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Developmental changes in amniotic fluid vascular endothelial growth factor levels of chick embryos

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that increases peripheral oxygen delivery by stimulating angiogenesis. VEGF is known to be expressed in several fetus tissues and placenta. VEGF is initially expressed in the yolk sac and in embryonic sites of vessels formation to support the assembly of a cardiovascular system. It is instructive not only for cardiovascular development, but also for the development of organ systems. The amnion forming the amniotic cavity, providing an aqueous environment for the embryo. A wide range of proteins including growth factors and cytokines has been identified in human amniotic fluid (AF). In this study, total protein concentration (TPC) and VEGF level in the amniotic fluid samples from chick embryos were measured using dye-based protein assay and enzyme linked immunosorbent assay (ELISA). TPC decreased from days E6 to E10 and increased from E11 to E15. However, the amount of AF VEGF was increased from day E6 to E11 and thereafter the levels decreased from days E11 to E15. Since VEGF is important in the development of the organ systems, changes in the TPC and VEGF levels in AF during chick embryonic development may be correlated with organ development. We have also concluded that VEGF is a constant component of chick AF.

Keywords: amniotic fluid, vascular endothelial growth factor, concentration, chick.

INTRODUCTION

During pregnancy, the fetus develops within a fluid space surrounded by the amniotic fluid and chorionic membrane. With advancing gestation, the membranes expand and increase in surface area to accommodate the growing fetus [1]. The importance of amniotic fluid (AF) growth factor for normal embryonic growth has been demonstrated in numerous studies [2]. In the human,

epithelial growth factor (EGF), transforming growth factor -α (TGF-α) and hepatocyte growth factor are present in the AF and are thought to play in the development of respiratory system and stomach [3; 4]. In the ship, blocking of AF ingestion by the fetus results in abnormal development of the liver and gastrointestinal tract, and inhibit placental growth [5; 6]. It has been demonstrated that the swallowing of AF growth factors is important in normal foetal development [7]. A wide range of protein has been identified in AF. In humans, there are dynamic temporal patterns with AF total protein concentrations from 7 to 20 weeks of gestation [8]. Foetal swallowing represents the principle mechanism of clearance of AF protein, which is thought to have a half-life of 1 to 2 days in monkeys. Growth factors and proteins present in the AF can be taken up by the foetus [9] and can diffuse through the foetal skin and can be absorbed by the vascularized foetal surface of the placenta [10]. Changes in the concentrations of AF nerve growth factor (NGF) in the chick embryos and IGF-I and IGF-II in avian embryos has been demonstrated [11; 12]. Vascular endothelial growth factor (VEGF) is the most potent direct stimulator of vascular endothelial cell growth. VEGF also induces vascular permeability and its expression is enhanced by hypoxia [13]. VEGF is known to be expressed in the placenta [14] and in several fetus tissues [15]. Two specific endothelial cell receptors, VEGFR-1 and VEGFR-1 are known to bind VEGF and a soluble form of VEGFR-1 has been identified [16; 17]. VEGF is initially expressed at high level in the yolk sac and in embryonic sites of vessels formation to support the assembly of a cardiovascular system. However, VEGF continues to be expressed after the onset of blood vessel formation, consistent with the idea that it is instructive not only for cardiovascular development, but also for the development of organ systems. In the adult, VEGF expression becomes restricted to specialized cell types in organs containing fenestrated endothelium, for example, the kidney and pituitary [18]. Several major growth factors upregulate VEGF, including epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1) and transforming growth factor-α and -β (TGF-α and TGF-β)[19]. Moreover, inflammatory cytokines and hormones have been shown to induce VEGF expression [20].

A wide range of proteins including growth factors has been identified in AF [21]. These proteins can enter the AF from the amniotic fluid cells, fetal urine, and of the foetal secretions that include transduction through foetal skin [22]. As AF is in contact with the embryo, then biochemical modifications in the embryo could be detected in the AF. In this study, total protein concentration (TPC) and VEGF levels in the AF from chick embryos were studied using Bradford dye procedure and enzyme linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Amniotic fluid samples

Fertile white Leghorn eggs were incubated at 38^{o}C in a humidified atmosphere to obtain chick embryos at different stages of development. The amniotic fluid was carefully aspirated using a pulled tip glass microcapillary pipette (Drummond Scientific Company, 20 μL) from incubated chicks embryos from day 6 to day 15 (E6 to E15).

