Int. J. Exp. Path. (1995), **76**, 271–276

# Expression of xenobiotic metabolizing enzymes in tumours of the urinary bladder

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Received for publication 3 April 1995
Accepted for publication 23 May 1995

**Summary.** The cytochromes P450, epoxide hydrolase and glutathione S-transferases are several of the major groups of enzymes involved in the metabolism of xenobiotics and these enzymes may have a role in influencing the response of tumours to anti-cancer drugs. In this study the cell specific expression of individual xenobiotic metabolizing enzymes has been investigated using immunohistochemistry in primary transitional cell tumours of the urinary bladder. The cytochromes P450 CYP1A, CYP2C and CYP3A, were present in 68, 28 and 68% of tumours respectively and the expression of CYP1A correlated with bladder tumour grade ($P$=0.03). Epoxide hydrolase was identified in 84% of tumours while the $\alpha$, $\mu$ and $\pi$ forms of glutathione S-transferase were expressed in 56, 72 and 52% of tumours respectively. In normal bladder epoxide hydrolase and glutathione S-transferase $\pi$ were the main enzymes expressed while there was no expression of CYP2C.

*Keywords:* bladder, cytochrome P450, glutathione S-transferase, neoplasm

Cancer of the urinary bladder is one of the commonest malignant tumours and is characterized by multiple recurrences and may have a poor response to anti-cancer drugs (Thrasher & Crawford 1993). The functionally related groups of enzymes, the cytochromes P450 (P450), epoxide hydrolase and glutathione S-transferases (GST), have a central role in the metabolism of a diverse range of xenobiotics. Generally, metabolism by these enzymes results in detoxification of xenobiotics and the expression in tumours of xenobiotic metabolizing enzymes, especially expression of GSTs, has been proposed as one possible mechanism as a result of which tumours fail to respond to anti-cancer drugs (Graham *et al.* 1991; Le Blanc & Waxman 1989; Tew 1994; Tsuchida & Sato 1992).

The P450s are a multi-gene family of constitutive and inducible enzymes with specific regulatory mechanisms that have a major role in the oxidative metabolism of a wide range of environmental chemicals including therapeutic drugs and several groups of biologically active endogenous chemicals (Gonzalez & Gelboin 1994; Kawajiri & Fujii-Kuriyama 1991; Nelson *et al.* 1993). The epoxide hydrolases are a group of enzymes which metabolize toxic and potentially carcinogenic and mutagenic epoxides produced by oxidative P450 metabolism to less toxic dihydrodiols although subsequent re-oxidation can produce highly reactive diol epoxides (Seidgard & DePierre 1983).

The GSTs like the P450s are a multi-gene family of enzymes which conjugate reduced glutathione with a

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variety of electrophilic compounds, often produced by P450 metabolism, generally to form inactive non-toxic glutathione conjugates (Board *et al.* 1990). The main families of GST are $\alpha, \mu, \pi$ and $\theta$ which are all cytosolic, while there is also a microsomal form of GST. The GSTs have a wide tissue distribution and specific forms of GST are present in many normal tissues (Campbell *et al.* 1991; Terrier *et al.* 1990). The expression of particular forms of GSTs has been identified in various tumours, frequently at higher levels than in corresponding normal tissues, and in most types of tumour GST-$\pi$ is the major form of GST expressed (Cairns *et al.* 1992; Ranganathan & Tew, 1991; Di Ilio *et al.* 1991; Green *et al.* 1993).

The overall contribution of xenobiotic metabolizing enzymes (XMEs) to the response of anti-cancer drugs requires knowledge of the cellular localization of individual enzymes and since there is little information regarding the expression of XMEs in tumours of the urinary bladder we have investigated the cell specific expression of individual XMEs in bladder tumours.

