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ORIGINAL ARTICLE

Quantitative analysis of nerve growth factor in the amniotic fluid during chick embryonic development

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Abstract Nerve growth factor (NGF) and most neurotrophic factors support the proliferation and survival of particular types of neurons. Besidesthe pivotal role of NGF in the development of neuronal cells, it also has important functions on non-neuronal cells. The amnion surrounds the embryo, providing an aqueous environment for the embryo. A wide range of proteins has been identified in human amniotic fluid (AF). In this study, total protein concentration (TPC) and NGF level in AF samples from chick embryos were measured using a Bio-Rad protein assay, enzyme linked immunosorbent assay (ELISA) and Western blot. TPC increased from days E10 to day E18. There was a rapid increase in AF TPC on day E15 when compared to day E16. No significant changes in NGF levels have been seen from day E10 to day E14. There was a rapid increase in NGF content on days E15 and E16, and thereafter the levels decreased from day E16 to day E18. Since, NGF is important in brain development and changes in AF NGF levels have been seen in some CNS malformations, changes in the TPC and NGF levels in AF during chick embryonic development may be correlated with cerebral cortical development. It is also concluded that NGF is a constant component of the AF during chick embryogenesis.

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1. Introduction

Nerve growth factor (NGF), originally identified as a neurite-promoting factor in peripheral sensory and sympathetic neurons, has been shown to function in the central nervous system (Spranger et al., 1990; Chiaretti et al., 2008). NGF, discovered almost half a century ago, is the founding and best-characterized member of neurotrophin family (Levi-Montalcini, 1987; Chao, 1992). The biological function of NGF is the maintenance and survival of the nervous system. Besides the pivotal role of NGF in the development of neuronal cells, it also has important functions on non-neuronal cells. For example, it is

an autocrine survival factor for memory B lymphocytes (Torcia et al., 1996).

The neurotrophins represent a family of structurally and functionally related, homodimeric proteins, including NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4, NT-4/5 and NT-6 (Götz et al., 1994). NGF exerts its differentiating and proliferation effects on the CNS during embryonic development. Some non-neuronal cell types can release NGF in a paracrine/autocrine fashion (Jiang et al., 1997).

Biological actions of NGF are mediated by two distinct receptors, the high-affinity $trkA^{NGFR}$ receptor and the low-affinity $p75^{NTR}$ receptor (Yano and Chao, 2000). It has been shown that NGF exerts indirect pro-angio-genetic effects mediated by specific angiogenic factors in the developing CNS (Calza et al., 2001) and that it induces increased expression of inflammatory markers in skin vessels (Raychaudhuri et al., 2001).

The amnion, which is the inner membrane, surrounds the embryo, forming the amniotic cavity, providing an aqueous environment for the embryo. The amnion contains a fluid to protect the embryo from infection, mechanical injury, and adhesion. The chick begins imbibing the amniotic fluid (AF) around day 13 of incubation and continues until day 19 of incubation. Hence, the embryo is exposed to and swallows the fluid containing proteins, minerals, water, hormones and any other nutrients needed for growth and development (Karcher et al., 2005).

AF is crucial to fetal health because it forms a protective sac around the infant that prevents mechanical and thermal shock, possesses antimicrobial activity, assists in acid/base balance, and contains nutritional factors. A wide range of proteins has been identified in human AF (Burdett et al., 1982). In human, these proteins can enter the amniotic fluid from the maternal uterine tissues, umbilical cord, amniotic fluid cells, fetal urine, meconium, and other fetal secretions that include transudation through fetal skin (Jauniaux et al., 1998). There is also a dynamic temporal pattern, with AF total protein concentrations rising from 7 to 20 weeks gestation (Benzie et al., 1974).

In this study, the total protein concentration (TPC) in chick amniotic fluid was determined by the Bio-Rad protein assay based on the Bradford dye-binding procedure. The presence and level of NGF in chick amniotic fluid was measured by enzyme linked immunosorbent assay (ELISA) and Western blot.

2. Material and methods

2.1. Amniotic fluid samples

Fertile white Leghorn eggs were incubated at 38 °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 chick embryos from day 10 to day 18 (E10–E18).

Amniotic fluid for each analysis was collected from 28 chick embryos. The 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 15,000 rpm at 4 °C for 10 min 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 °C until future analysis. Twenty eight samples from each time point were used for analysis of total protein and NGF concentration.

2.2. Total protein and NGF analysis

The total protein concentration of proteins in the amniotic fluid was determined by the Bio-Rad protein assay based on the Bradford dye procedure. NGF in amniotic fluid was measured using the sensitive two-site ELISA and antiserum against chick NGF. Microtiter plates (Dynatech, Canada) were first coated with 80 ng primary anti-NGF 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 (8 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 h 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 $MgCl_2$ buffer and the amount of fluorescence was measured after 50 min incubation at 37 °C using a fluorimeter (Dynatech, Canada).

For Western Blot analysis, aliquots of amniotic fluid from the embryos were mixed with a sample buffer containing 3.2% SDS, 15% glycerol, 2.8 M β-mercaptoeyhanol and 0.0015% bromophenol blue. Samples were applied to a 5–20% gradient SDS–PAGE gel (Bio-Rad) according to Lamely and the proteins obtained were transferred to nitrocellulose sheets, pore size 0.45 μm (Bio-Rad). After incubation for 2 h at room temperature in the blocking solution (PBS containing 5% skimmed milk), the nitrocellulose sheets were exposed overnight, at 4 °C, to anti-NGF monoclonal antibody and identified with a peroxidase-labeled mouse IgM PK 4010 Vectastain Avidin Biotin complex kit (Vectorlab.). The peroxidase activity was revealed with diaminobenzidine (0.5 mg/ml in PBS with 0.02% hydrogen peroxide).

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 $P \leqslant 0.05$ were considered statistically significant.

3. Results

3.1. Total protein concentration

The total protein concentration in AF in embryos aged E10–E18 was determined by Bio-Rad protein assay. The total protein concentration increased from day E10 to day E18. There was a rapid increase in AF TPC on day E16 when compared to day E15 (Fig. 1).

3.2. NGF concentration

The presence of NGF in the AF samples was shown using Western blot (Figs. 2 and 3). Using ELISA, we have also