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Tumour-derived fibroblast growth factor-2 exerts lymphangiogenic effects through Akt/mTOR/p70S6kinase pathway in rat lymphatic endothelial cells

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ABSTRACT

Fibroblast growth factor-2 (FGF-2) has been shown to induce both angiogenesis and lymphangiogenesis in the mouse corneum; however, the signalling mechanism underlying FGF-2-induced lymphangiogenesis remains unknown. Here we investigated the effect of FGF-2 on newly developed temperature-sensitive rat lymphatic endothelial (TR-LE) cells. The supernatant of PC-3 prostate cancer cells facilitated tube-like formation in TR-LE cells, and formation was inhibited by neutralising antibodies against FGF-2. The addition of FGF-2 stimulated tube-like formation as well as proliferation and chemotactic migration of TR-LE cells. Blockade of the Akt signalling pathway by LY294002 abolished the elongation of tubes induced by FGF-2, whereas inhibition of the extracellular signal-regulated kinase (ERK) signalling pathway had no effect. Rapamycin abrogated the phosphorylation of p70S6kinase and consistently inhibited the formation of tubes induced by FGF-2. Furthermore, tube-like formation induced by the supernatant of PC-3 cells was inhibited by LY294002 or rapamycin. These data suggest that FGF-2 exerts lymphangiogenic effects by activating the Akt/mammalian target of rapamycin (mTOR)/p70S6kinase pathway in lymphatic endothelial cells, and that the pathway provides a potent target for tumour lymphangiogenesis.

1. Introduction

The lymphatic vasculature is an important route for the metastatic spread of human cancer. Clinico-pathological studies have revealed that lymphangiogenesis can occur adjacent to or within human cancers, and that it correlates with metastasis to lymph nodes in many human tumours including prostate cancer.[1,2]

Fibroblast growth factor-2 (FGF-2) is a heparin-binding protein that induces the proliferation or differentiation of a variety of cell types.[3] In the corneal neovascularisation assay, FGF-2 can induce both angiogenesis and lymphangiogenesis.[4] FGF-2-induced lymphangiogenesis is blocked by neutralising vascular endothelial growth factor receptor-3 (VEGFR-3) antibodies, indicating that FGF-2 promotes lymphatic growth via the VEGF-C/VEGFR-3 signalling system in the corneum.[5,6] However, the corneal stroma does not express heparin sulfate, which is critical for efficient binding and signalling of heparin-binding growth factors such as FGF-2. Therefore, an indirect effect of FGF-2 mediated by non-heparin-binding factors such

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as VEGF-C may be predominant in promoting the lymphangiogenesis. Thus, the direct lymphangiogenic effect of FGF-2 in the tumour microenvironment remains controversial.

A culture of lymphatic endothelial cells is important to obtain a better understanding of their respective roles in vascular physiology and pathology including lymphangiogenesis. Recently, we succeeded in establishing a rat lymphatic endothelial cell line (TR-LE) from the thoracic duct of a transgenic rat harbouring a temperature-sensitive simian virus 40 (SV40) large T-antigen and enhanced green fluorescent protein (EGFP).[7,8] TR-LE cells maintain cobblestone-like morphology and express lymphatic endothelial markers VEGFR-3 (vascular endothelial growth factor receptor), LYVE-1, Prox-1, and podoplanin, together with endothelial markers CD31, Tie-2, and VEGFR-2. This lymphatic endothelial cell line enables lymphangiogenesis experiments *in vitro*.

In this study, we used TR-LE cells to identify the possible signalling targets regulating the lymphangiogenic effects of FGF-2, and to assess the involvement of FGF-2 in the interaction between cancer cells and lymphatic endothelial cells in tumour lymphangiogenesis.

2. Materials and methods

2.1. Materials

LY294002, SU4984, U0126, SP600125, rapamycin, and VEGF-C were purchased from Calbiochem (Darmstadt, Germany). Wortmannin and anti-FGF-2 antibody were purchased from Alomone Laboratories (Jerusalem, Israel) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Reagents were dissolved in dimethyl sulfoxide, PBS supplemented with 0.1% bovine serum albumin w/v or distilled water and stored at −20 or −80 °C.