## Materials and methods

### *Antibodies*

CYP1A, CYP2C and epoxide hydrolase were identified using rabbit polyclonal antibodies which have been raised and characterized in our laboratories, while CYP3A was identified using a monoclonal antibody (HL3) also produced in our laboratories (Murray *et al.* 1988; 1993a; Weaver *et al.* 1993). Three major cytoplasmic forms of GST (GST-$\alpha$, GST-$\mu$ and GST-$\pi$) were identified using rabbit polyclonal antibodies obtained from Novocastra Laboratories, Newcastle upon Tyne. All the antibodies used in this study have been well characterized immunohistochemically with each antibody giving identical results using both frozen sections and sections of formalin fixed wax embedded tissue (Murray *et al.* 1988; 1993a; McKay *et al.* 1995).

### *Tissue*

Samples of primary bladder tumours were obtained from trans-urethral resections of bladder tumours (age range of patients 29–92, 16M : 9F) submitted to the Department of Pathology, University of Aberdeen for diagnostic purposes. Samples of normal bladder (age range of patients 19–70, 1M : 4F) were obtained from patients undergoing bladder biopsy for possible bladder inflammation. All the tissue samples had been fixed in 10% neutral buffered formalin for 24 hours at room temperature and then routinely processed to paraffin wax.

### *Immunohistochemistry*

Formalin fixed wax embedded sections (4 $\mu$m) of bladder were dewaxed in xylene, rehydrated in alcohol and then washed sequentially in cold water and 0.05 M Tris-HCl (pH 7.6) containing 0.15 M sodium chloride (TBS). The sections were then immunostained with the antibodies to the different xenobiotic metabolizing enzymes. No antigen retrieval step was used as both heat pretreatment and proteolytic enzyme digestion significantly decrease the immunoreactivity of the antibodies (Murray *et al.*, 1988; McKay *et al.* 1995).

Sections of tissue were incubated for 1 hour with each of the primary antibodies at the following dilutions: anti-CYP1A, 1/320, anti-CYP2C, 1/500, anti-CYP3A (HL3) as undiluted hybridoma culture supernatant and anti-epoxide hydrolase 1/50. The polyclonal antibodies to CYP1A, CYP2C and epoxide hydrolase were prepared from 20 mg/ml stock solutions of 50% ammonium sulphate precipitates of the respective immunized rabbit sera. Antibodies to the different classes of GST were applied at the following dilutions of a stock solution (protein concentration 1 mg/ml): GST-$\alpha$ 1/100; GST-$\mu$ 1/50; GST-$\pi$ 1/200. Sites of antibody binding were demonstrated using an alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. Monoclonal mouse anti-rabbit (1/100, Dako Ltd, High Wycombe, Bucks; omitted for monoclonal antibody HL3), rabbit anti-mouse immunoglobulin (1/100 containing 1% normal human serum, Dako) and monoclonal APAAP (1/100, Dako) were sequentially applied to the tissue sections for 30 minutes each. Between antibody applications the sections were washed with TBS to removed unbound antibody. Sites of bound alkaline phosphatase were identified using an enzyme substrate solution containing 3 mg bromochloroindolyl phosphate (Sigma Chemical Co Ltd, Poole, Dorset), 10 mg nitro blue tetrazolium (Sigma), 6 mg sodium azide and 4 mg levamisole (Sigma) in 10 ml 0.05 M Tris-HCl buffer (pH 9.0) containing 0.2% magnesium chloride. After incubating the sections for 30 minutes at room temperature, the reaction was stopped by washing the sections in cold tap water. The slides were then air-dried and mounted in glycerine jelly.

Both negative and positive controls were incorporated in the immunohistochemical procedure. Negative controls used in place of the primary antibody were TBS (control for monoclonal and polyclonal antibodies), or normal rabbit immunoglobulins (control for polyclonal

antibodies). The positive control was sections of formalin fixed wax embedded normal liver as this tissue expresses all the enzymes being studied. The sections were examined by one observer using light microscopy in order to establish the presence or absence of immunostaining, and its distribution. Correlations between the expression of the different xenobiotic metabolizing enzymes and tumour grade or stage were determined using the $\chi^2$-test with Yates' correction.

