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Is there a pAkt between VEGF and oral cancer cell migration?

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ABSTRACT

The PI3K-Akt signalling pathway is a well-established driver of cancer progression. One key process promoted by Akt phosphorylation is tumour cell motility; however the mechanism of VEGF-induced Akt phosphorylation leading to motility remains poorly understood. Previously, we have shown that Akt phosphorylation induced by different factors causes both stimulation and inhibition of motility in different cell types. However, differential phosphorylation of Akt at T308 and S473 residues by VEGF and its role in head and neck cancer cell motility and progression is unknown. The cell lines investigated in this study exhibited a change in phosphorylation of Akt in response to VEGF. However, in terms of motility, VEGF stimulated oral cancer and its associated cell lines, but not normal keratinocytes or oral mucosal fibroblasts. The addition of a PI3 kinase and mTOR inhibitor, inhibited the phosphorylation of Akt and also effectively blocked VEGF-induced oral cancer cell motility, whereas only the PI3 kinase inhibitor blocked oral cancer associated fibroblast cell motility. This study therefore discloses that two different mechanisms of Akt phosphorylation control the motility potential of different cell lines. Akt phosphorylated at both residues controls oral cancer cell motility. Furthermore, immunohistochemical analysis of VEGF positive human head and neck tumour tissues showed a significant increase in Akt phosphorylation at the T308 residue, suggesting that pAkt T308 may be associated with tumour progression *in vivo*.

1. Introduction

Cell motility or migration is an essential part of most tumour pathways. Cells need to migrate away from their microenvironment to enable the tumour to spread or metastasise. Growth factors and matrix macromolecules are essential for the movement of cells [1]. Such movement requires a reorganisation of the actin cytoskeleton, which is under the control of many pathways including the PI3 kinase signal transduction pathway. The Class 1 PI3 kinases are a set of lipid kinases that phosphorylate the relatively abundant membrane phospholipid, phosphatidylinositol 4, 5 biphosphate (PIP2), generating small quantities of phosphatidylinositol 3, 4, 5 triphosphate (PIP3). This latter lipid signal controls a diverse set of effector molecules including the Akt group of oncogenic kinases (also known as protein kinase B) [2]. Activation of Akt, a 60 kDa serine/threonine kinase, depends on PI3K [3]. Increase of cellular PIP3 by PI3K eventually allows the activation of Akt by phosphorylation at residues T308 and S473 [4]. This activation is completed by structural modification stimulated by PI3K-dependent kinase-1 (PDK-1)-dependent phosphorylation at T308 and stabilisation by mTORC2-dependent phosphorylation at S473 [5].

We have previously shown that the PI3 kinase and Akt pathways are essential for the migration of fibroblasts in response to added factors such as Epidermal growth factor (EGF) and Transforming growth factor alpha (TGFα) [6]. The addition of PI3 kinase inhibitors blocks the migration stimulating activity of EGF and TGFα, the data indicating that both growth factors increase phosphorylation of Akt. Inhibition of PI3K activity blocks migration stimulated by G protein-coupled receptors or by receptor tyrosine kinases, signifying that PI3K has a vital function in cell migration [7]. Higher expression of phosphorylated Akt has also been reported in oral cancer, in comparison to normal mucosa and pre-cancerous tissue [8]. Vascular endothelial growth factor (VEGF) has been reported to stimulate the proliferation of endothelial cells and to enhance vascular permeability and survival [9]. In addition, it has also been shown that VEGF positivity, as assessed (or identified) by immunohistochemistry, is a functional indicator of poor prognosis in oral cancer.

