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Focal adhesion kinase inhibitors are potent anti-angiogenic agents

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ABSTRACT

Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase and scaffold protein localized to focal adhesions, is uniquely positioned at the convergence point of integrin and receptor tyrosine kinase signal transduction pathways. FAK is overexpressed in many tumor cells, hence various inhibitors targeting its activity have been tested for anti-tumor activity. However, the direct effects of these pharmacologic agents on the endothelial cells of the vasculature have not been examined. Using primary human umbilical vein endothelial cells (HUVEC), we characterized the effects of two FAK inhibitors, PF-573,228 and FAK Inhibitor 14 on essential processes for angiogenesis, such as migration, proliferation, viability and endothelial cell tube formation. We observed that treatment with either FAK Inhibitor 14 or PF-573,228 resulted in reduced HUVEC viability, migration and tube formation in response to vascular endothelial growth factor (VEGF). Furthermore, we found that PF-573,228 had the added ability to induce apoptosis of endothelial cells within 36 h post-drug administration even in the continued presence of VEGF stimulation. FAK inhibitors also resulted in modification of the actin cytoskeleton within HUVEC, with observed increased stress fiber formation in the presence of drug. Given that endothelial cells were sensitive to FAK inhibitors at concentrations well below those reported to inhibit tumor cell migration, we confirmed their ability to inhibit endothelial-derived FAK autophosphorylation and FAK-mediated phosphorylation of recombinant paxillin at these doses. Taken together, our data indicate that small molecule inhibitors of FAK are potent anti-angiogenic agents and suggest their utility in combinatorial therapeutic approaches targeting tumor angiogenesis.

Abbreviations: ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FAK, focal adhesion kinase; FI14, FAK Inhibitor 14; GST, glutathione-S-transferase; HBSS, Hank's buffered saline solution; HUVEC, human umbilical vein endothelial cells; IB, immunoblot; IGF-1R, insulin-like growth factor receptor 1; IP, immunoprecipitation; LB, Luria Bertani; PBS, phosphate-buffered saline; PF-228, PF-573,228; TBST, tris-buffered saline/Tween-20; TRITC, tetramethylrhodamine B isothiocyanate; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

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1. Introduction

Angiogenesis, or the growth of new blood vessels arising from pre-existing ones, is a complex process directed by growth factors, receptors, extracellular matrix (ECM)-to-cell and cell-to-cell interactions. Tumor-associated angiogenesis is necessary for sustaining tumor growth beyond 1 mm^3 (Gimbrone et al., 1972; Hanahan and Weinberg, 2000). Due to its central role in tumor growth, therapeutic targeting of angiogenesis has become a major focus in recent years. Although angiogenesis can be modulated by various growth factors, vascular endothelial growth factor (VEGF) has been shown to play a predominant role in tumor-associated angiogenesis (Smith et al., 2010; Veikkola et al., 2000). Thus, numerous agents targeting VEGF ligand or its receptors (VEGFR) have been developed and tested as anti-cancer therapies alone or in combination in various cancer types (Ellis and Hicklin, 2008). Currently, there are four anti-angiogenic agents approved for clinical use and many more being investigated in clinical trials, however, it is clear that many patients do not initially respond to and others acquire resistance to these modalities (Ebos and Kerbel, 2011). Resistance to VEGF pathway inhibitors, can arise from either evasive resistance (e.g. where alternate angiogenic signaling pathways are invoked) or intrinsic resistance (e.g. where redundant angiogenic signaling exists) (Bergers and Hanahan, 2008). Given these observations and clinical challenges, other targets involved in angiogenesis need to be examined to realize the full benefits of anti-angiogenic therapy.

