Biomedicine & Pharmacotherapy 68 (2014) 611–617

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Original article

Inhibition of phosphoinositide 3-kinase is associated with reduced angiogenesis and an altered expression of angiogenic markers in endothelioma cells

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ARTICLE INFO

Article history:
Received 28 February 2014
Accepted 17 March 2014

Keywords:
PI3k
Endothelioma
Angiogenesis
Akt
LY294,002

ABSTRACT

The phosphoinositide 3-kinase (PI3k) signaling pathway is involved in the regulation of numerous cellular activities. The pathway has also been implicated in the development of various tumors. In the context of vascular tumors, the role of the PI3k signaling still needs to be established. In the present study, the effects of blocking PI3k activation on endothelioma cells derived from mice with vascular tumors were investigated using the crystal violet assay, real-time cell analysis, light microscopy, the aorta ring assay and antibody arrays. The suppression of PI3k led to the inhibition of cell growth, cell migration, as well as angiogenesis. The inhibition of these processes correlated with low Akt activity. Antibody array analysis revealed that there was a suppression of several proangiogenic molecules, including Eotaxin-1 and basic fibroblast growth factor (bFGF) in cultures treated with LY294,002, an inhibitor of PI3k. At the same time, LY294,002 increased the expression of platelet factor 4 (PF4) and the Fas ligand (FasL), molecules which have antiangiogenic properties. The results suggest that PI3k may play a role in the expression of some of the key regulatory molecules involved in angiogenesis, and perhaps in the growth of endotheliomas. As such, it is plausible that the PI3k/Akt pathway may be a target for therapeutic molecules designed for the treatment of endothelial tumors.

1. Introduction

Vascular tumors are endothelial cell neoplasms characterised by dysregulated angiogenesis [1]. In chicks, the over-expression of P3k, an oncogene coding for the homologue catalytic subunit of PI3k, has been shown to lead to the development of vascular tumours known as hemangiosarcomas [2]. In a recent study, we showed that the growth of vascular tumors in mice correlated with increased Akt activation [3]. The significance of the PI3k/Akt pathway in the pathogenesis of vascular tumors requires elucidation, and the key role players in PI3k signaling need to be established.

The phosphoinositide 3-kinases, also known as phosphatidylinositol-3-kinases (PI3ks), are a family of enzymes that regulate a network of cellular processes [4,5]. In mammalian cells, PI3ks exist in three classes, I, II, and III, with class I being the most studied in the context of the regulation of cell growth, survival and proliferation [5–7]. Class I PI3ks are further divided into the IA and IB subgroups based on the receptors that activate them [5–7]. The former, which transmits signals from receptor tyrosine kinases (RTKs), has been implicated in tumorigenesis and is thus the focus of attention in this study [5,6].

Upon binding of a growth factor or signaling compound to a receptor tyrosine kinase (RTK), PI3k is recruited to the internal site of the cell membrane and activated [5]. Such activation produces 3′ phosphoinositide lipids which act as second messengers by activating diverse cellular target molecules [5,7]. One such example is the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) by PI3ks to form phosphatidylinositol 3,4,5-trisphosphate (PIP3) [5].

Phosphatidylinositol 3,4,5-trisphosphate initiates a cascade of events that lead to the recruitment and activation of protein kinase B (PKB), also known as Akt [4]. The protein kinase is one of the key downstream effectors of PI3k [4,6]. When activated, Akt regulates a broad array of cellular functions, including cell growth, attachment, proliferation, migration, and apoptosis. On the other hand, the subversion of the functions of PI3k is observed in a myriad of disorders that include cancer, diabetes mellitus and inflammation [6]. Following the establishment of the importance of PI3k in physiological processes and in disease, a number of inhibitors,

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http://dx.doi.org/10.1016/j.biopha.2014.03.017
0753-3322/

most notably Wortmannin and LY294,002, were developed to aid in studying the role of PI3k in the different settings. LY294,002 is a derivative of the broad spectrum kinase inhibitor quercetin and unlike Wortmannin, it's effects are reversible [6].

In this study, we investigated the effects of LY294,002 on endothelioma (sEnd.2) cells, which are endothelial cells derived from the subcutaneous tissue of mice with Pym T-induced vascular tumors. It is envisaged that better understanding of the involvement of the PI3k signaling pathway in vascular tumor growth may provide useful targets for therapeutic intervention. In the present study, the effects of the drug on endothelioma cell growth and migration, and on angiogenesis and the expression of angiogenic markers were investigated.

2. Materials and methods

2.1. Cell culture maintenance procedure

Endothelioma cells (obtained from Prof M.S. Pepper, University of Pretoria) were maintained at a temperature of 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in Dulbecco's modified eagle's medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat inactivated fetal calf serum (Invitrogen, USA), 2 mM L-glutamine (Invitrogen, CA, USA), and 1% Penicillin-streptomycin (Whitehead Scientific, Johannesburg, South Africa).

