Pituitary Autoantibodies in Lymphocytic Hypophysitis Target
Both γ- and α-Enolase – A Link with Pregnancy?

D.T. O’Dwyer1,2, V. Clifton3, A. Hall1, R. Smith1, P.J. Robinson1 and P.A. Crock1,2

1Paediatric Endocrine Unit, John Hunter Children’s Hospital, 2The Mothers’ and Babies’ Research Centre, 3Newcastle Protein, Biomolecular Research Facilility, University of Newcastle, NSW; and 4Cell Signalling Laboratory, Children’s Medical Research Institute, Westmead, NSW, Australia

Abstract
The first target autoantigen to have been identified in lymphocytic hypophysitis is a 49 kDa protein, identified as alpha-enolase. Pituitary autoimmunity is strongly associated with pregnancy and we have shown that pituitary autoantibodies from patients with peripartum lymphocytic hypophysitis also recognise enolase in the placenta. Enolase exists in different forms as a number of isoenzymes, which are homo- or heterodimers of three subunits, α, β and γ. αα-enolase is ubiquitous, ββ-enolase is muscle-specific and γγ-enolase, which is restricted to neuronal tissue and neuroendocrine cells, is known as neuron-specific enolase (NSE). NSE is expressed in normal human pituitary and pituitary neoplasms. The current study investigated which isoforms of enolase in pituitary and placenta reacted with the sera of patients with lymphocytic hypophysitis. Immunoblotting of two-dimensional gels of human pituitary cytosolic proteins showed that autoantibodies in patient sera react with both an acidic form, and more neutral forms of enolase. Immunoblotting with a monoclonal antibody to NSE confirmed the identity of the acidic enolase isofrom as the γγ-isofrom in both pituitary and placental samples. Gamma-enolase, i.e. NSE, was detected by immunohistochemistry in term placenta in decidua, syncytiotrophoblasts, anchoring villi and terminal villi. Our study is the first to describe the cellular localisation of NSE in normal human placenta, thus establishing a direct link between pituitary and placental autoantigens. This link provides a theoretical basis for the strong predilection of lymphocytic hypophysitis to occur during or after pregnancy.

Keywords: Pituitary autoimmunity, pituitary autoantibodies, enolase, neuron-specific enolase, lymphocytic hypophysitis, immunohistochemistry.

Introduction
Lymphocytic hypophysitis is a pituitary autoimmune disease that often presents in the peripartum period. Women constitute approximately 85% of cases (Beresi et al., 1999) and over half the female cases are pregnancy-related (Hashimoto et al., 1997 and Crock, 1997). We hypothesised that one cause of this relationship with pregnancy could be the sharing of target autoantigens between the pituitary and placenta. Up to 70% of patients with biopsy-proven lymphocytic hypophysitis have autoantibodies to a 49 kDa pituitary cytosolic protein (Crock, 1998). We have recently isolated this same immunoreactive protein from placenta and identified it as αα-enolase by amino acid sequencing (O’Dwyer et al., 2001).

Enolase (2-phospho-D-glyceratehydrolase; EC 4.2.1.11) is a dimeric glycolytic enzyme encoded by three genes: alpha (α), beta (β), and gamma (γ) enolase. These give rise to three subunits which combine to form five homodimeric or heterodimeric isoenzymes. αα-Enolase is ubiquitous and is known as nonneuronal enolase (NNE), ββ-enolase is muscle-specific enolase (MSE), and γγ-enolase is called neuron-specific enolase (NSE) (Marangos et al., 1987). The γγ-enolase-isoenzyme has been detected in neuronal and neuroendocrine cells as well as neuroendocrine tumours (Cunningham et al., 1992). Two heterodimeric enolase isoenzymes have also been described, αγ and αβ (Royd’s, 1982).