Focal adhesion kinase (FAK) is a 125-kDa non-receptor tyrosine kinase, which acts as a scaffold at sites of cell attachment to the extracellular matrix (ECM) and is activated following binding of integrins to ECM or upon growth factor stimulation including that mediated by VEGF (Abedi and Zachary, 1997; Mitra and Schlaepfer, 2006). FAK has been implicated as an important modulator of angiogenesis, as transgenic mouse models have indicated that endothelial FAK expression and activity are essential for the formation of new blood vessel networks during embryonic development (Braren et al., 2006; Ilic et al., 2003; Shen et al., 2005). More recently, using a tissue-restricted knockout mouse model, it was demonstrated that endothelial FAK was essential for tumor growth and tumor-associated angiogenesis, as mice lacking endothelial-specific FAK expression exhibited reduced tumor angiogenesis and hence reduced tumor growth *in vivo* (Tavora et al., 2010). FAK activity is also modulated following the activation of growth factor receptors including VEGFR2, which upon activation by VEGF ligand can recruit and activate Src kinase which subsequently phosphorylates focal adhesion kinase (FAK) at tyrosine 861 and modulates endothelial cell migration and survival (Abu-Ghazaleh et al., 2001). In addition to its putative role in angiogenesis, altered FAK activity and expression have been directly linked to tumorigenesis and metastasis since interference with FAK signaling led to decreased metastasis in a variety of tumor models, including breast and lung cancer (Golubovskaya et al., 2009; Zhao and Guan, 2009).

Given that FAK has been shown to have aberrant activity and/or expression in many cancers [reviewed in (McLean et al., 2005)], it has been described as a "druggable" target. Hence, there has been a surge in the discovery and preclinical development of pharmacological inhibitors of FAK activity, such as NVP-TAE-226, PF-562,271, PF-573,228 and FAK Inhibitor 14 (also known as Y15) [reviewed in (Schultze and Fiedler, 2010; Schwock et al., 2010)]. To date the effectiveness of these inhibitors has predominantly been examined in cancer cell lines and murine tumor models, where FAK inhibitor treatment resulted in reductions in tumor growth and metastatic burden (Bagi et al., 2008; Beierle et al., 2008). However, little consideration has been given to the effect that these inhibitors may have on normal cells in the tumor microenvironment, such as endothelial cells. We thus investigated the direct effects of FAK inhibitors on various processes important to angiogenesis, namely endothelial cell viability, survival, migration and vessel formation. To this end, we examined the direct effects of two FAK inhibitors, PF-573,228 (PF-228) and FAK Inhibitor 14 (FI14) on primary human endothelial cells. We present results suggesting that both of these FAK inhibitors have direct potent anti-angiogenic activities, and inhibit endothelial cell viability, migration and sprout formation along with the added ability to induce endothelial cell apoptosis in the case of PF-228. Thus, their observed efficacy in tumor models may in part be a result of their ability to potently inhibit tumor-associated angiogenesis.

2. Materials and methods

2.1. Reagents and cells

All chemical reagents were obtained from Sigma (Oakville, ON) or Fisher Scientific (Ottawa, ON) unless otherwise stated. The FAK inhibitors, PF-573,228 (PF-228) and FAK Inhibitor 14 (FI14), both from Tocris Bioscience (Ellisville, MO), were dissolved in dimethyl sulfoxide (DMSO) and then subsequently diluted to the indicated concentrations. Recombinant human vascular endothelial growth factor (VEGF) ($rhVEGF_{165}$; R&D Systems, Minneapolis, MN) was reconstituted according to the manufacturer's instructions. Human umbilical vein endothelial cells (HUVEC; Cambrex/Lonza, Allendale, NJ) were cultured in endothelial cell growth media (Singlequot-supplemented EGM2 media; Cambrex/Lonza) and used from passages 6–10. All cells were grown at 37 °C and 5% CO_2.

2.2. Proliferation/viability assay

HUVEC were seeded at 5×10^3 cells/well in a 96-well plate. The following day, cells were washed once with MCDB-131 (Invitrogen, Burlington, ON) and then incubated in MCDB-131 + 1% FBS containing either PF-228 or FI14 at various concentrations in the presence of 50 ng/ml VEGF. Cells treated with equivalent volumes of DMSO were used as a vehicle control in these experiments. After 72 h, media was removed and replaced with MCDB-131 + 1% FBS + 10% alamarBlue (AbD Serotec, Raleigh, NC). Plates were read (excitation 530 nm/emission 590 nm) using a Fluoroscan fluorescence plate reader (Thermo Scientific, Rockford, IL) 6 h post addition of alamarBlue.