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An endothelial-cell-enriched primary culture system to study vascular endothelial growth factor (VEGF A) expression in a teleost, the Japanese eel (*Anguilla japonica*)

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Abstract

A partial gene for eel (*Anguilla japonica*) vascular endothelial growth factor (VEGF) has been cloned and an endothelial-cell-enriched primary culture derived from rete mirabile established to study regulation of the expression of the eel VEGF gene. Cells were cultured in M199 medium containing 0.1% fetal calf serum (FCS) and serum-free M199 medium for long-and short-term experiments, respectively. Cells were separately treated with cobalt ions (Co^{2+}), basic fibroblast growth factor (bFGF), and estradiol (E2), which have been demonstrated to stimulate mammalian VEGF A expression, followed by quantification of the VEGF mRNA levels by real-time reverse transcription polymerase chain reaction. Our results show that: (1) the deduced eel VEGF protein encoded by the cloned gene is about 130 amino acids in length, and is closely related to a zebrafish (*Danio rerio*) VEGF A; (2) the endothelial-cell-enriched rete mirabile primary culture containing mainly (over 70%) the capillary endothelial cells; (3) the expression levels of the eel VEGF transcript were increased by Co^{2+}, bFGF, and E2 treatments in a dose-and time-dependent manner. Our data demonstrate that an eel partial VEGF gene has been cloned and its regulation of expression in endothelial-cell-enriched rete mirabile cell culture is similar to that in higher vertebrates.

Keywords: bFGF; Capillary endothelial cell; Eel; Fish; Hypoxia; Nitric oxide; Rete mirabile; Sex steroid; VEGF

1. Introduction

Vascular endothelial growth factor (VEGF) is one of the two potent endothelial mitogens (the other one is basic fibroblast growth factor, bFGF), while only VEGF makes the vascular permeability (reviewed by Ferrara, 2004). These two biological properties, mitogenicity and permeable, are important in the cascade of events leading to angiogenesis (reviewed by Ferrara, 2001, 2004). In humans, alternative splicing of the single VEGF gene results in six different VEGF isoforms, while the roles of individual isoforms have not been fully determined (Bruick and McKnight, 2001). VEGF expression is known to be regulated by oxygen tension (hypoxia), growth factors, and hormones (reviewed by Ferrara and Davis-Smyth, 1997; Ferrara, 2004). VEGF was first isolated from bovine pituitaries (Ferrara and Henzel, 1989). The mRNA of VEGF is detected in tumor cells or tissues, and in certain normal tissues or cells, including macrophages, lung epithelial cells, kidney epithelial cells, follicular cells in the pituitary, corpus luteum cells, and aortic smooth muscle cells (reviewed by Ferrara et al., 1992). Two VEGF receptor tyrosine kinases, flt-1 and flk-1, have been identified (reviewed by Klagsbrun and D'Amore, 1996; Zachary and Gliki, 2001). Recent works have demonstrated that VEGF gene is highly conserved from teleostean fishes (zebrafish and fugu) to mammals in the coding region, while no obvious sequence conservation in the 5′ flanking regions was observed suggesting the appearance of divergent regulatory mechanisms in the course of the evolution (Gong et al., 2004). On the other hand, in invertebrates, VEGF-induced

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neoangiogenesis in leeches (*Hirudo medicinalis*) has been demonstrated (Tettamanti et al., 2003). Invertebrate homologues of VEGFs and VEGF receptors have been also identified in fly, nematode and jellyfish, where they function in developmental cell migration and neurogenesis. The existence of VEGF-like molecules and their receptors in invertebrates without a vascular system suggests that this family of VEGF emerged at a very early stage in the evolution of multicellular organisms to mediate primordial developmental functions (reviewed by Holmes and Zachary, 2005).