Amniotic fluid for each analysis was collected from 28 chick embryos. The average amount of 0.5 ml amniotic fluid was collected from each embryo. To minimize protein degradation, amniotic fluid samples were kept at 4 °C during collection. Amniotic fluid samples were centrifuged at 15000 rpm at 4 °C for 10 minutes to remove any contaminating cells. The samples

that we used for analysis had no visible sign of contaminating red blood cells that we could detect under the microscope. The supernatant was frozen immediately and stored at -70 ^{o}C until future analysis. Twenty eight samples from each time point were used for analysis of total protein concentration and NGF level.

Total protein and NGF analysis

The total protein concentration of proteins in amniotic fluid was determined by the Bio-Rad protein assay based on the Bradford dye procedure. VEGF in amniotic fluid was measured using the sensitive two-site ELISA and antiserum against chick VEGF. Microtiter plates (Dynatech, Canada) were first coated with 80 ng primary anti-VEGF antibody (Abcam) per well in 0.1 M Tris buffer. After overnight incubation, the plates were blocked with EIA buffer (50 mM Tris pH 7.5, 0.3 M NaCl, 0.1% Triton X-100, 1% BSA and 1% gelatin). The samples and standards were placed in triplicate wells and incubated overnight at room temperature. After washing with phosphate buffered saline (PBS) a biotinylated secondary antibody (7 ng/mL) was added to each well and incubation was carried out overnight at room temperature. β-galactosidase coupled to avidin was then added for 2 hours followed by washing. Finally, 200 µM 4-methylumbelliferyl-β-galactoside (Sigma-Aldrich, Poole, UK) in 50 mM sodium phosphate were added as well as 10 mM MgCl2 buffer and the amount of fluorescence was measured after 50 minutes incubation at 37 ^{o}C using a fluorimeter (Dynatech, Canada).

All animal procedures were carried out in accordance with the Animals, Act, 1986. All values were expressed as mean±standard error of the mean (SEM). In all experiments, a minimum of 28 measurements were made in order to calculate a mean±SEM. Statistical analysis was performed using Student's t test and only values with $P \leq 0.05$ were considered statistically significant.

RESULTS

Total protein concentration

The total protein concentration in AF in embryos aged E6 to E15 was determined by Bio-Rad protein assay. The total protein concentration decreased from day E6 to day E10 and after that the levels increased from E11 to E15 (Figure 1).

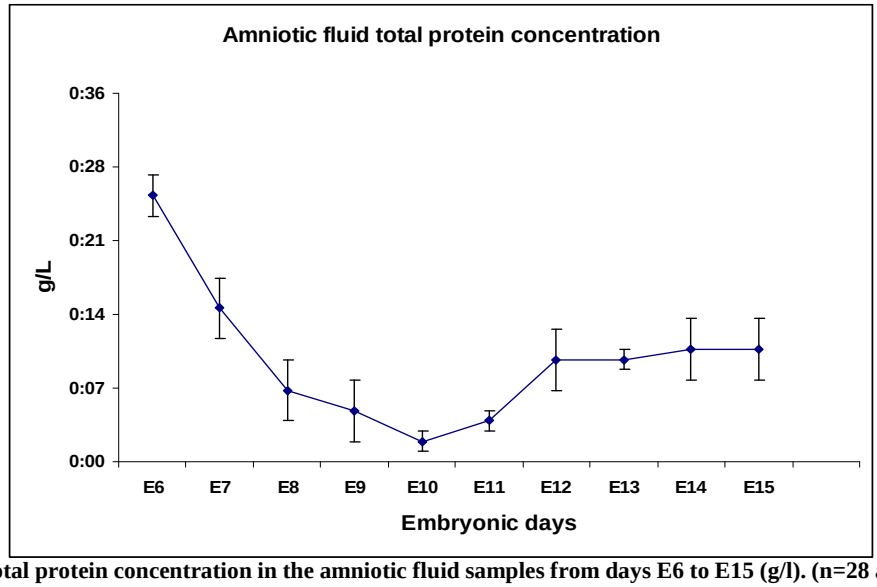

Figure 1. Total protein concentration in the amniotic fluid samples from days E6 to E15 (g/l). (n=28 at each time point)

VEGF concentration

Using ELISA, we have also analyzed VEGF concentrations in the AF samples from chick embryos aged E6 to E15. The amount of AF VEGF was increased from day E6 to E11 and thereafter the levels decreased from days E11 to E15 (Figure 2).