VEGF status may become a significant prognostic factor in head and neck cancer [10,11]. Over-expressed VEGF acts as an effective angiogenic cytokine, stimulating endothelial cells thus promoting angiogenesis in solid tumours such as breast or ovarian carcinomas [12]. VEGF is a disulphide-linked dimeric glycoprotein which has six isoforms generated by alternative splicing, typically the 121 and 165 isoforms are the most common. These VEGF isoforms vary in their heparin binding capacity, in addition to their ability to bind the tyrosine-kinase receptors

Abbreviations: pAkt T308, phosphorylated Akt at threonine 308; pAkt S473, phosphorylated Akt at serine 473; VEGF, vascular endothelial growth factor; PI3K, phosphatidylinositide 3-kinases; HNSCC, head & neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma.

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VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) and to neuropilin-1 and neuropilin-2 [13]. $VEGF_{121}$ is considered to be more angiogenic and tumourigenic than the other isoforms [14].

Oral cancer, a malignant neoplasm that affects the tissues of the mouth, is the eighth most common cause of cancer-related deaths worldwide [15,16]. Globally, more than 90% of these malignancies are squamous cell carcinomas (SCC) occurring in the mucous membranes and oropharynx [17]. There were some 399,546 new cases of oral cancer and other pharyngeal cancers according to the GLOBOCAN 2008 database, collated by the International Agency for Research on Cancer [18]. This incidence is thought to be due to increased use of alcohol and tobacco. Oral cancer still has a poor survival rate, with a high occurrence of metastases, even though there has been significant progress in cancer treatment over the past few decades [19].

In this study, we aimed to establish the role of the PI3K-Akt pathway in $VEGF_{121}$ induced migration of oral cancer cells. The resultant data will help us to extend the spectrum of known biological activities of this pathway and to propose that inhibition of this pathway will be a suitable target for chemotherapeutic drug design to control oral cancer cell metastasis.

2. Materials and methods

2.1. Reagents, antibodies and inhibitors

The primary antibodies used were: rabbit monoclonal anti-pAkt T308 (# 2965), anti-pAkt S473 (# 4060), anti-pan Akt (# 4691) (all Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal anti-VEGF A(A-20) (# sc152, Santa Cruz Biotechnology, TX, USA) and mouse monoclonal anti-GAPDH (# MAB374, Millipore, Darmstadt, Germany). The secondary antibodies used were goat anti-rabbit HRP conjugated (# 7074, Cell Signaling Technology), rabbit anti-mouse HRP conjugated (# P0260, Dako, Cambridgeshire, UK) and biotinylated anti-rabbit (BA-2020, Vector Laboratories, CA, USA). Recombinant Human $VEGF_{121}$ (# 10–1296) was purchased from Insight Biotechnology Ltd., Middlesex, UK. The PI3K-Akt pathway inhibitors LY294002 (# 9901) and PI103 (# 528100) were purchased from Merck Calbiochem, Darmstadt, Germany. The blocking peptides for pAkt S473 Ab (#1140) and pAkt T308 Ab (#1145B) were also purchased from Cell Signaling technology, Inc.

2.2. Cell culture

The highly differentiated oral mucosal squamous cell carcinoma cell line (TR-146) originated from cheek mucosa and was derived from lymph node. The stromal line (PM1) originated from forehead skin and was derived from dysplastic lesion. These lines were a kind gift from Dr. Dorothy Couch, Dundee Dental School. The oral adenoid squamous cell carcinoma (OASCC) cell line (TYS), derived from a minor salivary gland was a kind gift from Dr. Koji Harada, University of Tokushima, Japan. Normal adult keratinocytes (HaCaT) and normal oral mucosal fibroblasts (MM1) were a kind gift from Prof S.L. Schor and Dr. M. Macluskey, Dundee Dental School, UK, respectively. Mouth cancer-associated fibroblast cell line, COM D25 was isolated in-house from explant culture of a biopsy from the Oral Surgery Clinic, Ninewells Hospital, Dundee. All the cells were cultured at 37 °C and 5% CO_2 in MEM media supplemented with 10% (v/v) foetal calf serum (FCS) and 200 mM glutamine.

