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Research Article

Glipizide suppresses embryonic vasculogenesis and angiogenesis through targeting natriuretic peptide receptor A

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ABSTRACT

Glipizide, a second-generation sulfonylurea, has been widely used for the treatment of type 2 diabetes. However, it is controversial whether or not glipizide would affect angiogenesis or vasculogenesis. In the present study, we used early chick embryo model to investigate the effect of glipizide on angiogenesis and vasculogenesis, which are the two major processes for embryonic vasculature formation as well as tumor neovascularization. We found that Glipizide suppressed both angiogenesis in yolk-sac membrane (YSM) and blood island formation during developmental vasculogenesis. Glipizide did not affect either the process of epithelial to mesenchymal transition (EMT) or mesoderm cell migration. In addition, it did not interfere with separation of smooth muscle cell progenitors from hemangioblasts. Moreover, natriuretic peptide receptor A (NPRA) has been identified as the putative target for glipizide's inhibitory effect on vasculogenesis. When NPRA was overexpressed or activated, blood island formation was reduced. NPRA signaling may play a crucial role in the effect of glipizide on vasculogenesis during early embryonic development.

Introduction

In embryos, blood vessels form through two major processes, vasculogenesis and angiogenesis. During the initial stages of embryogenesis, endothelial cell precursors differentiate and assemble into the first polygonal network known as the primary vascular plexus by the process of vasculogenesis. The primary vascular plexus is then pruned and remodeled through the process of angiogenesis by sprouting and recruiting mural cells to establish mature blood vessels. Many of the vascular events that occur during embryonic development may be recapitulated in the adult during neovascularization in both physiological situations such as female reproductive cycle and pathological process of various diseases such as solid tumors [1,2].

The concept of inhibiting tumor neovascularization has been proposed as a valid strategy for antitumor treatment [3,4]. Tumor growth, invasion and metastasis require the development of a

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new vascular supply, which ensures the delivery of oxygen, nutrients, and growth factors. Angiogenesis is provoked early during tumor progression and occurs in response to environmental cues, in particular hypoxia, which regulates the expression of angiogenic factors [5,6]. Correspondingly, almost all the proteins engaged in the regulation of developmental angiogenesis are likewise involved in tumor vascularization in mouse models [7]. Meanwhile, some pro-angiogenic factors can stimulate both vasculogenesis and angiogenesis, while angiogenesis inhibitors can often inhibit angiogenesis as well as vasculogenesis [8]. Therefore, studies on developmental vasculogenesis and angiogenesis would provide important clues for therapeutic control of tumor neovascularization [9].

Chick embryo models have been useful tools for angiogenic studies due to the adaptability to manipulate and the similarity to mammalian embryos at early stages [10]. The chick embryo chorioallantoic membrane is a specified, highly vascularized extra-embryonic membrane and thus serves as an ideal indicator of the anti-angiogenic or pro-angiogenic properties of tested compounds. The chick embryonic yolk-sac membrane (YSM) is another extra-embryonic structure and is also the first site where angioblasts develop into blood vessels. Previous findings indicate that chick area vasculosa capillaries on the yolk-sac membrane share similar structural and growth characteristics to those associated with tumor vascularization, thus making the chick embryo YSM another useful model system for assessing the effects of putative angiogenic inhibitors and promoters in pathological angiogenesis [11]. A variety of angiogenic factors, including both inhibitory and stimulatory factors, from growth factors to antibiotics, have been discovered using bioassay systems based on the early chick extra-embryonic membranes [12,13]. In fact, vasculogenesis occurs earlier than angiogenesis at the site of yolk-sac. In the developing chick embryo, vasculogenesis involves the differentiation and migration of mesodermal cells to form the blood islands during gastrulation and the formation of primary capillary plexuses from angioblasts at the site of blood islands. Vascular endothelial (VE)-cadherin, an endothelial-specific cell–cell adhesion protein localized at junctions, plays a key role in multiple aspects of endothelial cell biology [14].

