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ARTICLE

Regulation of trophoblast migration and survival by a novel neural regeneration peptide

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Ambika Singh graduated with a Masters in Science (first class Honours) in 2006 from the University of Auckland, New Zealand. Her project involved studying the novel functions of a recently discovered neuroprotective peptide in human placental development during term pregnancy. She is currently in the final stages of her PhD investigating the role of sphingolipid compounds in the regulation of trophoblast differentiation, at the University of Western Australia, Australia.

Abstract Although placental trophoblast migration is tightly controlled in an autocrine/paracrine manner, the nature of chemoattractive factors facilitating and directing this biological activity remains largely elusive. Neural regeneration peptides (NRP), a recently discovered peptide family, stimulate neuronal migration, differentiation and survival of post-natal neurons within the murine central nervous system. Based on the neural-repair related activities of these peptides and parallels between neuronal and placental cell behaviour patterns, this study postulated that they play a role in placental development, in particular trophoblast migration and survival and investigated the role of a newly discovered NRP motif (NNZ-4920), which exhibits about 70% homology to the mouse NRP motif sequence and is homologous to a 13-mer fragment within the N-terminus of human *CAPS2*, in trophoblast migration and survival regulation. NNZ-4920 significantly enhanced trophoblast migration by 51% ($P < 0.01$) compared with controls and protected against stress induced by serum withdrawal and tumour necrosis factor-α/interferon-γ treatment, at femtomolar concentrations, with efficacy similar to epidermal growth factor. *CAPS2* expression was detected in purified term trophoblast and decidual cells. In conclusion, the placenta may be a source of NRP-related gene expression. Its encoded peptide products exert biological effects on term trophoblast migration and survival *in vitro*.

KEYWORDS: NRP, placenta, trophoblast migration, trophoblast survival

Introduction

During embryonic brain development, proliferative zones are formed where neuroblasts are generated. Proliferating cells subsequently migrate to their destined positions, where they differentiate into physiologically active neurons. A recently described factor, termed neural regeneration peptide (NRP), has been shown to effectively enhance murine neuroblast survival, proliferation, migration and differentiation at femtomolar concentrations (Gorba et al.,

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2006). Its effects were blocked by administration of a neutralizing antibody against the chemokine receptor CXCR4, implying it may exert its actions via this plasma membrane receptor. The novel human-derived synthetic peptide NNZ-4920 (H-REGRRAAPGRAGG-NH_2) exhibits 69.5% homology to the mouse orthologue NRP sequence (a fragment from the 135-amino-acid NRP encoded by the *Nrp* gene (GenBank NM_001013372) (Gorba et al., 2006). NNZ-4920 represents the amino acid residues 38—50 within the N-terminus of the human protein calcium-dependent activator protein for secretion (CAPS-2), which is strongly expressed in the brain and endocrine cells. *CAPS2*, one of three CAPS gene isoforms, displays differential spatiotemporal expression to that of *CAPS1* in adult and fetal tissues (Cisternas et al., 2003), indicating differential cellular function of these two molecules despite sharing more than 93% homology (Sadakata et al., 2004). The only major site of non-homologous resides is in their N-terminal regions i.e. the NNZ-4920 location. Neuronal studies have demonstrated CAPS2-mediated enhanced survival of Purkinje cells by promoting a depolarization-induced release of neurotrophins, such as neurotrophin 3 and brain-derived neurotrophic factor (Sadakata et al., 2004). Interestingly, the NNZ-4920 peptide portrays strikingly similar chemoattractive characteristics to stromal cell-derived factor 1/chemokine (C-X-C motif) ligand 12 (SDF1/CXCL12), the primary agonist of chemokine (C-X-C motif) receptor 4 (CXCR4) (Gorba et al., 2006).

The placenta expresses many peptides and receptors that are traditionally thought of as being neuronal in terms of origin and function. However, although the profile and expression may be similar, the function and regulation of these peptides often varies considerably. Similar to embryonic brain development, trophoblasts undergo continuous proliferation before migrating to their zone of maturation/differentiation (Mayhew and Barker, 2001). In addition, placental trophoblasts and neuronal cells both exhibit CXCR4 and SDF1 responsiveness. The factors and processes involved in the regulation of human placental development are complex and only partially understood. Trophoblast function is stringently regulated in an autocrine/paracrine manner by growth factors, binding proteins and matrix constituents such as proteoglycans. Disordered decidual—trophoblast interactions have been implicated in spontaneous early pregnancy miscarriages, while insufficient trophoblast invasion of spiral arteries has been closely linked with pregnancy complications such as pre-eclampsia and intrauterine growth restriction (Brosens et al., 1972; Hustin et al., 1990; Robertson et al., 1975; Smith et al., 1997).