## Results

The expression of xenobiotic metabolizing enzymes was investigated in 5 normal bladder samples and 25 primary transitional cell tumours of the urinary bladder consisting of 6 grade 1 tumours, 11 grade 2 tumours and 8 grade 3 tumours. There were 14 non-invasive tumours, 6 stage 1 (T1) tumours, 4 stage (T2) tumours and 1 stage (T3) tumour.

The main XMEs expressed in normal bladder were epoxide hydrolase and GST-$\pi$ while there was no expression of CYP2C (Tables 1 and 2). Immunoreactivity for each XME in normal bladder was present as homogeneous cytoplasmic staining of the transitional epithelium.

All the bladder tumours examined expressed at least one drug metabolizing enzyme and two of the tumours expressed all the enzymes studied (Table 2). There was frequent expression of both CYP1A and CYP3A while there was a much lower frequency of expression of CYP2C and a high proportion of the bladder tumours displayed immunoreactivity for epoxide hydrolase while each of the GSTs was expressed in at least half of the tumours (Table 1). Expression of CYP1A correlated with bladder tumour grade ($\chi^2 = 7.38$, $P = 0.03$; Table 3).

Immunoreactivity for each XME except GST-$\mu$ was present as uniform cytoplasmic staining of all tumour cells (Figures 1 and 2); in addition, several of the tumours also showed nuclear staining for GST-$\pi$. In contrast to the other XMEs GST-$\mu$ showed marked cell to cell variation in the intensity of immunoreactivity with adjacent tumour cells often showing either strong immunoreactivity or absent immunoreactivity (Figure 3).

**Table 1.** Percentage (number) of bladder samples expressing specific xenobiotic metabolizing enzymes

| Enzyme | Normal bladder ($n = 5$) % ($n$) | Bladder tumour ($n = 25$) % ($n$) |
|---|---|---|
| CYP1A | 20 (1) | 68 (17) |
| CYP2C | 0 (0) | 28 (7) |
| CYP3A | 20 (1) | 68 (17) |
| Epoxide hydrolase | 100 (5) | 84 (21) |
| GST-$\alpha$ | 20 (1) | 56 (14) |
| GST-$\mu$ | 60 (3) | 72 (18) |
| GST-$\pi$ | 80 (4) | 52 (13) |

**Table 2.** Percentage (number) of normal and bladder tumour samples expressing different numbers of xenobiotic metabolizing enzymes

| Number of enzymes expressed | Normal bladder ($n = 5$) % ($n$) | Bladder tumour ($n = 25$) % ($n$) |
|---|---|---|
| 0 | 0 (0) | 0 (0) |
| 1 | 20 (1) | 8 (2) |
| 2 | 0 (0) | 16 (4) |
| 3 | 40 (2) | 20 (5) |
| 4 | 40 (2) | 12 (3) |
| 5 | 0 (0) | 8 (2) |
| 6 | 0 (0) | 28 (7) |
| 7 | 0 (0) | 8 (2) |

## Discussion

The expression in tumours of individual xenobiotic metabolizing enzymes is potentially a factor in determining the response of tumours to anti-cancer drugs. Moreover, XMEs may also be involved in tumour cell growth and regulation through their role in the metabolism of biologically active endogenous chemicals especially steroid hormones and arachidonic acid (Nebert 1994). Many studies of tumour cells *in vitro* have shown resistance to particular anti-cancer drugs and in some cases this has been correlated with the deactivation of these compounds by specific XMEs expressed in tumour cells (Tew 1994). For example, in bladder tumour, cell lines which have the highest content of GST-$\pi$ display the greatest resistance to the effects of mitomycin C (Xu *et al.* 1994). However, the *in vivo* situation is likely to be considerably more complex and the outcome of xenobiotic metabolism in terms of either activation (toxicity) or detoxification will depend upon several different factors, particularly the cell specific expression of

**Table 3.** Correlation of CYP1A expression with grade of bladder tumour. The table gives the number of bladder tumours in different tumour grades expressing CYP1A

| | CYP1A | |
|---|---|---|
| Tumour grade | + | − |
| G1 | 2 | 4 |
| G2 | 6 | 5 |
| G3 | 8 | 0 |

$\chi^2 = 7.38$; 2d.f.; $P = 0.03$.