The pituitary has been shown to contain NSE by immunohistochemistry (Asa et al., 1984). We have previously reported the 49 kDa autoantigen in monkey pituitary, brain, thyroid and adrenal (Crock, 1998), all of which express NSE or γγ-enolase (and the ubiquitous αα-enolase) and in lung, liver and spleen (Crock, 1998), which only express αα-
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enolase (Pancholi, 2001). Thus autoantibodies against the 49 kDa-protein could be recognising either or both of these enolase isofoms. The relationship between pregnancy and the development of hypophysitis suggests that the placenta may express a key autoantigen also present in the pituitary. We sought to identify the enolase isoforms in the pituitary recognised by the sera of patients with hypophysitis and to determine whether similar forms were expressed and recognised in the placenta.

Materials and methods

Cytoplasmic proteins were prepared from human pituitary and placenta as previously described (O’Dwyer et al. 2001; Crock et al. 1993) and in accordance with guidelines from the Human Research Ethics Committees of the Hunter Area Health Service and the University of Newcastle.

Two-dimensional gel electrophoresis of placental and pituitary cytosol extracts

Human pituitary and placental cytosol preparations were enriched for proteins between 30-kDa and 100-kDa by centrifugal ultrafiltration (Millipore, Bedford, MA USA). Samples were acetone precipitated to desalt and then re-dissolved in the same rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG Buffer (Amersham-Pharmacia-Biotech, Uppsala, Sweden), 40 mM Tris, 0.3% dithiothreitol (DTT), bromophenol blue) used to equilibrate the Immobiline IPG Drystrip™ (Amersham-Pharmacia-Biotech, Uppsala, Sweden). The samples were then subjected to isoelectric focusing (IEF).

IEF was run on 11-cm IPG strips, pH 3–10 (Pharmacia Multiphor apparatus). Following IEF, the IPG strips were pre-equilibrated in the SDS-PAGE sample buffer, laid on a 10% polyacrylamide gel, and overlayed with 1% agarose. Second-dimension gels were run at 8 mA for 16 h. The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels were used in immunoblotting experiments and were pre-stained with zine to check the quality of the resolution before electro-transfer to nitrocellulose.

Immunoblotting of 2D-gels to identify enolase subunits in human placenta and pituitary

2D-PAGE from human pituitary and placental cytosol preparations were incubated with the following antibodies diluted in 1% BLOTTO (Skim milk powder in PBS): (A) polyclonal goat anti-enolase antibody 1:2500 (Santa Cruz Biotechnology Inc, CA, USA), that recognises all enolase isoenzymes, (B) monoclonal mouse anti-human NSE antibody 1:5000 (NSE-1G4 clone, Zymed Laboratories, San Francisco, CA, USA), that recognises only the γ-enolase subunit, and (C) sera from patients with peri-partum lymphocytic hypophysitis with high titre autoantibodies to the 49 kDa pituitary autoantigen we now know as αα-enolase. Primary antibody incubations were for 2.5 hours at 25°C, after which blots were washed with three changes of 0.05% Tween 20 in phosphate buffered saline (PBS) and three changes of PBS.

The blots were then incubated for 1.5h at 25°C with the following secondary antibodies, diluted 1:2500 in 1% BLOTTO: (A) rabbit anti-goat IgG-conjugated to alkaline phosphatase (AP) (Southern Biotechnology Associates Inc. Birmingham, Alabama, USA); (B) AP-conjugated rabbit anti-mouse IgG antibodies (Silenus, Melbourne, Australia); and (C) AP-conjugated sheep anti-human IgG (Silenus, Melbourne, Australia), respectively. The blots were washed as above, and immunoreactivity detected with BCIP (5-bromo-4-chloro-3-indolyl-phosphate tolune salt) and NBT (p-nitro blue tetrazolium chloride) substrates (Bio-Rad, Richmond, CA, USA).

