Enolase isoenzymes in uveal melanomas—a possible parameter of malignancy

JANICE A. ROYDS,¹ I. G. RENNIE,² M. A. PARSONS,³ W. R. TIMPERLEY,⁴ AND C. B. TAYLOR

From the Departments of ¹Biochemistry, University of Sheffield; ²Ophthalmology, Royal Hallamshire Hospital, Sheffield; ³Histopathology, University of Sheffield; and ⁴Neuropathology, Royal Hallamshire Hospital

SUMMARY Seven uveal melanomas were stained for γ enolase by an immunoperoxidase PAP (peroxidase-antiperoxidase) technique, and biochemical assays were carried out on tissue homogenates. A correlation between the biochemical assay and the immunoperoxidase staining was demonstrated. Two tumours with the highest biochemical assays showed positive staining for the enzyme, and 2 tumours with the lowest levels showed no appreciable staining. The highest level of enolase was present in a tumour which both clinically and histologically appeared to be benign, and the lowest level occurred in a mixed cell tumour which was large in size; the low level presumably related to its relatively fast rate of growth. Estimation of γ enolase activity in ocular melanomas may provide an accurate quantitative method for assessing the malignant potential of these tumours.

Recent changes in attitudes to the treatment of uveal melanomas have heightened the need for an accurate method of histological assessment of their prognosis. Most pathologists use a modification of the classification devised by Callender in 1931.¹ This system consists of 4 histological groups—spindle cell A, spindle cell B, mixed cell, and epithelioid cell. The determination of the cell type, based on this classification, appears to be the most important parameter in assessing prognosis.²⁻⁵ Tumours composed in part (mixed cell) or completely of epithelioid cells have a relatively poor prognosis. Unfortunately it has been shown that there is often a considerable disparity in the histological grading of these tumours when they are examined by different pathologists.

In a study performed by Gass⁶ 15 pathologists were asked to grade 15 uveal melanomas independently. In only 4 cases was there unanimous agreement on the appropriate histological grade. The problem of accurate classification most commonly arises from the difficulty in identifying epithelioid elements within the tumour. Furthermore uveal melanomas often show a marked difference in cell type from one area to another, and the pathologist may fail to classify the

tumour correctly because the section examined may not be representative of the tumour as a whole.

Enolase (E.C.4.2.1.11) is a dimeric cytoplasmic enzyme which catalyses the interconversion of 2-phospho-d-glycerate to phosphoenolpyruvate in the glycolytic pathway. It has been shown to contain 3 distinct subunits designated α , β , and γ , and 5 isoenzyme types have been demonstrated.⁷⁻⁹ These include the 3 homodimers $\alpha\alpha$, $\beta\beta$, and $\gamma\gamma$ and 2 hybrids $\alpha\beta$ and $\alpha\gamma$.

α Enolase is the commonest form in most adult tissues and is probably the sole isoenzyme present in early fetal tissue.^{10,11} Studies on monkey and rodent tissues indicate that γ enolase is confined to neurones and cells of the amine precursor uptake and decarboxylation (APUD) system,^{12,13} and our own personal observations indicate that this is also true in the human. Melanocytes, a component of the APUD system¹⁴ have been shown to contain γ enolase.¹⁵

Royds *et al.*¹⁶ suggested that with increasing de-differentiation of cutaneous melanocytic lesions there is a reversion to the fetal enzyme pattern and a comparative loss of γ enolase from the neoplastic tissue. The presence of epithelioid elements appears to have an adverse effect on the prognosis of uveal melanomas. Thus one might anticipate a reduction in the level of γ enolase in tumours containing these elements, and estimation of the amount of γ enolase

Correspondence to Dr W. R. Timperley, Department of Neuropathology, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF.

within uveal melanomas may be useful in assessing prognosis.

This paper presents the results of the localisation and assay of γ enolase in 7 uveal melanomas by means of the immunoperoxidase PAP technique on formalin-fixed paraffin sections and direct biochemical assay of tissue homogenates.

Patients and methods

PATIENTS

Specimens were obtained from 7 patients who had an eye enucleated for a primary choroidal melanoma. The age of the patients in the study ranged from 40 to 88 years (mean 64.7 years). Patient 1 had been observed for 2½ years prior to the enucleation. The tumour was relatively small, and little growth had been observed during that period. In view of the small size of the tumour and the patient's age enucleation was not proposed at the time of diagnosis. The eye was eventually enucleated because a painful thrombotic glaucoma had developed. Patient 3 had been observed for one year prior to enucleation. The

tumour appeared to have arisen from a choroidal naevus. The eye removed from patient 7 had been diagnosed as having melanosis oculi with external pigmentation, heterochromia iridis, and a deeply pigmented choroid. The tumour in this eye was large, occupying at least half the globe.

METHODS

Immediately after enucleation the globe was opened, and the tumour was identified and biopsied, where possible from more than one site. The remainder of the tumour was left in situ in the globe and fixed in formol saline. The biopsy material removed was rapidly frozen and stored at -80°C until biochemical assay was performed.

