Electrophoretic assessment of aqueous and serum neurone-specific enolase in retinoblastoma and ocular malignant melanoma

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Abstract
The isoenzyme pattern of enolase was examined in the aqueous humour and serum of patients with retinoblastoma (10 aqueous, 8 sera), malignant melanoma (4 aqueous, 25 sera), and normal subjects undergoing cataract surgery (25 aqueous, 30 sera). The assay we used allowed assessment of all three major isoenzymes, including the γγ isoenzyme (neurone-specific enolase). No enolase was detectable in normal aqueous; αα isoenzyme was present in the aqueous of one patient with malignant melanoma, while aqueous from all patients with retinoblastoma contained both αα and γγ. Normal serum contained only an αα band, while serum from patients with retinoblastoma contained αα, γγ, and γγ bands (7 sera, 87-5%), or αα only (1 patient, 12-5%). All sera from patients with malignant melanoma contained the αα band, with low levels of γγ in 16 (60%). In a single patient with Coats’s disease αα was present in the serum, but no enolase was detected in aqueous. Increased amounts of γ-containing isoenzymes of enolase are found in both serum and aqueous from patients with retinoblastoma. In malignant melanoma there is often an increase in serum γγ enolase. The assessment of aqueous and serum enolase patterns may be of value in the diagnosis of retinoblastoma and malignant melanoma.

The neurone-specific isoenzyme (γγ) of enolase is demonstrable in tumours of neuroectodermal origin, such as retinoblastoma and neuro-blastoma,1,2 in aqueous humour from patients with retinoblastoma,1 and in serum and urine1 from patients with neuroblastoma. Specific information about isoenzymes is yielded by measurement by radioimmunoassay,3 enzyme immunoassay,4 and electrophoretic separation,9 but not by assays based on spectrophotometry10,11 or luminescence.12,13 Modification11 of the electrophoretic assay, with a fluorescent endpoint, makes it more sensitive and rapid, allowing its application to small samples.

We wished to investigate the patterns of enolase isoenzymes in aqueous and serum from patients with malignant ocular tumours, and we describe here our clinical experience with electrophoretic assessment of this enzyme.

Patients, materials and methods
Aqueous humour samples were collected by paracentesis with a tuberculin syringe and disposable 25 gauge needle, with avoidance of blood contamination because of the high enolase activity in erythrocytes.3 Samples were collected from enucleated eyes of patients with retinoblastoma and ocular malignant melanoma. A simultaneous serum specimen was drawn, care being taken to avoid haemolysis. Control aqueous samples were taken from patients undergoing cataract extraction at the time of entry into the anterior chamber. All samples were stored at −40°C until assay.

Adenosine diphosphate (ADP), adenosine monophosphate (AMP), nicotinamide adenine dinucleotide phosphate (NADP), 2-phosphoglycerate, hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from yeast), and pyruvate kinase (EC 2.7.1.40, from rabbit muscle) were obtained from Boehringer-Mannheim (Mannheim, FRG). Magnesium chloride hexahydrate, potassium chloride, and sucrose were obtained from Sigma Ltd, Poole, England. Cellulose acetate plates (ISO Flur Titan III) and electrophoretic buffer (Electra HR) were obtained from Helena Laboratories, Beaumont, Texas, USA. Electrophoresis tank and power pack (Vokam 2541) were obtained from Shandon Southern Products Ltd, Runcorn, Cheshire, England.

The visualisation reagent contained (per litre) trometamol (Tris) HCl (pH 7-4, 50 mmol), MgCl2 (2 mmol), KCl (20 mmol), glucose (2 mmol), ADP (90 μmol), AMP (20 mmol), NADP+ (0-5 mmol), phosphoglycerate (1-2 mmol), pyruvate kinase (4 KU), hexokinase (7 KU), and glucose-6-phosphate dehydrogenase (3-5 KU). To minimise diffusion, sucrose was dissolved in this reagent to give a final concentration of 300 g/l. Aliquots of 1 ml of the solution were stored at −20°C and thawed immediately before use.

For isoenzyme analysis, two cellulose acetate plates (76 by 60 mm) were soaked for 30 minutes in tris barbital sodium buffer (pH 8.6–9.0), removed, and blotted. To one (the sample plate), 1 μl of sample was applied near the cathodic end of the plate, and electrophoresis was carried out in tris barbital sodium buffer at 300 V for 7 minutes at room temperature. At the same time, the other (substrate) plate was placed membrane side up on a clean glass slide and 1 ml of isoenzyme reagent was poured on to the surface and allowed to soak for the period of electrophoresis. Following electrophoresis the sample plate was carefully placed on the substrate plate with the two wet surfaces apposed. The plates were compressed between prewarmed (37°C) glass plates and incubated at 37°C for 25 minutes. The plates were separated and dried at approxi-
Table 1. Samples analysed for enolase isoenzymes

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Fluid</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Nomal'</td>
<td>Serum</td>
<td>30</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>Serum</td>
<td>25</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Serum</td>
<td>25</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td>Serum</td>
<td>4</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Serum</td>
<td>8</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Serum</td>
<td>1</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Serum</td>
<td>1</td>
</tr>
</tbody>
</table>

Remarkably 57°C for 10 minutes. The fluorescence of NADPH thus produced was stable for at least 60 minutes at room temperature and for up to 1 month at −20°C. The fluorescent intensity of the bands was assessed visually with a UV lamp with maximum output at 365 nm. Plates were also scanned by means of a Perkin Elmer LS-5 fluorometer, with scanning attachment and output to a chart recorder (λ_{ex} 360 nm, λ_{em} 440 nm).

