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# FLUORESCENT LOCATION OF HUMAN COLONIC TUMOUR CELLS BY MEANS OF AN ENZYME-INHIBITOR COMPLEX

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We studied the enzymic status of the tumour cell surface protease, guanidinobenzoatase (GB) in frozen sections of a human colonic tumour grown in nude mice and also in human colons. Active enzyme was demonstrated by the binding of a synthetic fluorescent probe for the active centre of guanidinobenzoatase (GB). It was observed that tissue derived inhibitors of GB blocked the binding of this fluorescent probe and that enzyme inhibitor complex formation could be controlled by lowering the pH of the medium with lactic acid. The presence of an inhibitor of GB in the mouse tumour extract was taken advantage of by making two fluorescent derivatives of this inhibitor; both of which located GB on colonic tumour cells in frozen sections of human colon.

KEY WORDS: Tumour protease, Inhibitors, Regulation, Fluorescence.

## INTRODUCTION

Guanidinobenzoatase (GB) is a protease which is present on the surface of tumour cells.[1,2] This enzyme can be located with the low molecular weight probe 9-amino-acridine (9-AA) which is a competitive inhibitor[2,3], such that cells possessing active fluoresce yellow. In the present study we have followed the interaction of GB with an inhibitor protein extracted from the tumour tissue of a nude mouse carrying a tumour derived from SW707 human colonic tumour cells. This interaction was monitored with 9-AA as a competitive inhibitor of GB. When 9-AA failed to bind to GB this demonstrated that the active centre of GB was masked by the tissue protein inhibitor. The use of two inhibitors of GB, one of which is fluorescent, enabled the interaction of the non-fluorescent protein inhibitor with GB to be studied in an indirect manner. This interaction between GB and protein inhibitor was shown to be pH regulated. The results suggest that colonic tumour cells may regulate the activity of cell surface GB by using the end product of glycolysis, lactic acid. The presence of the tissue protein inhibitor in the frozen sections and the pH regulation of latent enzyme formation were not known when a previous study reported that GB of colonic tumour cells was inhibited *in vivo*.[4]

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*Abbreviations*: 9-AA, 9-aminoacridine; GB, guanidinobenzoatase; TR-E, Texas red labelled mouse tumour extract.



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In this earlier study, it now seems likely that the tissue inhibitor had complexed with the GB during the preparation of the frozen sections.

The knowledge that the tumour sections contained protein inhibitors enabled us to prepare a fluorescent labelled extract of soluble tissue proteins from these solid colonic tumours obtained from the mouse. When this fluorescent labelled extract was placed on frozen sections of human colon, the tumour cell surface GB recognised and bound the fluorescent labelled inhibitor selectively. Thus, the crude extract of the mouse tumour was able to provide a convenient source of a protein for the selective fluorescent location of human colonic tumour cells. At the same time this technique clearly confirmed the presence of both the cell surface GB and the inhibitor, by the direct visualisation of the fluorescent inhibitor.

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## MATERIALS AND METHODS

Human colonic tumour cells SW707 were kindly provided by Dr. M. Herlyn, The Wistar Institute, Philadelphia, Pa., USA and the solid tumours grown in nude mice were generously provided by Professor Dr. G. Sauer of the Deutsches Krebsforschungszentrum, Heidelberg, West Germany. We studied the frozen sections of the solid tumours obtained from the nude mice and frozen sections of 23 different human colonic specimens obtained at surgery. The human colonic sections contained a range of normal colonic mucosal cells, polyps and carcinoma of the colon. 9-aminoacridine, rhodamine B isothiocyanate and Texas red acid chloride (sulfoforhodamine 101 acid chloride) were purchased from Sigma Chemical Company, St. Louis, Mo, USA. Rhodamine and Texas red derivatives of glycine were prepared to establish the degree of fluorescent labelling achieved in the tissue extracts (see later).

