epithelial cells seeded at low density developed into polarized acinar units when EGF is withdrawn. Two populations of cells within each acinus became evident from day 6 to 8: An outer layer of cells that was in direct contact with the ECM, and an inner set of cells not in contact with the matrix that underwent apoptosis (Figures 1 and 7(b)). This emulated the ductal structures of breast epithelial cells found in vivo. In the presence of EGF, normal breast epithelial cells exhibited severe morphological deformities, including colony overgrowth, luminal filling, and resistance to apoptosis (Figure 7(c)). It was observed that EGF disrupted the formation of luminal structures of normal breast epithelial cells either in monolayer or 3D cultures with BME (Figures 7(a) and 7(c)). We also found evidence that rapamycin and its derivative can revert cells’ structure from disorganized to organized (Figures 3, 4, and 7).

Our criteria for phenotypic reversion are: increased structural organization, limited colony size/proliferation, and the presence of basolateral polarity. Immunofluorescence staining of F-actin and -catenin showed a much higher occurrence of morphologically spherical colonies, indicating structural reorganization. This was also supported by the increase in expression of cell-cell adhesion molecules, transmembrane molecules, and ECM proteases shown by the RT-PCR array data (Table 1). Smaller mean area of RAP-treated cell colonies provided strong evidence of limited proliferation. A decreased number of total cell viability over time and increased level of caspase activity confirmed that RAP had a growth-limiting effect on cancer cells. The presence of centrally located cell death indicated the process of lumen formation, directed by caspase-induced programmed apoptosis, and was a result of polarization of the epithelial layers. Upmodulation of caspase, in addition to increased gene expression of basement membrane components and cell-matrix adhesion molecules such as integrins, gave evidence that RAP exposure promoted development of basolateral polarity (Figure 8).

In previous studies, RAP has been seen to prevent morphological disruption and inhibit runaway proliferation normally caused by Akt activation in breast epithelial cells (MCF-10A) [18], and to significantly inhibit growth of human lung cancer cells [14]. The value of inhibiting mTOR as a potential cancer treatment was further validated by this study. However, due to its poor water solubility and limited stability, analogues of RAP have been developed with improved pharmaceutical properties for parenteral delivery, including CCI-799 (tensirolimus), RAD-001 (everolimus), and AP-23573 (ARIAD) [15,19,20], which also inhibit mTOR. The effects of these analogues on the phenotypic reversion of malignant breast epithelial cells cultured in 3D BME should also be studied. Synergistic or additive effects of RAP with other cancer drugs have been studied, including paclitaxel, carboplatin and vinorelbine [21]; mellerian inhibiting substance [22]; doxorubicin [23]; and gemcitabine [24]. Synergistic or additive effects of RAP with other drugs on the phenotypic reversion of malignant cells in 3D BME culture should also be investigated. This study can be used as an experimental template for such future investigation.

In conclusion, by showing that RAP can revert malignant breast epithelial cells to a normal phenotype, our study has provided supporting data for the value of mTOR inhibition as a potential anti-tumor strategy. To provide further insight, two further directions of research should be taken: Further studies on the effects of RAP’s analogues on phenotypic reversion, and studies on the synergistic effects of mTOR inhibition with other cancer targets.

5. ACKNOWLEDGEMENTS

Funding for this project was provided by NIH Grant No. 1 R21 CA 131798-01A1. Special thanks to Dr. Timothy Doyle and Dr. David Britt for their contributions.

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