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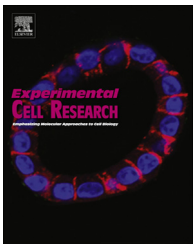

Research Article

Dual contribution of MAPK and PI3K in epidermal growth factor-induced destabilization of thyroid follicular integrity and invasion of cells into extracellular matrix ☆

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ABSTRACT

Normal thyrocytes grown as reconstituted follicles in collagen gel were evaluated for drug effects of small molecule kinase inhibitors on growth factor-induced cell migration in a 3D context. MEK inhibition by U0126 only partially antagonized EGF/serum-induced cell migration from the basal follicular surface into the matrix. Combined treatment with U0126 and LY294002, a PI3K blocker, was necessary to abolish migration. However, exposure to only LY294002 facilitated the response to EGF by breakdown of the original follicular structure. In the same time EGF promoted thyroid cell survival that was compromised by LY294002 in absence of EGF. Cells treated with EGF and LY294002 retained the ability to form follicles. The findings indicate that dual inhibition of MAPK and PI3K/AKT pathways is required to fully block matrix invasion of EGF-stimulated thyroid cells. Conversely, single drug treatment with PI3K inhibitor adversely promotes invasiveness probably by destabilizing the follicular epithelium.

Introduction

The phosphoinositide 3-kinase (PI3K)-AKT/protein kinase B signaling pathway is frequently deregulated in cancer inferring multiple roles in tumor growth and progression with potential implications for targeted therapy [12]. Also in thyroid there is compelling evidence that PI3K contributes to cancer development. Activating mutations within the PI3K catalytic subunit (PI3KCA) is restricted to anaplastic cancer [17]. However, increased PI3K signaling associated with other primary oncogenic signals and conferring a higher proliferation rate likely occurs also in differentiated tumors [37,46,54]. In fact, PI3K signaling mediates oncogenic transformation in *Kras* mutant mouse thyroid epithelial cells and dual inhibition of MAPK and PI3K is required to inhibit growth of cancer cell lines in which both pathways are constitutively activated [36]. Treatment with LY294002, a potent

Abbreviations: TSH, thyroid stimulating hormone; EGF, epidermal growth factor; TGF-β, transforming growth factor-beta; MAPK, mitogen activated protein kinase; ERK, extracellular-regulated kinase; MEK, MAPK/ERK kinase; PI3K, phosphoinositide 3-kinase; EMT, epithelial–mesenchymal transition; mTOR, mammalian target of rapamycin; ZO-1, zonula occludens-1 protein; FBS, fetal bovine serum; 2D, two-dimensional; 3D, three-dimensional

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pan-PI3K inhibitor, effectively reduced tumor growth in a mouse model of follicular thyroid cancer [15]. The importance of the PI3K pathway is further supported by observations of reduced thyroid cancer cell proliferation and induction of autophagic death when the mammalian target of rapamycin (mTOR), a downstream target of AKT, is inhibited [34].

PI3K/AKT signaling participates in the manifestation of epithelial–mesenchymal transition (EMT) implicated in tumor progression and dissemination of cancer cells [30]. This is largely an unexplored field in thyroid cancer, although recent findings from experimental studies on thyroid cancer cell lines suggest that PI3K signaling contributes to metastatic spreading [3]. A great number of reports infer multiple roles of PI3Ks in cell migration depending on cell type and context [4], indicating that PI3K inhibitors with broad specificity as LY294002 likely involves multiple targets that may vary spatiotemporally in different stages of the migrating process. Recently, a diametrically opposed effect of PI3K inhibition promoting colon cancer cell survival and metastasis in tumors with increased Wnt–β-catenin signaling was reported [49], conveying a cautionary remark that subversive effects of small molecule inhibitors to PI3K may be encountered depending on co-activation of other signaling pathways.

Microenvironment may exert profound effects on intracellular signaling and modify drug responses [20,42,52]. Accordingly, tumor cell responses may differ in many aspects depending on whether cells are conventionally cultured or embedded in extracellular matrix. 3D culture was recently adopted to decipher novel mechanisms of drug resistance [31]. Considering the prominent role of PI3K in epithelial morphogenesis [48] and formation of the epithelial junction complex [44], we investigated in this study the possible impact of a preserved epithelial phenotype on drug response to PI3K and MEK inhibitors during growth factor-induced cell migration in reconstituted thyroid follicles embedded in collagen gel. The working model, which is known to promote and preserve epithelial polarity of thyroid cells in culture [16], was previously successfully used to monitor invasiveness [41,53] and dedifferentiation [23] following epidermal growth factor (EGF) stimulation.