Endothelial cells are the cells encircling blood vessels (endothelium), in an adult human: the whole endothelium consists of approximately 1×10^{13} cells forming an almost 1 kg "organ" (Sumpio et al., 2002). Endothelial cells are involved in a wide range of biological processes including reproduction, development, and tumor invasion. For example, angiogenesis (new blood vessel forming), an important process in reproduction and development, begins with the degradation of the basement membrane of the capillary by activated endothelial cells that will migrate and proliferate, leading to the formation of solid endothelial cells sprouts (reviewed by Folkman and Shing, 1992). The majority of angiogenesis seen in vertebrates occurs during embryogenesis (reviewed by Risau and Flamme, 1995), while in adult humans, the proliferation rate of endothelial cells is very low compared with that of other cell types, labeling studies estimated turnover times from 47 to over 23,000 days (Hobson, 1984). One of the physiological exceptions in which angiogenesis is found in the female reproductive system. Thus, the female reproductive system provides a special model to study the regulation of normal angiogenesis (Reynolds and Redmer, 1998; Liekens et al., 2001). On the other hand, an ample evidence demonstrates that a sexual or hormonal bias to cardiovascular abnormalities (reviewed by Mendelsohn and Karas, 1999), so endothelial cells obtained from the female reproductive system may have a certain sexual bias. It seems that, since estrogen receptors have been also identified in the cardiovascular system (Jesmin et al., 2002); capillary endothelial cells derived from non-reproduction systems may be more suitable for studying effects of steroids on the circulation physiology. Indeed, various systems have been established to study capillaries endothelial cells from non-reproduction tissues (reviewed by Bachetti and Morbidelli, 2000). There are many potential applications of capillary endothelial cell cultures, because tumor pathological angiogenesis takes place at the level of capillary endothelium, not aortic endothelium (Folkman et al., 1979). However, HUVEC (human umbilical vein endothelial cells, from a transitory circulatory tissue) is widely used to study the physiology of vascular endothelial cells because they are more closely associated with the microvasculature in term of growth and usually not observed in the large blood vessels (reviewed by Manconi, 2000). However, it is possible that HUVEC may not be real capillary endothelial cells. Studying capillary endothelial cells from the lower vertebrates can give certain hints as to their higher counterparts, since the basic essential roles and/or functions of capillary endothelial cells as well as VEGF are conserved during evolution.

Fish rete mirabile consisting of a rich network of blood capillaries associated with the swim bladder (gas gland) in teleosts is one of the most accessible and cleanest sources of capillaries in vertebrate circulatory systems. Among the physostome teleosts, the eel (*Anguilla* spp.) has particularly well-developed gas gland rete mirabile (Wanger et al., 1987). The capillaries in the eel's rete mirabile are straight unbranched segments which can be as long as 10 mm, are eight times longer than muscle capillaries which are otherwise the longest capillaries in vertebrates (reviewed by Wanger et al., 1987 and references therein). Indeed, the rete mirabile is predominantly composed of endothelial cells (Krogh, 1959, cited by Wanger et al., 1987 and references therein), which provides an excellent mode to study capillary as well as endothelial cell physiology (e.g. Buchanan and Wagner, 1990; Bendayan and Rasio, 1997; Schlezinger and Stegeman, 2000); a cell culture system of capillary endothelial cell from the American eel (*Anguilla rostrata*) rete mirabile has been reported (Garrick, 2000). The author provided a method to increase the purity of endothelial cells either by fibronectin-hyaluronic acid or by *B. simplicifolia* lectin coated matrix while in that paper an application of this system on endothelial cell research is not performed.

The purpose of this work is to validate VEGF expression in an endothelial-cell-enriched primary culture from a lower vertebrate, Japanese eel (*Anguilla japonica*), to understand the control of VEGF expression and capillary endothelial cell physiology during vertebrate evolution. Capillary endothelial cells in rete mirabile primary culture are enriched by chymotrypsin, gelatin matrix and a two-step cell culture procedure. Partial VEGF gene cDNA has been cloned, RT-real-time PCR was employed to assay VEGF expression. The results suggest that VEGF and its expression regulation in eel endothelial-cell-enriched primary culture are similar to those in higher vertebrates.

2. Materials and methods

2.1. Animals

Cultured eels (*A. japonica*) were purchased from aquaculture farms. Their body masses were 550–620 g. The animals were reared in running tap water for no more than two weeks. Gender was not identified prior to sacrifice, GSI (gonadal-somatic index) is below 1% for females and 0.1% for males. Three pairs of rete mirabile from three eels were employed for each in vitro test.

2.2. Chemicals

Gelatin (from cold water fish skin); Trypan blue solution; 0.25% Trypsin-EDTA solution; DAF (4,5-diaminofluorescein diacetate solution); α-chymotrypsin, collagenase; β-estradiol (1,3,5(10)-estratriene-3,17β-diol) were purchased from Sigma-Aldrich, USA. PBS (phosphate buffered saline, pH 7.2, without calcium chloride and magnesium chloride); 7.5% (w/v) Sodium bicarbonate solution; Medium199 (with Earle's