Immunohistochemical localisation of NSE in the human placenta

Tissues were formalin fixed and paraffin embedded. Sections of placenta were mounted on slides coated with 3-aminopropyltriethoxy-silane (AAS) (Sigma Chemical Co. St Louis, MO, USA). The anti-γ-enolase monoclonal antibody (Zymed) and the polyclonal goat anti-enolase antibody (Santa Cruz) were used in conjunction with avidin-biotin-peroxidase reagents (Universal LSAB+, DAKO USA). Briefly, the sections were deparaffinised and endogenous peroxidase activity was quenched with hydrogen peroxide (1% in PBS) for 10 minutes. Non-specific binding was reduced by blocking sections with 20% normal horse serum in a moist chamber, at 4°C. The sections were then incubated overnight with the primary antibody or the appropriate substitute control. All antibodies were diluted in 1% bovine serum albumin (BSA) in PBS buffer. Following incubation, sections were washed with PBS (10 min) and incubated for 2h at room temperature with a biotinylated anti-goat secondary antibody, washed for 10 min in PBS and then incubated with avidin-biotin peroxidase complex solution for 2h at room temperature. Specific immunostaining was visualised using diaminobenzidine (Sigma Immunochimicals, DAB peroxidase substrate tablet set). The sections were counterstained with Carazzi’s haematoxylin (0.5 g haematoxylin, 100 ml glycerol, 25 g aluminium potassium sulfate, 0.1 g potassium iodate and 400 ml distilled water) for 3min and washed in running tap water for 5 min. Sections were then dehydrated in ethanol and mounted using DePex (BDH Chemicals).

Negative controls were conducted in which placental sections were incubated with non-immune goat serum or antibody dilution buffer alone or antibody pre-absorbed with the homogenised rat brain. Positive controls consisted of rat brain that were stained for enolase and γ-enolase. All experimental sections were stained simultaneously to allow direct comparison between samples. All sections were examined by light microscopy and qualitatively assessed. Sections were examined by at least two individuals with ten sites examined...
on each section. A positive result was recognised when at least 80% of sites examined contained positive, preabsorbable staining.

Results

Immunoblotting of 2D-gels identifies enolase subunits in human placenta and pituitary

The polyclonal anti-enolase antibody detected two different enolase isoforms on 2D-PAGE from human pituitary and human placental cytosol, a single protein at acidic pH 4.9 but a number of proteins around pH 6.5 (Fig. 1A and 1D). The most acidic enolase isoform was identified as γ-enolase by monoclonal anti-NSE antibodies (Fig. 1B and 1E). The isoelectric point (pl) of γ-enolase estimated from the 2D-array as 4.9 is identical to the theoretical pl of 4.94 (using ExPASy, Compute pI/Mw tool). A number of immunoreactive enolase-subunits were detected around pl = 6.5, which is close to the theoretical pl for α-enolase of 6.99. α-enolase has a number of potential phosphorylation sites (Cooper et al., 1984), and it is assumed that the subunits detected in this region represent different phosphorylation states.

Patient sera react to both α- and γ-enolase subunits in human placenta and pituitary

The 2D-PAGE immunoblot using patient serum showed reactivity directed against both the γ- and α-enolase subunits in the pituitary. The corresponding Coomassie blue-stained gel

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**Fig. 1.** Two-dimensional western blots of cytosolic proteins from human pituitary and placenta.

2D arrays of pituitary (panels A to C) and placental (panels D to F) cytosolic proteins were transferred to nitrocellulose and immunoblotted. Panels A and D were probed with polyclonal goat anti-enolase antibody; B and E with monoclonal mouse anti-enolase antibody and; C and F probed with positive control patient serum. Secondary antibodies were conjugated to an alkaline phosphatase detection system.
Immunohistochemical localisation of NSE in the human placenta

Immunoreactive (IR) enolase and NSE were detected in the control tissue, rat brain. Preabsorption of the sections with the polyclonal anti-pan-enolase and the monoclonal anti-NSE antibodies resulted in a significant reduction in specific staining for enolase and NSE in the rat brain sections. This method was used to detect the enolase isoforms in term placenta with an anti-enolase polyclonal antibody and an anti-NSE monoclonal antibody. The immunoreactivity demonstrated by the anti-enolase polyclonal antibody reinforced the ubiquitous expression of enolase (mainly due to α-enolase reactivity). Using the monoclonal antibody to γ-enolase, NSE reactivity was localised to the decidua, vascular smooth muscle and vessels of anchoring and terminal vilii, and especially to the syncytiotrophoblast cells. Pre-absorption of the monoclonal antibody to NSE on rat brain prior to application to the placental section resulted in a significant reduction in specific staining for NSE in the placenta (Fig. 2).

