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Original article

Targeting expression of adenosine receptors during hypoxia induced angiogenesis – A study using zebrafish model

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ABSTRACT

Hypoxia is known to be a major player during pathological angiogenesis and adenosine as a negative feedback signaling to maintain oxygen delivery in pathological ischemic condition. We mimicked hypoxic condition and studied angiogenesis by inducing adenosine receptors using forskolin, a plant compound and NECA analogue of adenosine using zebrafish model. Vascular endothelial growth factor (VEGF) is known to play a key role during pathological angiogenesis and regulated by the factors HIF1a under hypoxic condition and recently Notch is proposed to play a negative feedback loop mechanism along with VEGF signaling but the role of adenosine receptor during the process is not known. We evaluated the mRNA expression of adenosine receptors (A1, A2a.1, A2a.2, A2b), HIF1a, VEGF A, VEGF R2, NRP1a, NOTCH 1a and DLL4 and the phenotypic variations of zebrafish embryos when treated with DAPT, γ-secretase inhibitor of Notch in addition to treating the embryos with SU5416, a VEGF receptor inhibitor. Upregulation of adenosine receptors (A1, A2a.1, A2a.2, A2b), HIF1a, VEGF A, VEGF R2, NRP1a, NOTCH1a and DLL4 was observed embryos were when treated with forskolin and NECA could possibly mimic hypoxic condition. Hatching and heart rate also increased with NECA and forskolin. SU5416 showed decreases in blood vessel formation and decreased adenosine receptors, VEGF, VEGFR2, HIF1a and NRP1a expression and DAPT, exhibited decreases in blood vessels and decreased NRP1a, NOTCH1a, DLL4 expression. These embryos developed with poor vasculature, tail bending, abnormal phenotypes and developmental delay. Forskolin treated with inhibitors showed increased blood vessel formation, normal phenotype, development and adenosine receptors (A1, A2a.1, A2a.2, A2b), HIF1a, VEGF A, VEGF R2, NRP1a, NOTCH 1a and DLL4 gene expression suggesting that adenosine mediated Notch and VEGF could play an important role during development and angiogenesis. Targeting VEGF and Notch signaling with adenosine receptors inhibitors which might have a therapeutic significance during hypoxia and abnormal angiogenesis.

1. Introduction

The blockade of abnormal angiogenesis in pathological conditions such as diabetes, tumor is gaining lot of significance during drug development. Abnormal angiogenesis was observed under numerous conditions such as solid tumour growth, diabetic retinopathy, psoriasis and rheumatoid arthritis [1–3]. Angiogenesis proceeds with capillary formation and initiation of sprouting in response to diverse cytokines and metabolic stimulus. Vascular Endothelial Growth Factor (VEGF), an important regulator of normal and pathological angiogenesis [4]. It is released immediately after the angiogenic stimuli and signals vascular endothelial cells proliferation and migration to form new capillary tubes. More recently, the interactions between Notch signaling pathway and VEGF have been described during angiogenesis as VEGF is known to induce expression of Notch receptors and ligands in a gradient dependent manner [5]. Nude mice study with targeted deletions of NOTCH1 and DLL4 resulted in death due to defects in proper formation of angiogenic vascular remodeling failure. Notch was reported to be in feedback loop link with VEGF, that upregulates DLL4 by VEGF in development and angiogenesis [6]. VEGF is highly regulated by hypoxia through adenosine receptors and HIF1a, which can react with hypoxia response elements and induce transcriptional activity. Therefore VEGF is focused as a potential target for treatment of various tumors and angiogenic disorders under hypoxic condition [7,8].

Adenosine receptors are proposed to play proangiogenic role in vascular and immune cells within microenvironment of hypoxic tissues to maintain tissue oxygenation in chronic ischemic condition [9]. Adenosine also stimulates the production of angiopoetin-1, VEGF and Interleukin-6 (IL6) via adenosine receptor on vascular cells that may contribute to angiogenesis [3,10,11]. It is also fairly well established that other growth factors such as tissue growth factor-β (TGF-β) [12], epidermal growth factor (EGF) [13] and platelet-derived growth factor

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BB (PDGF-BB) activates VEGF expression [14] to stimulate angiogenesis to supply oxygen and nutrition for wound healing, developing tumour and tissue regeneration. However, the specific function of adenosine receptor and its isoforms are not known [4,15]. Numerous studies have found that 5′-N ethylcarboxamidoadenosine (NECA) promotes a response similar to hypoxia by inducing adenosine receptor, an agonist that significantly increases intracellular cAMP levels and VEGF [16,17]. Also plant extract diterpene Forskolin (FSK), a potent and unique activator of adenylyl cyclase, enhanced various endothelial events, including angiogenesis by elevating the intracellular cAMP level and adenosine receptors. These processes were mediated by modulating adenosine receptor and VEGF expression in angiogenic pathways [18]. Hence we have used FSK to induce adenosine and its receptor to study role of VEGF and Notch signal pathways during adenosine induced angiogenesis using zebrafish model.

Zebrafish (*Danio rerio*), Indian teleost has excellent utility as a human disease model system. It offers several advantages, including ease of experimentation, drug administration, rapid development, optical transparency and amenability to *in-vivo* genetic manipulation indeed when compared to other vertebrate model system [19,20]. This is an excellent model for studying angiogenesis, since genetic studies have revealed conservation of the molecular pathways between teleost and mammals [20]. The pattern of angiogenesis is simple and at molecular level angiogenesis in zebrafish is similar to other vertebrates [21]. Recently, zebrafish embryonic models have been developed to dissect the detailed events of hypoxia-induced tumour cell invasion and metastasis in association with angiogenesis under normoxia or hypoxic conditions [22,23]. The results are found to have positive impacts on embryos by altering the angiogenesis patterns during hypoxic condition suggesting that zebrafish is a predictive model for testing angiogenesis modulators.