Materials and methods

3D culture and treatments

Porcine thyroid follicles were isolated as previously described [40]. For 3D culture, collagen type I solution (Purecol®; Advanced Biomatrix Inc, San Diego, CA, USA) was neutralized according to the manufacturers instruction and mixed with follicle segments in suspension to form 100 μl gels in 96 well plates. To prevent unwanted sedimentation of follicles culture plates were repeatedly rotated during collagen polymerization. Cells were cultured in Earle's minimum essential medium (PAA Laboratories GMbH, Pasching, Austria) supplemented with 5% FBS (Sigma Aldrich, Taufkirchen, Germany), penicillin (200 U/ml), streptomycin (200 μg/ml) and amphotericin B (2.5 μg/ml) (PAA Laboratories) for 6 days allowing reconstitution of follicles. For experiments cells were either kept in 5% FBS or switched to 0.5% FBS followed by stimulation with 1 mU/ml TSH (Sigma Aldrich) and 10 ng/ml EGF (Roche Diagnostics, Mannheim, Germany) without or with 10 μM U0126 (Promega, Madison, WI, USA) and 10 μM LY294002 (Sigma-Aldrich) for 48 or 96 hours (h). All reagents were added at 2× concentration taking the gel volume into account.

Direct microscopy

Collagen gel-embedded follicles were routinely viewed for morphological changes during treatment and imaged with a Zeiss Axiovert CFL 40 light microscope or a AMG EVOS™ fl digital inverted fluorescence microscope equipped with a Sony® ICX285AL CCD camera. Follicle profiles with evident signs of radial cell migration into surrounding matrix were quantified in a standardized manner from four fields of view at 20× ensuring that no objects were double counted. Cells considered migratory comprised both cells that evidently were separated from the nearby follicles and cells with clearly visible basal projections that extended into matrix but retained a connection to the follicular wall.

Immunolabeling and fluorescence microscopy

Cells in collagen gels were fixated with 4% formaldehyde for 20 min and thereafter permeabilized with 0.5% Triton-X in phosphate buffered saline (PBS) for 10 min and incubated with blocking solution consisting of 2% normal donkey serum in 0.1% Triton-X/PBS followed by rabbit anti-human ZO-1 antibody (Invitrogen, Camarillo, CA, USA) diluted 1:200 in blocking solution both overnight at 4 °C. Rhodamine red-conjugated secondary antibody diluted 1:200 (Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA) was added for 2 h followed by extended rinsing in PBS overnight to reduce background fluorescence in gels. Specimens were counterstained with DAPI nuclear stain, mounted on glass with coverslips and Fluorescence Mounting Medium (Dako, CA, USA), and viewed and photographed in a Zeiss Axioscope 2 plus fluorescence microscope equipped with Nikon DS-Qi1Mc camera. Follicles with a discernible lumen enclosed by tight junctions (ZO-1) were counted as detailed above. In addition, specimens solely labeled with DAPI for counting of follicles containing pyknotic nuclei with condensed chromatin were evaluated in Zeiss Axioscope 2 plus fluorescence microscope.

Western blot analysis

Porcine thyroid cells grown in petri dish and stimulated for 5 min with EGF (10 ng/ml) in the presence U0126 (10 μM) or LY294002 (10 μM) were lysed in presence of 0.4 mM Pefablock (Roche diagnostics, Mannhein, Germany) 1 mM sodium orthovanadate, 5 μg/ml aprotinin and 5 μg/ml leupeptin (all from Sigma Aldrich). Protein concentration was determined using micro-BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were separated on 4–15% SDS-PAGE gels (Biorad Laboratories, Hercules, CA, USA) and transferred to PVDF membranes (Transblot, Biorad). After blocking in 1% BSA in TBST buffer (20 mM Tris, 137 nM NaCl and 0.l% Tween-20) membranes were probed overnight at 4 °C with antibodies against phospho-p44/42 MAPK (p-ERK1/2; Cell Signaling Technology, Danvers, MA, USA), p-44/42 MAPK (*t*-ERK1/2; Cell Signaling), phospho-AKT and AKT (4058 and 4691; Cell Signaling). After incubation with secondary HRP-conjugated antibodies membranes were developed with enhanced chemiluminescence (Luminata™ Forte Western; Millipore) and photographed in a LAS