Histological methods. After fixation in formol saline for a period of 24 to 48 hours sections from the globe containing the tumour were processed into paraffin wax. Standard 5 μm sections were cut and stained with haematoxylin and eosin. Histological grading by the modified Callender classification was performed independently by one of us (I.G.R.).

Immunohistochemistry. Paraffin sections were

Fig. 1 *Spindle celled melanoma stained for γ enolase. ($\times 800$).*

Table 1 *Gamma enolase in various types of uveal melanoma*

Series number	Age	Sex	Site	Cell type	PAP γ	γ	γ
						$\mu\text{g/g}$	$\mu\text{g/mg protein}$
1	88	F	Choroid	Spindle B (with areas spindle A)	1.5 + patchy	46.6	0.63
2	59	F	Choroid	Spindle B	1+	40.9	0.60
3	40	M	Choroid	Mixed, predominantly epithelioid	$\pm$	21.25	0.22
4	70	F	Choroid	Mixed, predominantly epithelioid	$\pm$	10	0.20
5	67	M	Choroid	Mixed, predominantly spindle	$\pm$	8.96	0.15
6	56	M	Choroid	Mixed, predominantly spindle	-ve	4.0	0.10
7	73	F	Choroid	Mixed, no predominance of cell type	-ve	1.86	0.04

stained for γ subunits of enolase by the peroxidase-antiperoxidase (PAP) technique. Sections (5 μm) were stained by the peroxidase-antiperoxidase technique using anti- γ enolase sera. Normal rabbit serum and adsorbed antisera were used as controls. The intensity of the staining for γ enolase was graded independently by one of us (W.R.T.).

Biochemical assay—treatment of tissue samples. Tissue samples were weighed, homogenised in 2 ml of 50 mM tris buffer, pH 7.1, containing 4 mM MgCl_2 , and then centrifuged at 40 000 g for 30 minutes at 4°C. The resulting supernatant fluids were assayed in

duplicate for γ immunoreacting enolase by radioimmunoassay.

Radioimmunoassay. The radiolabelled $\gamma\gamma$ enolase was prepared by the method of Bolton and Hunter¹⁷ using N-succinimidyl-3,4 hydroxy-5 (¹²⁵I) iodophenyl propionate (Amersham International Ltd., Amersham, Bucks HP7 9LL).

The primary antiserum used was produced in rabbits.¹⁸ The separation of the bound and free antigen in the assay was accomplished by precipitation with goat anti-rabbit IgG as a secondary antibody (Miles Laboratories Ltd., Slough, Bucks). The tubes

Fig. 2 *Spindle celled melanoma, negative control. Note the few cells containing granules of melanin pigment in the bottom right-hand corner. ($\times 800$).*

were then centrifuged at 2000 g for 30 minutes at 4°C in an MSE6L. The supernatants were removed by aspiration and the pellets counted in an Intertech-nique Well-type γ counter. The γ immunoreactivity in the melanomas became diluted in parallel with the purified $\gamma\gamma$ enolase used to generate the standard curve, providing evidence that the 2 cross-react immunologically.

Results (Table 1)

Histological grading. Five of the tumours were graded as mixed cell tumours, the other 2 being spindle cell B. In one case (patient 1) large areas of spindle cell A cells were present within the tumour. However, in keeping with the guidelines laid down by McLean *et al.*¹⁹ on the classification of spindle cell A tumours this tumour was classified as spindle cell B.

Identification of γ enolase in paraffin sections of uveal melanomas by the immunoperoxidase technique. The 2 spindle cell tumours with the highest γ enolase levels both stained positively for γ enolase (Figs. 1, 2). The strongest staining was found in patient 1, whose tumour was classified as spindle cell B containing spindle cell A elements. Of the 5 mixed cell tumours 3 were found to have equivocal staining for γ enolase, and in 2 the staining for γ enolase was absent.

Estimation of γ enolase by biochemical assay. γ Enolase was found in all 7 tumours studied (Table 1). However, a marked variation in the levels present was found. The highest levels of γ enolase, 46.6 $\mu\text{g/g}$ and 40.0 $\mu\text{g/g}$ were found in 2 of the spindle cell B tumours. All 5 of the mixed cell tumours had lower levels of the enzyme than the spindle celled tumours.

Discussion

Our results confirm the presence of γ enolase within melanocytic tumours of the uveal tract. Although the series is small, the more benign spindle cell tumours appear to have a higher level of γ enolase than those containing epithelioid cell elements. The highest level of enolase was present in a tumour which both clinically and histologically appeared the most benign. The lowest enolase level occurred in a mixed cell tumour (patient 7) which was large in size; its size may reflect a relatively fast growth rate. It has been established that large melanomas of the uveal tract carry a relatively poor prognosis.

A correlation between the biochemical assay and the immunoperoxidase staining was demonstrated. The 2 tumours with the highest biochemical assays both stained positively for the isoenzyme. The 2 tumours with the lowest levels of γ enolase showed no appreciable staining.

