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Glypican-1 nanoliposomes for potentiating growth factor activity in therapeutic angiogenesis

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ABSTRACT

Therapeutic angiogenesis is a highly appealing concept for treating tissues that become ischemic due to vascular disease. A major barrier to the clinical translation of angiogenic therapies is that the patients that are in the greatest need of these treatments often have long term disease states and co-morbidities, such as diabetes and obesity, that make them resistant to angiogenic stimuli. In this study, we identified that human patients with type 2 diabetes have reduced levels of glypican-1 in the blood vessels of their skin. The lack of this key co-receptor in the tissue may make the application of exogenous angiogenic growth factors or cell therapies ineffective. We created a novel therapeutic enhancer for growth factor activity consisting of glypican-1 delivered in a nanoliposomal carrier (a "glypisome"). Here, we demonstrate that glypisomes enhance FGF-2 mediated endothelial cell proliferation, migration and tube formation. In addition, glypisomes enhance FGF-2 trafficking by increasing both uptake and endosomal processing. We encapsulated FGF-2 or FGF-2 with glypisomes in alginate beads and used these to deliver localized growth factor therapy in a murine hind limb ischemia model. Co-delivery of glypisomes with FGF-2 markedly increased the recovery of perfusion and vessel formation in ischemic hind limbs of wild type and diabetic mice in comparison to mice treated with FGF-2 alone. Together, our findings support that glypisomes are effective means for enhancing growth factor activity and may improve the response to local angiogenic growth factor therapies for ischemia.

1. Introduction

Peripheral arterial disease (PAD) affects over 200 million people worldwide and is estimated to afflict approximately 16% of the general population over 65 years of age. Severe PAD has serious consequences for patients including the formation of non-healing ulcers, pain from intermittent claudication and, ultimately, increased risk for limb amputation. The current clinical treatments for PAD include surgical revascularization with bypass grafting or endartectomy, or percutaneous interventions such as angioplasty, stenting and atherectomy. However, these procedures cannot be performed in a significant portion of patients and many do not respond to these therapies [1]. An alternative approach for treating ischemic disease is to stimulate the body to create new vasculature to restore blood flow through its own regenerative processes. Several approaches have been explored to this end including the delivery of progenitor cells [2], viral vectors to express growth factor/angiogenic transcription factor genes [2–11] or through the delivery of growth factors [3,12,13]. Growth factors as protein therapeutics for ischemia have potential advantages from regulatory, production and delivery perspectives. However, in practice angiogenic growth factor therapies, both through delivered proteins and genes, have led to disappointing results in clinical trials [14]. Thus, while the concept of therapeutic angiogenesis has great

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promise, there are no current treatments that are capable of stimulating neovascularization in the context of human ischemic disease.

The presence of diabetes increases the risk of developing PAD by two-fold and increases the rate of progression of the disease [1]. Diabetic patients are 5–10 fold more likely to need limb amputation due to PAD [15]. The disease processes that drive development of ischemia may also induce disruptions in the pathways that are critical to mounting an effective angiogenic response to growth factor therapy. Insulin resistance is a hallmark of diabetic disease and, similarly, long-term ischemic vascular disease in aged humans may also represent a state in which the body can no longer respond effectively to growth factors such as FGF-2 and VEGF in ischemic tissues. We have recently shown that many of the heparan sulfate proteoglycans (HSPGs) that serve as co-receptors for the FGF-2 and VEGF families of growth factors are expressed at lower levels in ob/ob mice than wild type (WT) mice [16]. In addition, heparanase expression is increased in cells treated with fatty acids and in animals on a high fat diet [17–20], in atherosclerotic plaques [20–22] and following stenting or vascular injury [23,24]. Heparanase is an enzyme that can degrade the heparan sulfate chains on cell surface proteoglycans [25,26], ultimately leading to enhanced surface shedding of these molecules and loss of their activity as co-receptors for growth factor signaling [27,28]. Thus, many disease states and pathophysiological processes lead to the loss of HSPGs from the cell surface.