2.3. SDS-PAGE and Western blotting

Cells grown on 60 mm culture dishes were lysed on ice with RIPA buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4; 0.1% w/v SDS, 1% v/v Triton x-100, 1% w/v sodium deoxycholate and 5 mM EDTA) containing protease inhibitors (Roche Applied Science, IN, USA). RIPA buffer with added phosphatase inhibitors (Roche Applied Science, IN, USA) was used to lyse the cells treated with different concentration of VEGF. Lysates were clarified by centrifugation at 13,000 rpm for 5 min. Samples were then mixed with an equal volume of Laemmli sample loading buffer (BioRAD, CA, USA) including 5% (v/v 2-mercaptoethanol). Samples were heated at 95 °C for 5 min and loaded onto 'Any kD' SDS-PAGE BioRad TGX precast gels. After completion of SDS PAGE, proteins were electro-transferred onto nitrocellulose transfer membrane (0.45 μm, Whatman, Buckinghamshire, UK) and then immunoblotted with anti-pAkt T308 (1:1000), anti-pAkt S473 (1:2000) , anti-pan Akt (1:1000), anti-GAPDH (1:500), goat anti-rabbit HRP conjugated secondary antibody (1:2000) and rabbit anti-mouse HRP conjugated secondary antibody (1: 10,000). Immunoblots were developed using Immun-Star WesternC Kit (BioRad). Loading was controlled against GAPDH expression.

2.4. Boyden chamber migration assay

A 48-well Boyden chamber (Neuroprobe, Inc., MD, USA) was used for the *in vitro* migration assays as previously described [20]. In brief, cells suspended in serum-free MEM with bovine serum albumin (2 μg/ml) (SF-BSA) were seeded into the upper compartment of the chamber. The lower compartment was filled with different concentrations of $VEGF_{121}$ and inhibitors, diluted with SF-BSA. The two compartments were separated by a porous membrane filter (8 μm, Costar, UK) coated with type 1 native collagen. The chambers were incubated for 5 h at 37 °C. The filter was then washed twice in PBS, fixed in cold methanol and stained either with Mayer's (#MHS 32, Sigma-Aldrich, MO, USA) or Gills 3 (#095903, Brunel Microscope, Wiltshire, UK) haematoxylin overnight. The cells on the upper surface of the filter were scraped off with a cotton swab. The membrane was then mounted onto a glass slide and examined under bright field illumination at a magnification of ×200. Six replicate wells were used per variable. The numbers of migrated cells adherent to the lower surface of the membrane were counted in 3 random fields per well *i.e.* 18 fields per variable. Data were expressed as mean cell number per field ± SEM. When comparing different variables, results were expressed as a percentage of the controls.

2.5. Collagen gel migration assay

The collagen gel migration assay was performed as previously described [2]. Type I collagen from rat tail tendons was used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as described earlier [21]. Collagen gels were overlaid with 1 ml of either serum-free MEM (SF-MEM) or SF-MEM containing 4× the final concentration of $VEGF_{121}$. Confluent stock cultures of cells were then harvested, resuspended in growth medium containing 4% (v/v) FCS at the desired concentration and 1 ml aliquots were added to the overlaid gels. Considering the 2 ml volume of gel, 1 ml medium overlay and 1 ml cell inoculum, this procedure gives a final concentration of 1% (v/v) serum in both control and test cultures. Cells attached to the surface of the gel within 1 h and started to migrate into the underlying 3D gel within 24 h. Four days after plating, the number of cells remaining on the surface or that had migrated into the gel were determined by microscopic observation of 10 randomly selected fields in each of the duplicate cultures. Cell migration was expressed by the number of cells that migrated into the 3D gel, as a percentage of the total number of cells present (Mean ± s.e.m). When comparing different variables, results were expressed as a percentage of the controls.

2.6. Immunohistochemistry

64 HNSCC, 22 dysplastic and 11 normal oral mucosal tissue samples were collected by Dr. M. Macluskey, after ethical approval was granted, at Ninewells Hospital (Dundee) and the Royal Infirmary (Aberdeen) and were stored at Tayside tissue bank.