Immunohistochemistry of neurone specific enolase with γ subunit specific anti-peptide monoclonal antibodies

G I Murray, M E Duncan, W T Melvin, J E Fothergill

Abstract

Aims—To investigate the application in immunohistochemistry of γ -subunit specific anti-peptide monoclonal antibodies to human neurone specific enolase (NSE); and to determine their reactivity with formalin fixed, wax embedded sections of normal tissue and neuroendocrine tumours.

Methods—Immunohistochemical staining was performed on sections of formalin fixed, wax embedded tissue with two monoclonal antibodies (NSE-P1 and NSE-P2) raised against different synthetic peptides specific for the γ subunit of human enolase (neurone specific enolase).

Results—Both antibodies gave strong immunostaining in normal tissues and cells known to contain NSE. There was no immunoreactivity in tissues containing either the $\alpha\alpha$ or $\beta\beta$ isozymes of enolase. The reactivity of the antibodies with a range of neuroendocrine tumours was also studied and both antibodies gave strong immunostaining of tumour cells in the different tumours.

Conclusions—The use of synthetic peptides from defined regions of a molecule as immunogens provides antibodies of high specificity. These monoclonal antibodies to NSE are ideally suited for immunohistochemical studies and they should be particularly useful in histopathology as they react with epitopes which are resistant to formalin fixation and wax embedding.

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Enolase (2-phospho-D-glycerate hydro-lyase, EC no 4.2.1.11) is a cytoplasmic enzyme of the glycolytic pathway which catalyses the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate. There are several different isozymes of enolase and structurally the enzyme consists of dimers of three different polypeptide chains designated α , β , and γ .^{1,2} The $\alpha\alpha$ isozyme is expressed in many tissues and the $\beta\beta$ isozyme occurs exclusively in muscle. The $\gamma\gamma$ isozyme is present in neurones and neuroendocrine tissues; this isozyme of enolase has been designated neurone specific enolase (NSE).^{3,4} The identification of NSE immunoreactivity, particularly by immunohistochemistry, has made it a general marker of neural and neuroendocrine differ-

entiation in both normal tissues and tumours.⁵⁻⁹

There have been few reports of the development of monoclonal antibodies to NSE¹⁰⁻¹³ presumably reflecting the difficulty of producing antibodies which are γ chain specific as the α , β , and γ polypeptides show considerable sequence homology.¹⁴ We have produced monoclonal antibodies to NSE using a novel approach with synthetic peptides specific for the γ chain of enolase as immunogens.¹⁵

Methods

Sequence analysis of the amino acid sequences of the α , β , and γ subunits of human enolase identified sequences specific for the γ chain of enolase. Peptides corresponding to these regions were synthesised, conjugated to carrier proteins and used as immunogens. One antibody corresponding to each of two peptides was finally produced and the antibodies were designated NSE-P1 and NSE-P2.¹⁵ Both monoclonal antibodies were of the IgG1 subclass. The antibodies recognised purified human NSE and showed no cross reaction with α enolase.¹⁵

Samples of various normal tissues, including brain, peripheral nerve, pancreas, adrenal gland, liver, skeletal muscle and specimens of different types of neuroendocrine tumours submitted to the Department of Pathology, University of Aberdeen, were used in this study. All the tissue specimens had been fixed in 10% neutral buffered formalin for 24 hours at room temperature and then embedded in wax.

Sections of formalin fixed wax embedded tissue, which had been mounted on slides coated with poly-L-lysine, were dewaxed in xylene and rehydrated in descending concentrations of alcohol. The sections were subsequently washed in tap water and 0.05M TRIS-HCl (pH 7.6) containing 0.15M sodium chloride (TBS) and then immunostained with NSE antibodies. Both antibodies were used as undiluted hybridoma culture supernatant. The NSE antibodies were applied to the rehydrated tissue sections for 1 hour at room temperature. TBS used in place of the primary antibodies acted as a negative control. Sections were then washed in TBS for two successive 5 minute periods and sites of antibody binding were then identified using an alkaline phosphatase anti-alkaline phosphatase technique (APAAP). Rabbit anti-mouse immunoglobulin (1 in 100 in TBS containing 1% normal human serum, Dako Ltd, High Wycombe, Bucks) and

Department of Pathology
University of Aberdeen AB9 2ZD
G I Murray

Department of Molecular and Cell Biology
M E Duncan
W T Melvin
J E Fothergill

Correspondence to:
Dr Graeme I Murray.
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monoclonal APAAP (1 in 100 in TBS; Dako) were sequentially applied for 30 minutes at room temperature. Between each antibody application the sections were washed in TBS to remove unbound antibody. Alkaline phosphatase was demonstrated using an incubating solution consisting of 10 ml 0.05M TRIS-HCl buffer (pH 9.0), 3 mg 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 10 mg nitro blue tetrazolium (Sigma), 20 mg magnesium chloride, 6 mg sodium azide to block endogenous electron transport and 4 mg levamisole to inhibit endogenous alkaline phosphatase. This enzyme substrate solution produces an insoluble blue-black final reaction product. The sections were incubated at room temperature in this solution for 15 minutes and the enzyme reaction was stopped by washing the sections in cold tap water. The sections were then lightly counterstained (optional) in Mayer's haematoxylin, air dried, and mounted in glycerine jelly. All sections were examined by transmitted light microscopy and the distribution and intensity of immunoreactivity recorded.