**Results**

A maximum of three bands were detected by this procedure. The slowest band represents the α isoenzyme, the intermediate band γγ isoenzyme, and the anodic band γγ isoenzyme. Bands were visible only if 2-phosphoglycerate was included in the substrate medium, indicating that the enzyme was enolase. Visual assessment of the levels in each band appeared to correlate well with those obtained by scanning.

In total, 64 serum and 39 aqueous samples were examined (Table 1). No enolase was detected in normal aqueous humour, and normal serum contained only the αα band with relative intensity ranging from 1+ to 2+. Aqueous humour from all eyes with retinoblastoma contained αα and γγ bands, with one sample also containing an intermediate (αγ) band (Fig 1). There was considerable variation in the relative intensities of the individual bands. Serum from all retinoblastoma patients contained an αα band, 7/8 (87.5%) also containing αγ and γγ bands (Fig 2).

An αα band was visible in aqueous humour from one patient of the four with malignant melanoma, but in none could αγ or γγ be demonstrated. The αα band was present in the serum of all 25 malignant melanoma patients, with relative intensities ranging from 1+ to 2+, and in 16 γγ (1+) was also present (Fig 3). In a single case of Coats’s disease, an αα band was present in the serum with no enzyme visible in the aqueous.

**Discussion**

Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is widely distributed in tissues, and reversibly catalyses the conversion of 2-phosphoglycerate to phosphoenol pyruvate. Its isoenzymes are dimers consisting of two subunits coded for by three gene loci (α, β, and γγ). In most adult tissues only the αα dimer is present, while in adult muscle and heart the ββ dimer predominates though αβ hybrids are demonstrable in the heart. Since the γ subunit was previously believed to be expressed exclusively in mature neurons, γγ isoenzyme was designated neurone-specific enolase (NSE). However, low levels of NSE are present in some non-neuronal tissues. The enzyme is not bound to cellular membranes and probably escapes freely following cell injury. γγ Subunits (that is, αγ and γγ) containing enolase isoenzymes have been...
reported to be increased in the serum of patients with neuroblastoma,14,15 medullary carcinoma of the thyroid,16 and small-cell carcinoma of the lung,17-20 in urine of patients with neuroblastoma,21 and in the aequous of patients with retinoblastoma.16

Methods for the measurement of enolase activity in tissues and biological fluids include spectrophotometric21-23 and bioluminescence24,25 assays for total enzyme, and radioimmuno-assay26 enzyme immunooassay,27 immunohistochemistry,28 and electrophoretic separation followed by colorimetric visualisation,29 for measurement of isoenzymes. A modification of the electrophoresis method30 with fluorescence scanning makes it sensitive enough for use on small samples, with assessment of all isoenzymes within one hour.

Retinoblastoma is the commonest malignancy of infancy. It has features of both glial and neuroectodermal origin1-13 and may be unilateral or bilateral. With appropriate therapy in the early stages the prognosis is good.24 Occasional spontaneous regression has been reported.35 Correct diagnosis, without recourse to biopsy, is vital, since it is undesirable both to enucleate an eye for a benign condition and to miss a fatal, though potentially curable, malignant tumour. Rates of error worldwide, in the diagnosis of retinoblastoma have been estimated to be 25%.36 If doubt about the diagnosis still exists after tests such as a CT scan have been performed, methods such as the assay of aqueous humour enzymes have been advocated.37-39 However, although increased levels of serum and aqueous humour lactate dehydrogenase are often present in retinoblastoma, raised aqueous enzyme levels may be found in other conditions, such as Coats' disease.39,40 Aqueous levels of γ-containing enolase isoenzyme have been shown by radioimmunoassay to be raised in retinoblastoma. Paraocentesis of the aqueous humour is sometimes necessary for the diagnosis of both primary38 and secondary39,40 anterior segment malignant tumours, though it is associated with a slightly increased risk of tumour dissemination.41 In contrast, aspiration of vitreous, subretinal fluid, or tumour cyst material has been strongly discouraged.42,43

Using an electrophoretic assay44 we have demonstrated different enolase isoenzyme patterns in aqueous humour and serum. Three bands were identified, corresponding to those previously reported to be αα, αγ and γγ isoenzymes. Aqueous from patients with retinoblastoma contains αα and γγ bands, while their serum often contains all three bands. The γ bands were absent from normal sera and normal aqueous but were faintly present as the γγ band in serum from two-thirds of patients with malignant melanoma. There were no differences between the sexes.

The presence of raised levels of NSE in serum and aqueous from patients with retinoblastoma is expected because of the presumed neuroectodermal origin of this tumour and its previous demonstration in tumour tissue.1,2 Malignant melanoma arises from pigmented cells, in which NSE has been reported not to occur,10 though Royds and colleagues8 have identified the enzyme in malignant melanoma tissue. The existence of a faint γγ band in serum from patients with malignant melanoma may be due to leakage from the tumour, direct damage to the retina from raised intraocular pressure, or to metastatic spread.

Retinoblastoma is associated with increased levels of γγ-enolase in serum and aqueous. While serum levels may be of diagnostic help, there may be occasions when aqueous paracentesis is necessary for diagnosis. Because of the risk of tumour spread it should be reserved for cases where there is serious doubt about the diagnosis. The presence of a faint γγ band in serum from patients with malignant melanoma may also be of diagnostic value. However, further studies (both quantitative and qualitative) need to be performed on a larger population to assess the full potential of the assay.