### *Frozen Sections*

In this study the human colonic tissues were collected at surgery, and frozen at $-70°$. The frozen specimens were transferred to the laboratory in dry ice and then sectioned on a freezing microtome. The sections were placed on polylysine coated glass slides and were then immediately returned to the microtome chamber. The object was to avoid moisture forming on the surface of the tissue which might allow the extraction of intracellular inhibitors. The sections were stored at $-20°$ until used for fluorescent microscopy.

### *Location of GB with 9-aminoacridine in Frozen Sections*

Sections were placed in a tank containing 9-AA ($10^{-3}$M) in isotonic saline for 2 min. The excess reagent was washed from the slide in isotonic saline followed by immersion for 1 min in a tank of isotonic saline. The sections were examined under a Leitz Diaplan fluorescent microscope employing filter cube [G] and photographed with ASA 400 colour film and filter K490. Cells possessing active GB appear yellow.

### *Inhibition of GB by Tissue Inhibitors*

Protein inhibitors of GB interact with the enzyme at a site distinct from the active centre with the result that the enzyme inhibitor-complex cannot bind[5] 9-AA. In order

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to extract inhibitor from the cut cells of the section, 25 $\mu$l of isotonic saline was placed on top of the frozen section in a wet box for 1 h prior to 9-AA location of active GB. The presence of inhibited GB was inferred when 9-AA failed to locate the tumour cells; this could be reversed by treatment with formaldehyde.[4]

### *Preparation of Fluorescent Proteins from an Homogenate of the Solid Tumour obtained from Nude Mice*

The tumour (about 10 mm diameter) was homogenised in 5 ml isotonic saline and the pH adjusted to 8.0 with sodium bicarbonate solution. The homogenate was filtered and treated with an excess of either Rhodamine B iso-thiocyanate or Texas-red acid chloride for 1 h. The products were then dialysed for 2 h against 300 ml isotonic saline containing 5 g activated carbon to adsorb the diffusing excess reagent. This Texas red extract (TR-E) contained 2 mg protein/ml[6] and the Texas red content was equivalent to $1.29 \times w^{-6}$ M Texas-red glycine as determined using a standard curve for Texas-red glycine.

### *Selective Location of Colonic Cells with Fluorescent Tissue Inhibitors*

Frozen sections from the mouse tumour and the human colons were treated with 10 $\mu$l of these fluorescent extracts (eg TR-E for 10 min), then washed in isotonic saline for 20 min. prior to fluorescent microscopy with the Leitz Diaplan microscope employing filter cube [$N_{2.1}$]. Under these conditions colonic tumour cells with GB (known to be active) fluoresced red on a black background. Inhibition of GB with unlabelled tissue inhibitors prior to the application of TR-E, and subsequent displacement of inhibitor with formaldehyde were carried out as for the 9-AA study.

## RESULTS AND DISCUSSION

### *The Mouse Model System*

Human colonic tumour cells grown in the nude mice possess active guanidinobenzoatase (GB) on their cell surface and fluoresce yellow when treated with 9-AA (Figure 1). The surrounding inflammatory cells from the host tissue do not possess GB and appear blue-green under these conditions of fluorescent microscopy. When the frozen sections of mouse tissue were exposed to 25 $\mu$l NaCl for 1 h, prior to 9-AA treatment, the tumour cells lacked active enzyme and appeared blue-green (Figure 2). Formaldehyde treatment[5] of this slide resulted in the displacement of inhibitor from the GB and a regain in the ability of 9-AA to locate the tumour cells (similar to Figure 1). The above results indicate that although the cell surface GB is active on the colonic tumour cells *in vivo*, the tissues contain easily extractable inhibitor which can interact with the cell surface GB to form a latent enzyme or GB-inhibitor complex.

Detailed experimental data have been presented elsewhere[5] which show that the interaction of the GB with the inhibitor is regulated by pH and that above pH 5.8 latent enzyme is formed; below pH 5.7 the inhibitor cannot react with GB. The presence of active GB on the colonic tumour cells *in vivo* may depend on the cell surface being at a pH of 5.7 or below. This may be achieved by the export of lactic acid produced in tumour cells which show a marked preference for glycolysis.