Although it is known that the Notch and VEGF signaling pathways are both involved in normal and pathological angiogenesis, little is known about crosstalk between them and the role of adenosine receptor in this process is not known. Hence, we investigated whether adenosine receptors when induced non-selectively due to FSK could mimic hypoxia in zebrafish and this could be used to understand gene expression during pathological condition. We used SU5416 (VEGF signaling inhibitor) and DAPT (Notch signaling inhibitor) to evaluate the expression of angiogenic factors such as adenosine receptors (A1, A2a.1, A2a.2, A2b), HIF1a, VEGF A, VEGF R2, NRP1a, NOTCH1a and DLL4 using zebrafish embryos at 48 and 72 h post fertilization (hpf). Phenotypic changes were recorded and major blood vessel like Intersegmental Vessels (ISV) were stained using alkaline phosphatase and red blood cell (RBC) staining techniques. VEGF signaling in association with Notch could be a determinant factor in the angiogenic process and hence its ligand, receptors, co-receptors mRNA expression have been evaluated in the present study.

2. Materials and methods

2.1. Zebrafish maintenance and embryo collection

Adult wild type zebra fish (*Danio rerio*) both male and female (6 months old) were obtained from local suppliers at Chennai, India. These were maintained at 28 ± 0.5 °C on a 14 h light/10 h dark cycle in 40 l glass tanks and kept at 2:1 ratio of male to female. Flake foods were obtained from local suppliers and fed during normal and during breeding period, fish were fed with live blood worms. The tank water was recycled regularly at fortnightly/weekly and continuously aerated with aquarium pumps.

Minimum 5 days prior to spawning, male and female were housed separately. Previous day before eggs requirement, 6 males and 3 females were placed in breeding tanks for natural spawning and equipped with as appropriate mesh size for eggs to be collected at the bottom of the tank. Overnight, fish were undisturbed and one hour after turning on light in next morning, eggs were collected. The eggs were washed 3–4 times with egg water to remove debris prior to starting of experiment to avoid contamination and then rinsed thrice with embryos medium. Embryos medium (Hanks medium) was prepared as per standard protocol of Kimmel et al. [24] along with 0.003% 1-phenyl 2-thiourea (PTU) for culturing embryos. Embryo stages were given as hours post fertilization (hpf).

2.2. Animal treatment

Forskolin (7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-on), DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), SU5416 (1,3-Dihydro-3-[(3,5-dimethyl-1H-pyrrol-2-yl)methylene]-2H-indol-2-one) and NECA (5′-N ethylcarboxamidoadenosine) were purchased from Sigma Aldrich, India and dissolved in 0.1% DMSO. Healthy embryos were dechorionated by enzymatic digestion with 1 mg/ml protease for 5–10 min at room temperature and then washed thrice in embryo medium. Around 30 embryos were treated with various concentrations directly to the 3mL embryo media during 22 hpf in a 6 well culture plate till 72 hpf.

Scoring system was developed for the range-finding experiment and compared with normal development of a zebrafish embryos up to 72 hpf as described by Kimmel et al. [24]. Experimental embryos were compared with reference embryos in the scoring matrix dependent on its stage of development. All deviations for instance defect in notochord formation, will result in a lower point score which corresponds to certain morphological defects. From this scoring matrix Concentration-Response curve has been plotted statistically (Data not shown) to derive LD50 and EC50 value of FSK. During range-finding study of FSK, embryos were maintained in embryo medium without PTU till 72 hpf.

2.3. Visual screening and photography

Embryos were visually inspected for viability, gross morphological defects, heart rate at 48hpf and 72 hpf. Control and treated embryos were examined using compound microscope [Euromax (4× and 10×)] at 48 hpf and 72 hpf and images were collected and stored using a digital camera and image acquisition software-Image J focus attached to a computer. The observed phenotype frequencies in each dose were entered after deducting dead embryos and embryos with normal phenotypes (multiple malformations were observed in same concentration on few sample). Scoring system have been used to study gross morphological defects and all the experiments were performed in triplicates and statistical analysis was performed.

2.4. Alkaline phosphatase (ALP) staining

Embryos after 72 hpf were washed with PBS 3–4 times and fixed in 4% paraformaldehyde to study the formation of Intersegmental vessel (ISV) as mentioned [25]. Prefixed embryos were again washed with Phosphate Buffered Saline (PBS) 3 times and dehydrated by immersing in 25%, 50%, 75% and 100% methanol each with 5 min in PBST. Embryos were then equilibrated in 0.1 M Tris-HCl; pH 9.5; 50mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween-20 (NTMT) buffer thrice each with 15 min duration at room temperature. After equilibrating in NTMT, 4.5 µL of 75 mg/mL NBT and 3.5 µL of 50 mg/mL BCIP was added. After staining for 20 min, the reaction was stopped by adding PBS with Tween-20 (PBST). Embryos were then immersed in a solution of 5% formamide and 10% hydrogen peroxide in PBS for 20 min which removed endogenous melanin in the pigment cells and allowed full visualization of stained vessels. It is then examined using compound microscope and photographed. Intersegmental blood vessel (ISV) formations were quantified using Angioquant software. Angioquant Toolbox, MATLAB 6.5 automated image analysis software was used to measure total length and size of blood vessels as fold increase in the length and size of tubule complex. Embryos treated with compounds have been