2.2. Cells

TR-LE cells, a conditionally immortalised rat lymphatic endothelial cell line, were maintained on culture dishes pre-coated with 10 μg/mL fibronectin (Iwaki Glass, Tokyo, Japan), in HuMedia-EG2 (Kurabo, Osaka, Japan) supplemented with 20% foetal bovine serum (FBS) v/v at a permissive temperature (33 °C).[7,8] PC-3 human prostate cancer cells were kindly provided by Dr. M. Nakajima (Novartis Pharma K.K., Takarazuka), and maintained in RPMI-1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS v/v.

2.3. Preparation of conditioned media (CM)

PC-3 cells were seeded in 10-cm culture dishes in 10 mL of RPMI1640 supplemented with 10% FBS v/v for 48 h to reach sub-confluence. The supernatants were centrifuged to remove debris and were used immediately in the tube formation assay.

2.4. Tube formation assay

TR-LE cells were cultured in DMEM (Invitrogen Corp.) supplemented 0.2% v/v FBS for 12 h, and were harvested with trypsin/EDTA. A cell suspension (1.1×10^4/ 200 μL) was prepared in CM supplemented with antibodies or DMEM supplemented with 0.2% FBS v/v. Heparin (Sigma; 10 μg/mL) was added to the suspension. After 5 min of treatment with inhibitors, the suspension was seeded in a 96-well plate that had been coated with 40 μl of 10 mg/ml Matrigel. After a 3-h incubation at 37 °C, cultures were photographed with a NIKON inverted microscope, and the length of the tube-like network was measured using MAPMETER PJ Type (UCHIDA YOKO, Tokyo, Japan).

2.5. Cell proliferation assay

TR-LE cells (6×10^3 cells/well) were seeded in 100 μl of DMEM containing 5% FBS v/v in 96-well plates pre-coated with 10 μg/mL laminin. Cells were allowed to adhere for 3 h, and incubated with human recombinant FGF-2 (Kaken Pharmaceutical Co., Ltd.) and heparin (10 μg/mL) for an additional 48 h at 37 °C. The cells were fixed with 2.5% glutaraldehyde v/v and stained with crystal violet (0.1% w/v in a 20% methanol solution v/v). After several washes, crystal violet was extracted with 30% acetic acid v/v, and the absorbance was measured at 595 nm.

2.6. Cell migration assay

The chemotactic migration of TR-LE cells was measured using Transwell cell culture chambers (Costar 3422, Cambridge, MA, USA) as described previously[9] with some modifications. The filter's lower surface was precoated with 5 μg of Matrigel (Collaborative Research Co., Bedford, MA). After a 12-h pre-incubation in DMEM supplemented with 0.2% FBS v/v, TR-LE cells were harvested with trypsin/EDTA. After several washes, a TR-LE cell suspension (1×10^5/100 μL) was added to the upper compartment of the chamber; FGF-2 and heparin (10 μg/mL) were added to the lower compartment. After a 9-h incubation at 37 °C, the filters were fixed with methanol, and cells that had migrated to the lower surface were counted by crystal violet assay.

2.7. Western blot analysis

After the indicated treatment, the cells were rinsed with ice-cold PBS and lysed in sample buffer [24 mM Tris-HCl (pH 6.8), 5% glycerol w/v, 1% SDS w/v, and 0.05% bromophenol blue w/v]. Cell lysates were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were incubated with Block Ace (Dainipponseiyaku, Suita, Japan) and probed with the indicated primary antibodies (1:1000). Protein content was visualised using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Phospho-Akt, phospho-ERK, phospho-p38 and phospho-p70S6kinase antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA), and FGFR-1 from SantaCruz (Santa Cruz, CA).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression of FGFRs by TR-LE cells and normal rat liver tissue (male 19-week-old Wistar rat; Japan SLC,