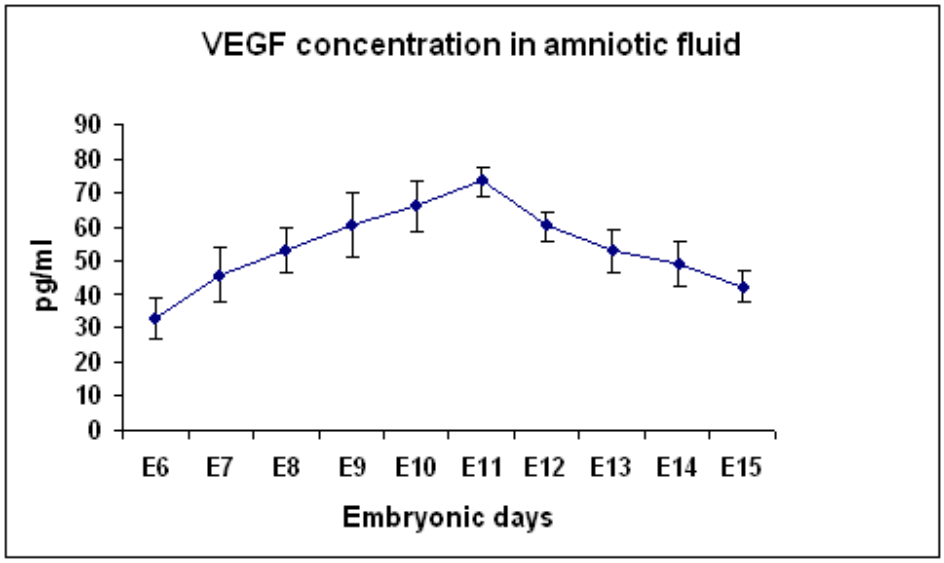

Figure 2. Vascular endothelial growth factor concentration in the amniotic fluid samples from days E6 to E15 (pg/ml). (n=28 at each time point).

DISCUSSION

In this study we investigated the TPC and VEGF levels in the chick AF. We investigated VEGF as it is one of the most important growth factor that plays a key role in the organogenesis including the development of cardiovascular system [23]. It has also been shown that VEGF signaling is important in the development of endoderm-derived organs including liver and pancreas [24].

VEGF is a potent stimulator of cardio- and angiogenesis. The extensive fetal and placental tissue growth of normal pregnancy is characterized by a strong local demand for vascular expansion, but so far little is known about factors regulating VEGF in human pregnancy. VEGF is essential for fetal development because the loss of only one copy of the VEGF gene leads to embryonic lethality [25]. VEGF exerts its biological effects through VEGFR-1 and VEGFR-2. VEGFR-2 is the major mediator of mitogenic, angiogenic and permeability enhancing and endothelial survival properties of VEGF [26]. VEGF was identified in human AF [27].

The amnion, which surrounds the embryo, forming the amniotic cavity, providing an aqueous environment for the embryo. The amnion contains a fluid to protect the embryo from mechanical and thermal shock, possesses antimicrobial activity and contains nutritional and growth factors. A wide range of proteins has been identified in human AF [21]. In humans, these growth factors can enter the AF from the maternal uterine tissues, umbilical cord, fetal urine and other fetal secretions that include transduction through fetal skin [22].

As AF contains growth factors and it has been shown that the swallowing of AF is important in normal fetal development [7], thus, in this study the changes in the total protein concentration (TPC) and VEGF levels have been studied. In this study, we showed that TPC in AF increases from day E6 to day E18. This result is consistent with the finding that human AF TPC rising as pregnancy proceeds [28]. We have also shown that there is a change in AF VEGF concentration during chick embryonic development. AF VEGF levels increases from E10 to E15 and decreased from E11 to E18. VEGF is important in the organogenesis and changes in AF VEGF concentration has been seen in some diseases [29]. Changes in the TPC and VEGF levels during chick embryonic development may be associated with organ development. Since AF is in contact with the developing embryo and the embryo swallow AF during development, changes in the VEGF and TPC in the AF could affect the developing embryo.

CONCLUSION

It is thus concluded that VEGF is a constant component of AF during chick embryonic development and it might also be involved in chick organogenesis.

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