Interestingly, *CAPS2* expression has been detected in the placenta (Cisternas et al., 2003), although the specific cell types expressing this gene in the placenta remain unknown. Based on the known activities of NRP, its localized expression within regions of elevated cell migratory activity and analogies between neuronal and placental cell morphology during migratory movement and *CAPS2* expression, this study postulated that NNZ-4920 may display chemoattractive and survival properties in trophoblast cells. To assess the ability of the human placenta to produce NRP-like peptides, expression of *CAPS2* was also investigated.

Materials and methods

The following reagents were purchased from commercial sources: DNase I and interferon γ (IFNγ) (Roche Diagnostics, Basel, Switzerland); Percoll (Amersham Biosciences, New Jersey, USA); M199 media, fungizone, trypsin-EDTA, fetal bovine serum, bovine serum albumin, laminin, phosphate-buffered saline (PBS), sodium dodecyl sulphate, fibronectin, syto-24 stain, Tris, acetic acid and EDTA buffer, dithiothreitol and penicillin/streptomycin (Invitrogen, Carlsbad, USA); GoTaq Flexi DNA polymerase, ImProm-II reverse transcription system and GoTaq Hot Start polymerase (Promega, Madison, USA); dNTP (Bioloine, Toronto, Canada); D-(+)-glucose and paraformaldehyde (Scientific Supplies, Auckland, New Zealand); tumour necrosis factor α (TNFα) (PreProTech, Canton, USA); epidermal growth factor (EGF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and poly-D-lysine (PDL; Sigma—Aldrich, Castle Hill, Australia); Cy3-labelled goat anti-mouse antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK); and anti-cytokeratin 7 antibody (DacoCytomation, Glostrup, Denmark).

Manufacture and stabilization of the peptide NNZ-4920

NNZ-4920 (H-REGRRAAPGRAGG-NH_2) was manufactured by Auspep (Parkville, Victoria, Australia) as a trifluoroacetate salt with an amidated C-terminus (theoretical molecular weight of 1285.5). The only difference to the human *CAPS2* sequence motif (amino acids 38—50) is a switch from aspartate (position 6) to alanine purely for stability purposes. D-(+)-trehalose was added to the peptide (w/w ratio 167:1) during lyophilization to prevent water uptake during storage. Prior to each experiment, NNZ-4920 was reconstituted in PBS at a concentration of 500 μmol/l. The peptide was stored at -80°C in a lyophilized state under argon pressure. Subsequent dilutions of peptides were made in PBS.

Cytotrophoblast culture

Normal delivered placentas were obtained with informed consent from women at Auckland Hospital by elective Caesarean section. Cytotrophoblasts were isolated as described earlier (Blumenstein et al., 2002). Briefly, cytotrophoblasts derived from term placenta ($n = 4$) were liberated by eight sequential digestions with 0.25% trypsin digestion, supernatants were collected in 50 ml Falcon tubes and centrifuged at 290*g* for 7 min. Erythrocytes were removed by incubation of cell pellet in a red cell lysis buffer (50 mmol/l NH_4Cl, 10 mmol/l $NaHCO_3$ and 0.1 mmol/l EDTA) and cytotrophoblasts were purified by centrifugation at 1200*g* for 20 min on a discontinuous Percoll gradient (20—60%). Cells migrating between the 40% and 50% Percoll bands were collected and plated either in 12-well transwell plates (5×10^4 cells/well) for migration assays or 96-well plates (9×10^4 cells/well) for viability studies. The cells were grown in M199 media, supplemented with 10% FCS and penicillin/streptomycin (100 U/ml) in a 5% CO_2 humidified atmosphere at 37°C. After 24 h, culture media was removed, cells were washed with PBS and media containing 1% FCS was added. Villous placental tissue was washed in saline to remove