Both methods of γ enolase estimation may be of value in estimating the malignant potential of a uveal melanoma. The biochemical assay, although requiring the acquisition of fresh material in order to perform it, has distinct advantages. Firstly, unlike the immunoperoxidase method, it is quantitative rather than subjective. Secondly, the larger amount of material analysed by this technique renders it less susceptible to sampling error. Obviously further work on γ enolase levels in uveal melanomas is necessary. This should include a prospective trial where enolase levels within uveal melanomas could be compared with long-term survival. Nevertheless we believe that γ enolase estimation may provide an accurate quantitative method for assessing the malignant potential of uveal melanomas.

The authors thank Messrs A. J. Dark, R. C. Gupta, J. F. Talbot, and A. Zaidi and Miss M. A. C. Jones for their assistance in obtaining the pathological material; Miss P. Little, Mr A. Buxton, Miss P. Mills, and Mr C. Day for technical assistance; and Mrs P. Kirk for typing the manuscript. The work was supported by a grant from the trustees to the former United Sheffield Hospitals (grant number 276).

References

- 1 Callender GR. Malignant melanotic tumours of the eye: a study of the histological types in 111 cases. *Trans Am Acad Ophthalmol Otolaryngol* 1931; **36**: 131–40.
- 2 Callender GR, Wilder HC, Ash JE. Five hundred melanomas of the choroid and ciliary body followed five years or longer. *Am J Ophthalmol* 1942; **25**: 962–7.
- 3 Flocks M, Gerende JH, Zimmerman LE. The size and shape of malignant melanomas of the choroid and ciliary body in relation to prognosis and histological characteristics. *Trans Am Acad Ophthalmol Otolaryngol* 1955; **59**: 740–58.
- 4 Paul E, Parnell BI, Fraker M. Prognosis of malignant melanomas of the choroid and ciliary body. *Int Ophthalmol Clin* 1962; **2**: 387.
- 5 McLean IW, Foster WD, Zimmerman LE. Prognostic factors in small malignant melanomas of choroid and ciliary body. *Arch Ophthalmol* 1977; **95**: 48–58.
- 6 Gass JP. Problems in the differential diagnosis of choroidal naevi and malignant melanoma. The XXXIII Edward Jackson Memorial Lecture. *Am J Ophthalmol* 1977; **83**: 299–323.
- 7 Rider CC, Taylor CB. Enolase isoenzymes in rat tissues. Electrophoretic, chromatographic, immunological and kinetic properties. *Biochim Biophys Acta* 1974; **365**: 285–300.
- 8 Fletcher L, Rider CC, Taylor CB. Enolase isoenzymes III. Chromatographic and immunological characteristics of rat and brain enolase. *Biochim Biophys Acta* 1976; **452**: 245–52.
- 9 Marangos PJ, Zis AP, Clark RL, Goodwin FK. Neuronal, non-neuronal and hybrid forms of enolase in brain: structural, immunological and functional comparisons. *Brain Res* 1978; **150**: 117–33.
- 10 Fletcher L, Rider CC, Taylor CB, Adamson ED, Luke BM, Graham CF. Enolase isoenzymes as markers of differentiation in teratocarcinoma cells and normal tissues of mouse. *Dev Biol* 1978; **65**: 462–75.
- 11 Schmechel DE, Brightman MW, Marangos PJ. Neurones switch from non-neuronal enolase to neurone-specific enolase during differentiation. *Brain Res* 1980; **190**: 195–214.
- 12 Schmechel DE, Marangos PJ, Brightman M. Neurone-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature* 1978; **276**: 834–6.

- 13 Schmechel DE, Marangos PJ, Zis AP, Brightman M, Goodwin FK. Brain enolases as specific markers of neuronal and glial cells. *Science* 1978; **199**: 313–5.
- 14 Teillet MA, Le Donarin N. La migration des cellules pigmentaires étudié par la méthode des greffes hétérospécifiques de tube nerveux chez l'embryon d'oiseau. *C R Acad Sci (D) (Paris)* 1970; **270**: 3095–8.
- 15 Parsons MA, Royds JA, Taylor CB, Timperley WR. Enolase isoenzymes as markers of cellular differentiation in normal human foetus and adult. *J Pathol* 1981; **134**: 314.
- 16 Royds JA, Parsons MA, Rennie IG, Timperley WR, Taylor CB. Enolase isoenzymes in benign and malignant melanocytic lesions. *Diag Histopathol* in press.
- 17 Bolton AE, Hunter WM. The labelling of proteins to high specific activities by conjugation to a ^{125}I -containing acylating agent. *Biochem J* 1973; **133**: 529–39.
- 18 Royds JA, Parsons MA, Taylor CB, Timperley WR. Enolase isoenzyme distribution in the human brain and its tumours. *J Pathol* 1982; **137**: 37–49.
- 19 McLean IW, Zimmerman LE, Evans RM. Reappraisal of Callender's spindle A type of malignant melanoma of choroid and ciliary body. *Am J Ophthalmol* 1978; **86**: 557–64.