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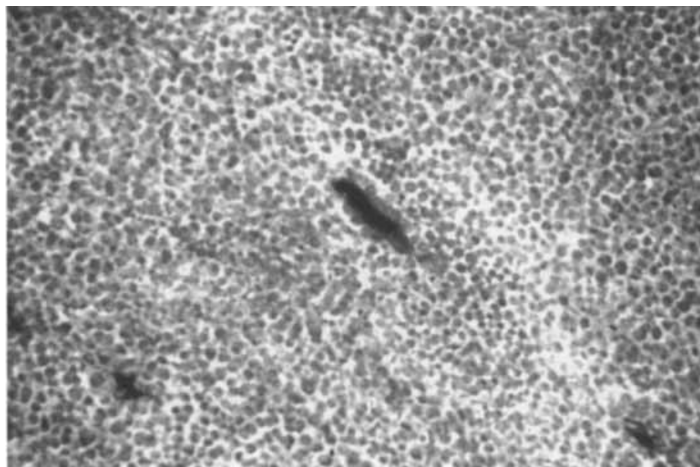

FIGURE 1 Human colonic tumour cells grown in a nude mouse treated with 9-AA. The surface of the tumour cells contains GB which binds 9-AA and the tumour cells exhibit yellow fluorescent ring staining. Magnification × 500. (See color plates at back of issue)

The significance of this pH regulation has only now become apparent. Tissue which is collected at *post mortem* or in which the tumour cells have been unable to maintain glycolysis might well contain latent GB rather than active GB due to the possibility of tissue inhibitors reacting with tumour cell GB. This new finding may explain why in an earlier publication on the enzymic status of GB in colonic tumours, the latent enzyme was observed.[4] We know that freshly excised colonic tumours exhibit active GB (see below) provided local inhibitors within the tissue do not react with the GB.

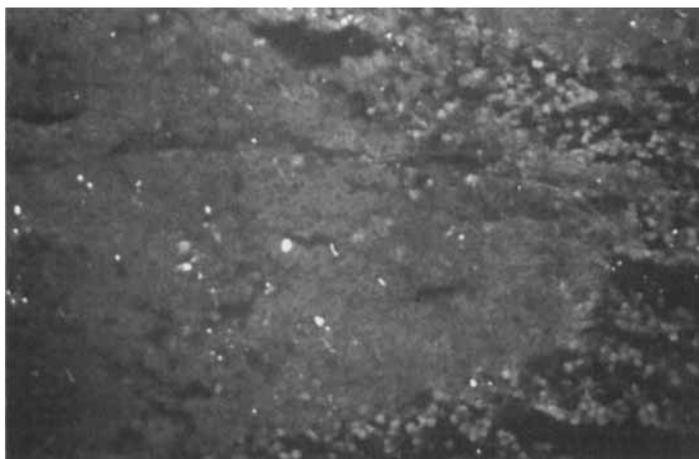

FIGURE 2 Human colonic tumour cells grown in a nude mouse, first treated with NaCl to extract inhibitor of GB and then treated with 9-AA. The tumour cells contain a protein inhibitor of GB which is solubilised in NaCl and then reacts with the tumour cell surface GB resulting in inhibition of GB and failure to bind 9-AA. The yellow dots remaining are mast cells which bind most polycyclic compounds non-specifically. Magnification × 500. (See color plates at back of issue)

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### *Human Colonic Tumour Sections*

We examined 23 different regions of human colonic tissue, frozen sections being prepared immediately prior to fluorescent location of tumour cells. We observed that the mucosal cells of normal colon bound 9-aminoacridine, as did mucosal cells of polyps and the cells of colonic carcinoma. In this paper we will present data largely from one patient which provided particularly clear evidence for the colonic carcinoma cells to illustrate the points we wish to make.