2.2. Cell growth assay

Cell viability was assessed using the crystal violet nuclear staining assay. Endothelioma cells were seeded in 24-well culture plates at a density of 10 000 cells per well for 24 hours, and then treated with LY294,002 (0–10 μM) or dimethylsulfoxide (DMSO) for 24–72 hours. At termination, cells were fixed with 1% glutaraldehyde in PBS for 15 minutes, followed by staining with a 0.1% crystal violet solution (Sigma-Aldrich, St Louis, MO, USA) for 30 minutes. The chromophore was extracted with 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) per well. The absorbance was read at 570 nm on an ELx 800 Universal Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA). Three wells were analysed for each concentration. The percentage of viable cells was calculated as follows: viability (%) = [A570 (treated) – A570 (blank)]/[A570 (control) – A570 (blank)] × 100.

2.3. Cell morphology

Endothelioma cells were seeded on cover slips in 6-well plates at a density of 3×10^5. After 24 hours, the cells were treated with LY294,002 (0–10 μM) over a period of 48 hours. The cells were fixed in Bouin's fluid. After 30 minutes of fixing, they were rinsed in 70% ethanol for a further 30 minutes, and then rinsed with water.

The cells were immersed in Meyer's haemalum for 20 minutes, rinsed with water, followed by 70% ethanol. The cells were incubated in 1% eosin for 2 minutes and then rinsed twice with increasing concentrations of ethanol (70%, 96%, and 100%). Xylol was used to rinse the cells twice before mounting them onto microscopic glass slides using resin. The slides were viewed with an Olympus BX63 light microscope and images were captured using the Olympus DP72 digital camera (Wirsam Scientific, Johannesburg, South Africa).

2.4. Caspase activity

Endothelioma cells were seeded at a density of 3×10^5 cells/well and allowed to attach overnight. The cells were treated with LY294,002 (10 μM) with or without the caspase inhibitor zVADfmk (50 μM) for 48 hours. Control cells were exposed to DMSO. Caspase-3 activity was measured using commercially available kits according to the manufacturer's instructions (BioVision, Mountain View, USA). In brief, control and drug-treated sEnd.2 cells were lysed in caspase lysis buffer, followed by centrifugation at 16,0009 g for 15 minutes. The supernatant was mixed with assay buffer in the presence of 200 mM substrate. Samples were incubated at 37 °C for 4 h and absorbance was read at 405 nm using an ELx 800 Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA).

2.5. Akt kinase activity

The activity of Akt kinase was measured in control and LY294,002-treated cells using an enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer (Stressgen, MI, USA). Cells were seeded at a density of 3×10^5 cells/well. After 24 hours, the cells were treated with LY294,002 at the indicated doses or with DMSO (control). After 48 hours, 50 μL of assay buffer was added to each well. Thirty μl of sample, purified active Akt, inhibitor diluent or dilution buffer were added to triplicate wells. Ten μl of diluted ATP were then added and the plate incubated for 90 min. Anti-phosphate specific substrate was added to each well and the plate was incubated at room temperature. This was followed by the addition of 40 μL/well of secondary antibody solution. The plate was incubated at room temperature for 30 min. After washing, tetramethylbenzidine was added to each well and the plate was incubated at room temperature for 45 min. The reaction was stopped by adding 20 μL/well of acid stop solution. The absorbance was read at 450 nm using the ELx 800 Universal Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA).

2.6. Cell migration assay

Cell migration experiments were performed using 16-well plates (CIM-16, Roche Applied Science, Johannesburg, South Africa). Prior to each experiment, cells were deprived of FBS for 24 hours. A volume of 160 μL serum free medium with or without 10 ng/ml bFGF as a chemoattractant was added to each well of the lower chamber of a CIM plate. Serum free medium (20 μL) was also added to the upper chamber and the plate was allowed to equilibrate for 1 hour. Endothelioma cells (6×10^3 cells/well) were seeded into the upper chamber of the plates and treated with LY294,002 at the indicated concentrations. Identical amounts of dimethyl sulfoxide were used as control. Each condition was performed in quadruplicate. The cells that migrated through the filter into the bottom chamber were counted with an xCELLigence RTCA DP instrument. The cell index (CI) was recorded by the instrument analyzer and analysis was performed with the supplied RTCA software (vs. 1.2.1). Results are based on raw data without CI-normalization.

2.7. Aorta ring assay

Aortas were harvested from 6–8 weeks old male Sprague-Dawley rats, cleaned, and cut into 1 mm wide rings. The rings were then embedded in fibrin gel in 24-well culture plates and maintained in MCDB-131 medium supplemented with 0.3 mg/mL amino caproic acid and 1% penicillin-streptomycin. The cultures were treated with either LY294,002 at the indicated doses or DMSO for 10 days. Medium and drug were renewed every 2 days. Cultures were monitored for growth every second day with a Zeiss Axiovert microscope (Carl Zeiss, Jena, Germany) attached to a digital camera. The area of neovessel growth was