Discussion

The striking clinical association of lymphocytic hypophysitis and pregnancy was noted even in the first case report by Goudie and Pinkerton in 1962 (Goudie & Pinkerton, 1962). They hypothesised that ‘the changes (of Hashimoto’s thyroiditis and anterior hypophysitis) are auto-immune reactions to the pituitary and thyroid antigens released during the puerperal involution of these glands’. Our aim was to explain this link in light of the current concept of shared tissue autoantigens and molecular mimicry in autoimmunity. We have identified one of the target autoantigens in lymphocytic hypophysitis as enolase, a glycolytic enzyme with a number of isoforms. The isoform we purified from placenta was α-enolase, the ubiquitous form (O’Dwyer et al., 2001). However, patient autoantibodies may be recognising other isoforms.

Two-dimensional PAGE of pituitary cytosolic proteins enabled us to identify γ-enolase as the main target antigen of autoantibodies in the sera of patients with peripartum lymphocytic hypophysitis. The sera also recognised the α-subunit but the reaction was weaker. This is intriguing, as α- and γ-subunits share 85% sequence homology. The γ-subunit, which dimerises as γ- or neuron specific enolase (NSE), is a cell-type-specific subunit and a marker of neuroendocrine tissue. This may explain the relatively high incidence of pituitary autoantibodies to the 49kDa protein (we now know as enolase) reported by our group in Hashimoto’s thyroiditis (19%) and Addison’s disease (43%) (Crock, 1998). We were interested to know if γ-enolase had been described in the human placenta, and to our knowledge it had not.

Probing two-dimensional PAGE of placental cytosolic proteins with polyclonal anti-enolase antibodies, we were able to identify α-enolase subunits as expected. A monoclonal antibody to γ-enolase identified this isoform clearly, although the corresponding Coomassie gel showed that this protein was far less abundant. Interestingly, the same patient serum which had bound strongly to γ-enolase in the pituitary 2D gels, now reacted more weakly to the placental protein. It is probable that the patient autoantibodies interact differently with the conformational epitopes in the placenta.

We confirmed our findings by immunohistochemistry. The current report is the first to describe the presence of γ-enolase.
in placenta. It was localised to decidua, the vascular smooth muscle of anchoring and terminal villi and syncytiotrophoblast cells. Placental syncytiotrophoblasts are known to produce the peptide-hormones corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), so the detection of γ-enolase in these cells is consistent with their neuroendocrine role. This finding highlights the concept of the ‘diffuse’ endocrine system (Carlei & Polak, 1984) in contrast to organ-specific autoimmune endocrinopathies with specific target enzymes such as thyroid peroxidase in Hashimoto’s disease (Czarnocka et al., 1985) and 21-hydroxylase in Addison’s disease (Winqvist et al., 1992). It has been suggested that one of the target autoantigens in autoimmune pituitary disease could be an enzyme involved in the production, or processing of pro-α-subunit of ACTH (pOMC-derived) peptides (Sauter et al., 1990; Thodou et al., 1995). By definition such an enzyme would be in the amine precursor uptake and decarboxylation (APUD) cells. The identification of γ-enolase (present in APUD cells) as a major antigen in lymphocytic hypophysitis could provide an explanation for why hypophysitis is often seen in association with adenitis and/or thyroiditis (Beressi et al., 1999).

We have demonstrated that γ-γ-enolase, is a target autoantigen shared by the pituitary and placenta and that it is recognised by sera from patients with peripartum lymphocytic hypophysitis. Placental γ-enolase was localised in syncytiotrophoblasts, which are the multi-nucleated cells on the maternal side of the placenta, and therefore exposed to the maternal circulation. It is conceivable that peri-partum lymphocytic hypophysitis may develop if the maternal immune system develops autoantibodies against placental γ-enolase, which may then react against γ-enolase containing cells in the pituitary. Alternatively, there may be cross-reactivity between αα- and γ-enolase epitopes as they are 85% homologous and antibodies initially elicited against αα-enolase could then react with γ-enolase by the phenomenon of ‘epitope spreading’. The immunological milieu of pregnancy is unique and we have now shown a link between the placenta and pituitary, which could explain the association between lymphocytic hypophysitis and pregnancy.

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References