Colonic carcinoma cells possess active GB (Figure 3) which binds 9-AA and

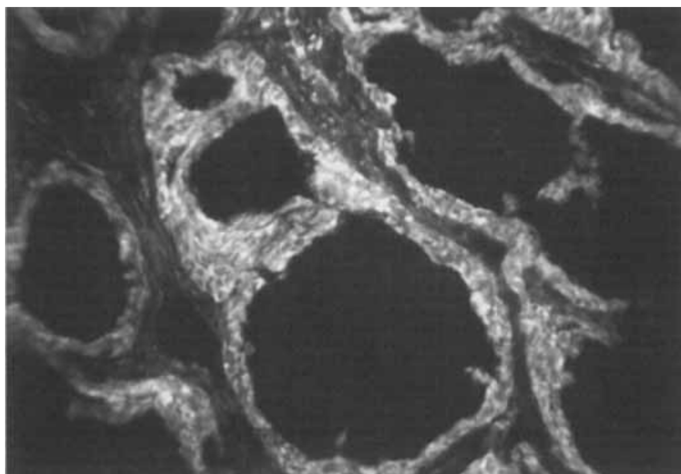

FIGURE 3 Colonic tumour cells in a frozen sections of human colon treated with 9-AA. The colonic tumour cells possess active GB and bind 9-AA which enable these cells to be located by their yellow fluorescence. Magnification × 250. (See color plates at back of issue)

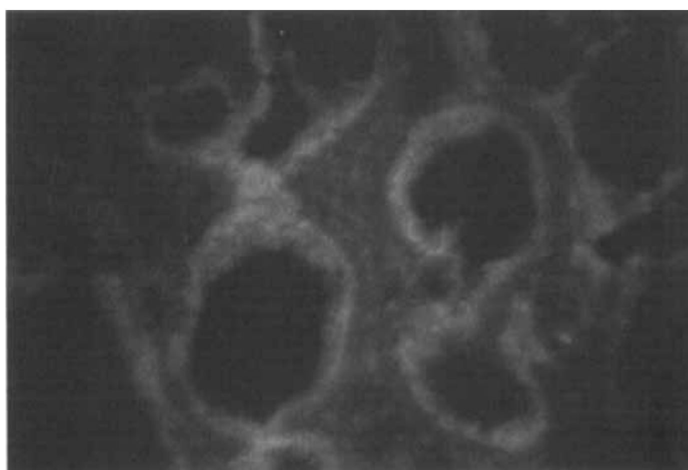

FIGURE 4 Colonic tumour cells in a frozen section of human colon treated with NaCl to extract inhibitor of GB and then treated with 9-AA. The binding of the protein inhibitor to the tumour cell surface GB prevents the binding of 9-AA. This is indirect evidence for the presence of a GB inhibitor protein in the NaCl soluble extract of this section. Magnification × 250. (See color plates at back of issue)

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fluoresces yellow. In an exactly similar fashion to the mouse studies, the human tissue contained readily extractable inhibitors which blocked the subsequent fluorescent location of colonic tumour cells with 9-AA (Figure 4). This inhibitor could be displaced with formaldehyde (data similar to Figure 3).

We used both rhodamine-labelled mouse extract and Texas-red extract (TR-E) to determine whether we could locate human colonic tumour cells selectively with a fluorescent labelled inhibitor extracted from the mouse tumour-bearing tissue. We

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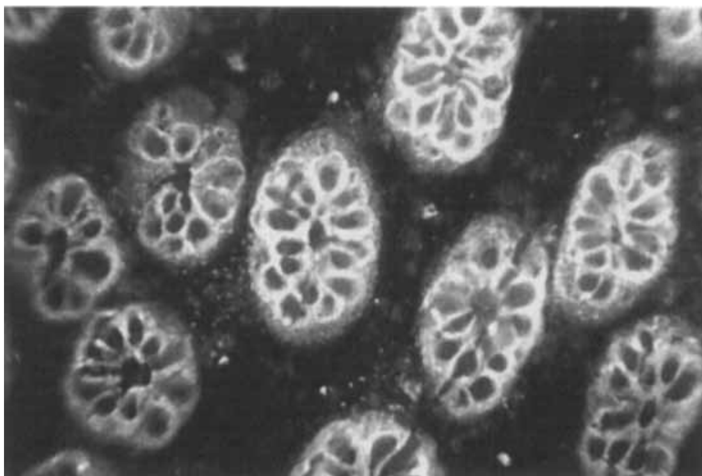