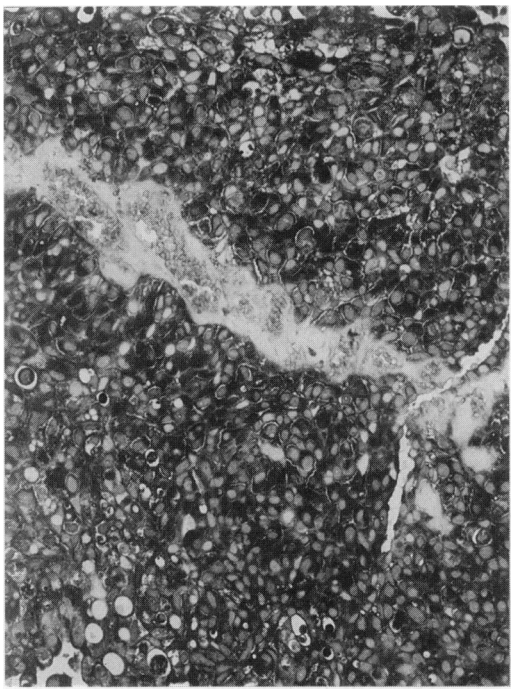

**Figure 1.** Immunoreactivity for CYP3A in a superficial papillary area of a transitional cell carcinoma. ×120.

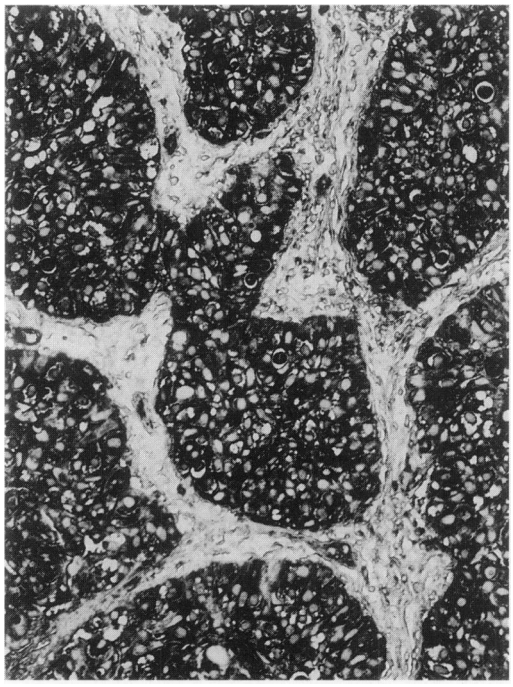

**Figure 2.** There is strong immunoreactivity for epoxide hydrolase in all the tumour cells of a poorly differentiated (G3) invasive (pT2) transitional cell carcinoma. ×120.

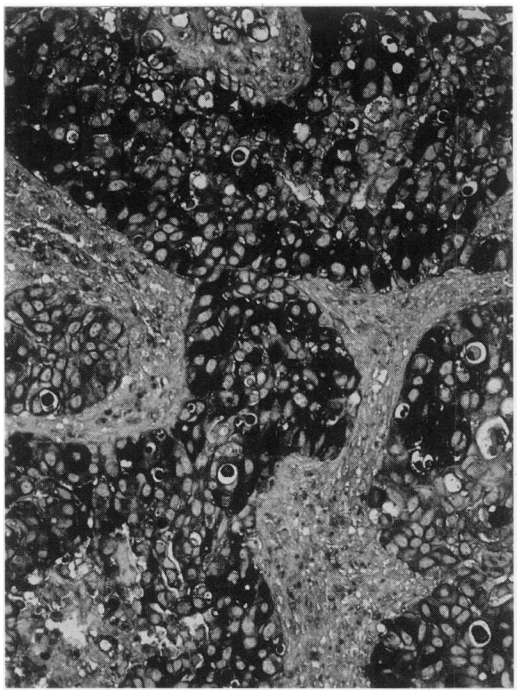

**Figure 3.** The same tumour as Figure 2 showing marked cell to cell variation in the immunoreactivity for GST-$\mu$. ×120.