In this study, we have examined the expression of the cell surface proteoglycan glypican-1 in disease and explored its use as a therapeutic enhancer for angiogenic growth factor delivery. The glypicans are distinguished from other cell surface HSPGs by their linkage to the membrane through a glycosylphosphatidylinositol (GPI) anchor. This GPI linker enables the phospholipase mediated-shedding of glypicans and drives preferential localization of the protein in cholesterol-rich lipid rafts [29]. These properties allow glypicans to associate with calveolae [30], control endocytosis/recycling and transcellular transport [31–34], regulate the formation of morphogen gradients [35,36] and facilitate growth factor cell signaling [37–40]. Glypican-1, in particular, is highly expressed in gliomas and their associated vasculature [41,42]. A hallmark of gliomas is vigorous angiogenic response to the tumor that drives neovascularization through multiple mechanisms [31–34]. Glypican-1 has a key role in the growth, metastasis and angiogenic properties of gliomas [41–44]. In addition, glypican-1 is the most prevalent member of the glypican family expressed in endothelial cells and the vascular system [45]. It can act as a co-receptor/modulator for many angiogenic factors including members of the FGF and VEGF growth factor families [41,45–48]. Several studies also have shown that glypican-1 can stimulate cell cycle progression in endothelial cells [44,49]. In this work, we show that glypican-1 is lost in the blood vessels of skin samples from human patients with type 2 diabetes. We developed a novel therapeutic that consists of glypican-1 embedded in a nanoliposomal carrier (a “glypisome”) that can be locally delivered to ischemic tissues from an alginate gel to potentiate the angiogenic response to localized growth factor therapies in diseased tissues. We demonstrate that this therapeutic enhancer improves the *in vitro* activity of FGF-2 and VEGF in endothelial cells through multiple mechanisms. When locally delivered from an alginate gel, glypisomes also enhance the therapeutic potential of FGF-2 therapy leading to increased revascularization of ischemic limbs in wild type and diabetic mice.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in MCDB-131 media with 7.5% FBS, EGM-2 supplements (Lonza), L-glutamine and antibiotics. Endothelial cells that were used for the experiments did not exceed passage six. HeLa cells (ATCC) were cultured in high glucose DMEM with 10% FBS, L-glutamine and antibiotics. For lentiviral production, HEK293T cells were cultured in DMEM with sodium pyruvate, 10% FBS, L-glutamine and antibiotics.

2.2. FGF-2 trafficking and Rab co-localization assays

Constructs for expressing GFP-Rab5, GFP-Rab7 and GFP-Rab11 were as described previously [50,51]. A construct to express GFP-Rab11 was created using similar methods. For studying the effect of glypisomes on endosomal trafficking, HEK 293 cells were plated on glass bottom dishes and transfected with the constructs using lipofectamine 2000 (Life Technologies, inc.). We conjugated Alexa Fluor 594 to FGF-2 while it was bound to heparin column to prevent loss of activity as described previously [52]. Twenty-four hours after transfection, 27 ng/ml of labeled FGF-2 was added. The cells were fixed at various time points after the addition of FGF-2 and then imaged using a laser scanning confocal microscope (LSM 710; Zeiss). The percentage Rab-positive endosomes that also contained FGF-2 was quantified using 10 cells at each time point (all Rab-positive endosomes were counted for each cell). The total fluorescence intensity and nuclear intensity of FGF-2 was calculated by selecting the whole cell or DAPI-stained nucleus and measuring intensity in exposure normalized images using Photoshop software (Adobe).

2.3. Cell proliferation assay

Endothelial cells were passaged into a 96-well plate and cultured in low serum media with 2% FBS for 24 h. Glypisomes and/or growth factors were then added to cells. After 24 h, BrdU was added to the cells and proliferation was assessed 12 h later using a colorimetric BrdU assay (Cell Signaling, Inc.). For the temporal bead release study 27,000 endothelial cells/well were plated in a 12 well plate and an alginate bead containing either FGF-2 or glypisomes was added to the well, at each time point the number of cells were counted using a hemocytometer and normalized to the initial cell count.

2.4. Wound healing cell migration assay

Endothelial cells were grown to confluence and then cultured in low serum media (2% FBS) for 24 h. A cell scraper was used to create a wound in each well. The glypisomes were added with FGF-2 (10 ng/ml) or VEGF-A (10 ng/ml) immediately after wounding of the monolayer. The wounds were imaged along their entire length using an inverted, phase-contrast microscope with digital camera immediately after wounding and six hours later. The average gap was calculated using Metamorph software (Molecular Devices) the rate of wound closure was calculated by taking the difference between the two measurements.

2.5. In vitro angiogenic differentiation assay

Multi-well culture plates were coated with growth factor depleted Matrigel (BD Biosciences) and allowed to gel for one hour at 37 °C. In each well, endothelial cells were plated at a concentration of