Glipizide is a second-generation sulfonylurea that stimulates pancreatic β cells to release insulin. Glipizide has recently been discovered to exhibit anti-angiogenic activities in the chick chorioallantoic membrane model and tumor-bearing mouse model [15]. In the present study, we investigated the effect of glipizide on early chick embryonic vascular development. The effect of glipizide on the yolk-sac blood vessel formation was determined using the YSM model and its impact on blood island formation during vasculogenesis was evaluated subsequently using VE-cadherin expression as an indicator of blood islands.

Along with the functions in relation to blood pressure regulation, atrial natriuretic peptide (ANP) and its receptor natriuretic peptide receptor A (NPRA)/guanylyl cyclase-A have recently been demonstrated to interrupt vascular endothelial growth factor (VEGF) signaling to angiogenesis and exhibit an inhibitory effect on cell growth and proliferation [16,17]. Our previous studies have found that the anti-angiogenic effect of glipizide is associated with up-regulation of NPRA. Therefore, we manipulated NPRA gene to investigate whether it could affect the vascular development in early chick embryo. In addition, ANP and a cell permeable analog of cyclic guanosine 3′, 5′-monophosphate (cGMP), were examined for their effects on the formation of blood islands. In this context, we establish that glipizide exerts its inhibition on vasculogenesis in early embryonic development through targeting ANP/NPRA/cGMP signaling pathway.

Materials and methods

Chick embryos

Fertilized leghorn eggs were acquired from the Avian Farm of South China Agriculture University (Guangzhou, China) and incubated in a humidified incubator (Boxun Medical Instruments, Shanghai, China) at 38 °C and 70% humidity until the desired developmental stage of chick embryos [Hamburger and Hamilton (HH)] is reached [18].

Gene transfection and RNA interference

HH2-3 chick embryos were prepared for early chick culture according to methods described by Chapman et al. [19]. Plasmid *NPRA*-pMES, for over-expressing NPRA, and *NPRA*-shRNA-pMES, for silencing NPRA expression, were purchased from Open Biosystems (now GE Healthcare, Lafayette, CO, USA). HH3 chick embryos were transfected with the plasmids carrying *GFP* reporter gene (pGEFP-N1, TaKaRa Bio, Shiga, Japan), *NPRA*-pMES or *NPRA*-shRNA-pMES by electroporation. Fast Green FCF (10 μg/ml, F-7252; Sigma-Aldrich, St. Louis, MO, USA) was added 1:10 to visualize injection of the construct. Briefly, approximately 2 μg plasmid DNA was microinjected in the space between the vitelline membrane and the epiblast of chick embryos during gastrulation. Electroporation was conducted on an electroporator (BTX-ECM399, Harvard Apparatus, Holliston, MA, USA) using a pair of parallel platinum electrodes at the previously described settings [20]. After electroporation, the chick embryos were incubated to desired stage for further manipulation. During incubation, transfection efficiency was directly assessed by the fluorescence of GFP from the pMES vector.

Tissue transplantation

The transfected embryos were incubated for 5 h before tissue transplantation experiment. The GFP labeled primitive streak tissue, roughly 200 × 200 μm, was excised from the posterior region of a transfected donor embryo and grafted into the same position of same-stage untransfected host embryos (HH3). After transplantation, embryos were incubated until the desired stage, and then photographed and fixed for in situ hybridization and immunofluorescent staining.

In situ hybridization

Whole-mount in situ hybridization of chick embryos was performed essentially as standard protocol. Digoxigenin-labeled riboprobes were generated using sequences for *VE-cadherin, VEGFR2*, and *NPRA* (Open Biosystems) fragment for antisense and sense probes subcloned into pSPT18 (Roche, Penzberg, Germany) using the PCR primer pairs 5′-GAATTCAGTGCCACCATCTTTTTCAGTG-3′ and 5′-GTCGACATAGTCTCCAATCTGCTCCA-3′. The whole-mount stained embryos were photographed and then frozen sections