individual XMEs and the spectrum of enzymes expressed in individual tumour cells (Graham *et al.* 1991).

It is therefore important to identify directly the expression in tumour cells of individual XMEs and in this study the cellular localization and distribution of several major XMEs in primary transitional cell tumours of the urinary bladder has been investigated. Immunohistochemistry was used to investigate the expression of XMEs in bladder tumours as this technique, which can be applied to formalin fixed wax embedded sections, has the sensitivity and specificity to show expression of specific enzymes in tumour cells. In contrast, techniques such as Northern blotting or immunoblotting, which potentially are quantitative techniques, are not capable of showing expression of specific enzymes directly in tumour cells and also require fresh tissue. This study showed that specific XMEs are expressed in bladder tumour cells, there is induction of expression of several enzymes in bladder tumours compared with normal bladder, particularly CYP1A, CYP3A and GST-$\alpha$, and that there is a complex relationship between the phenotypic expression of individual XMEs in bladder tumours.

All the P450s showed induction of expression in bladder tumours and the bladder tumours displayed a

relatively high frequency of expression of CYP1A and CYP3A; in contrast, CYP2C showed a relatively low frequency of expression, being identified in only about one-quarter of bladder tumours indicating there is differential regulation of specific forms of P450 in bladder tumours. Expression of individual forms of P450 including CYP1A, CYP2B and CYP3A has been identified in several distinct types of malignant tumour and the expression of some forms of P450 in tumours may be part of a pleiotropic response to malignancy (Murray *et al.* 1993b; Foster *et al.* 1993; Czerwinski *et al.* 1994). CYP1A is involved in the activation of a variety of potential bladder carcinogens especially those derived from tobacco smoke and the expression of CYP1A in bladder tumours suggests it may have a role in the progression of established tumours by its continued activation of these agents (Gonzalez & Gelboin 1994).

There was frequent expression of epoxide hydrolase in bladder tumours and expression of epoxide hydrolase appears to be characteristic of a variety of malignant tumours (Murray *et al.* 1993a, b). The presence of epoxide hydrolase in bladder tumours provides this type of tumour with a mechanism for removing toxic epoxides formed as a result of P450 metabolism, thus potentially contributing to the drug resistance observed in these tumours.

GST-$\pi$ was the main form of GST expressed in normal bladder and our results are consistent with a previous study which showed that GST activity in normal bladder was mainly due to GST-$\pi$ expression (Singh *et al.* 1991). GST activity has previously been identified in bladder tumours with some tumours showing a high level of GST activity while other tumours displayed low GST activity (Lafuente *et al.* 1990). The specific form or forms of GST responsible for GST activity in those tumours have not been identified and our results would indicate that GST-$\alpha$, GST-$\mu$ and GST-$\pi$ are all likely to contribute to total GST activity (Lafuente *et al.* 1990). However, in several cell lines derived from bladder tumours GST-$\pi$ has been identified as the major form of GST expressed and in each cell line there was much lower expression of GST-$\alpha$ while the expression of GST-$\mu$ was not investigated (Ahn *et al.* 1994). Those findings suggest that cell lines derived from bladder cancer may not accurately reflect the expression of individual GSTs in bladder tumours.

## Acknowledgements

This work has been funded by grants from the Scottish and Home Health Department and endowment funds from Aberdeen Royal Hospitals NHS Trust. RJW was a post-doctoral research fellow supported by The Wellcome Foundation.

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