In one experiment, before application of the anti NSE antibodies, sections were subject to proteolytic enzyme digestion with trypsin. Sections were digested with 0.1% trypsin (type 2, Sigma) for up to 20 minutes at 37°C. The trypsin was prepared as a solution in distilled water containing 0.1% calcium chloride and the pH of the solution adjusted to 7.8 with 0.1M sodium hydroxide immediately before use. The action of trypsin was stopped by washing the sections in cold tap water and TBS.

Results

NORMAL TISSUES

Both anti-NSE antibodies gave a positive

immunohistochemical signal on sections of formalin fixed wax embedded tissues. The intensity of the immunostaining with each NSE antibody was similar and the localisation and distribution of immunoreactivity with each antibody was identical. Digestion of sections with trypsin before the NSE antibodies were applied completely abolished all positive immunoreactivity and no immunostaining was observed when the primary antibodies were replaced by TBS.

In brain tissue (cerebral cortex and cerebellar cortex) strong immunoreactivity was identified within the cytoplasm of nerve cell bodies and their axons and processes (fig 1). There was no immunoreactivity in glial cells, arachnoid mater, or blood vessels. In the peripheral nervous system NSE immunoreactivity was identified in ganglion cells and peripheral nerve fibres (fig 2). Chromaffin cells of the adrenal medulla showed strong immunoreactivity while all zones of the adrenal cortex were entirely negative. In the pancreas NSE immunostaining was observed in all the cells within the islets of Langerhans (fig 3); there was no immunoreactivity present in the acini or ducts of the exocrine pancreas. There was no NSE immunoreactivity in either skeletal muscle or liver tissue.

TUMOURS

Different types of neuroendocrine tumours were studied, including islet cell tumours of the pancreas, pheochromocytomas of the adrenal medulla, carcinoid tumours of the small bowel and appendix and neuroblastomas of the adrenal gland. The various types of neuroendocrine tumours showed an identical localisation and distribution of NSE with the two different anti-NSE antibodies. There was generally strong diffuse cytoplasmic

Figure 1 Identification of NSE immunoreactivity in cerebellar cortex. Immunoreactivity is apparent in neurones of the cerebellar cortex.

Figure 2 NSE immunoreactivity in nerve fibres of a peripheral nerve. There is no immunostaining in the adjacent connective tissue.

Figure 3 Immunohistochemical localisation of NSE in pancreas. There is strong immunoreactivity in an islet of Langerhans and no immunostaining of surrounding exocrine acini.

Figure 5 NSE immunoreactivity in tumour cells of a small bowel carcinoid tumour.

staining of the tumour cells in the different types of neuroendocrine tumour studied (fig 4–6). Nerve fibres and ganglion cells adjacent to the tumours also showed strong NSE enolase immunoreactivity. There was no immunostaining of connective tissue, muscle, or blood vessels associated with the different types of tumour.

Discussion

The glycolytic enzyme enolase consists of

several isozymes composed of dimers of three closely related polypeptide chains, and the $\gamma\gamma$ isozyme has been designated neurone specific enolase as it is present in high concentrations in neurones.⁶ Two monoclonal antibodies were produced using synthetic peptides specific for distinct regions of the γ polypeptide chain of enolase.¹⁵ The peptides were from external hydrophilic regions which are most likely to be antigenic. An important advantage of using synthetic peptides from

Figure 4 Immunohistochemical detection of NSE in an islet cell tumour (insulinoma). There is strong immunoreactivity in all tumour cells.

Figure 6 Pheochromocytoma of the adrenal medulla displaying NSE immunoreactivity in tumour cells.

defined regions of a protein or polypeptide as immunogens is that it allows antibodies to be produced that are specific for one member of a closely related family of antigens. This is particularly applicable to the study of NSE as the different isozymes show considerable homology and the specificity of some of the currently available antibodies (polyclonal) to NSE has been questioned.¹⁶

NSE is present in both central and peripheral nervous tissue and this isozyme has also been identified in tissues and cells with a neuroendocrine function.³ The immunoreactivity of the two NSE antibodies was investigated by studying a range of normal tissues known to express the neurone specific isozyme of enolase and also tissues which express different isozymes of enolase. Both antibodies displayed strong immunoreactivity in tissues and cell types known to contain NSE and there was no immunoreactivity in tissues which contain other isozymes of enolase, especially liver cells which express the $\alpha\alpha$ isozyme, and skeletal muscle which contains the $\beta\beta$ isozyme of enolase. One advantage of immunohistochemistry is that the specificity of antibodies can be investigated by immunostaining a variety of tissues which are known to express¹ or not² the antigen being investigated. The results of the immunohistochemistry of normal tissues with the NSE antibodies helps confirm their specificity because immunohistochemical studies did not highlight any unexpected immunoreactivity.

In histopathology NSE is a frequently used general marker of neuroendocrine differentiation of tumours.⁹ NSE is a cytoplasmic enzyme and its use is complementary to other general markers of neuroendocrine differentiation, such as chromogranin, which depend on the presence of an adequate number of neurosecretory granules.^{17,18} As the neurone specific monoclonal antibodies showed intense immunoreactivity in sections of formalin fixed, wax embedded tissues the potential utility of these antibodies in histopathology was investigated by studying a range of neuroendocrine tumours derived from different tissues all of which have been shown to contain NSE.¹⁹⁻²¹ Strong immunoreactivity was identified with both NSE antibodies in a range of neuroendocrine tumours. There was no apparent difference in the intensity or distribution of immunostaining with either antibody in any of the tumours.

These antibodies are ideally suited for the immunohistochemical detection of NSE and there is no immunoreactivity with tissues or cell types known to contain other isozymes of

enolase. They should be particularly useful in histopathology as both NSE antibodies displayed strong immunoreactivity in formalin fixed, wax embedded section of tissue.

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