FIGURE 5 Normal human colon frozen section treated with TR-E. TR-E is the Texas-red labelled protein extract obtained from homogenising the human colonic tumour from the nude mouse. Normal mucosal cells possess cell surface GB which is recognised by the TR-E, only the mucosal cells bind the red fluorescent protein. Magnification × 250. (See color plates at back of issue)

FIGURE 6 Human colonic tumour frozen section treated with TR-E. The colonic tumour cells bind the Texas-red inhibitor protein in TR-E and enable the tumour cells to be located by their red fluorescence. Magnification × 250. (See color plates at back of issue)

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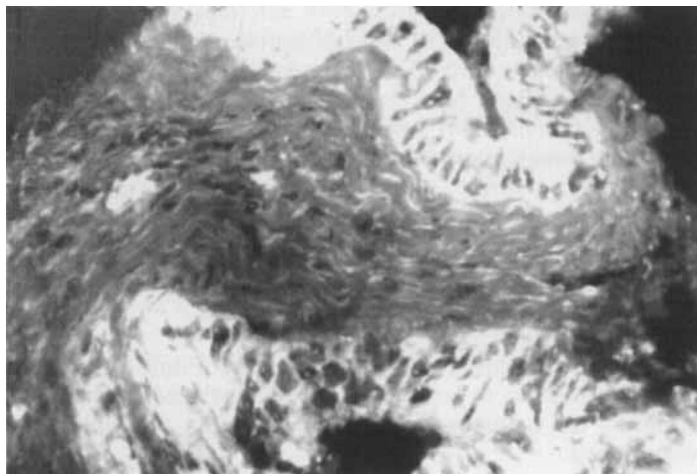

FIGURE 7 Human colonic tumour frozen section treated with TR-E. This is a higher power magnification to illustrate the red fluorescent ring staining of the tumour cell surface, due to the interaction of cell surface GB with the fluorescent inhibitor in TR-E. Magnification × 500. (See color plates at back of issue)

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observed that the rhodamine-labelled mouse extract did locate human colonic tumour cells but that the fluorescence was weak and faded rapidly. The TR-E selectively located mucosal cells; normal human mucosal cells and polyp cells were fluorescent (Figure 5) whilst the colonic tumour cells were strongly fluorescent (Figures 6, 7). Pretreatment of the colonic tumour sections with 25 $\mu$l isotonic saline for 1 h prior to treatment with TR-E resulted in no red fluorescent staining of the colonic tumour cells. This pre-saturation of the GB with unlabelled inhibitor prevented further binding of labelled inhibitor in TR-E. The evidence presented in Figures 5–7 clearly demonstrates that although the mouse tumour extract contained many fluorescent proteins, only the GB on the mucosal and tumour cells recognised a fluorescent protein. Thus the tumour cell surface GB acted as a receptor for its specific Texas-red labelled inhibitor, the latter being in the mixture of proteins referred to as TR-E.

This direct visualisation of GB interaction with a Texas-red protein inhibitor is compelling evidence of the potential role of protein inhibitors in the regulation of cell surface GB activity. We know from other studies[5] that once the enzyme inhibitor complex is formed, this cannot be dissociated by lowering the pH to below 5.7. Since the GB on the colonic tumour cells in the mouse (Figure 1) and human colon (Figures 6–7) is active, although the surrounding cells contain the inhibitor of this enzyme, we must tentatively conclude that the pH at the tumour cell surface is below 5,7 *in vivo*. The most likely mechanism for maintaining this cell surface pH *in vivo* is the export of lactic acid produced in glycolysis. We conclude that a tumour cell associated protease and a soluble protein inhibitor are present in tumour bearing tissues. The interaction of the enzyme with this inhibitor can be demonstrated indirectly by compitition with 9-AA (Figures 1 and 2) and directly by the selective binding of a Texas-red labelled inhibitor from a tissue extract containing many Texas-red labelled proteins. Thus the location of GB on tumour cells with a fluorescent cytosolic inhibitor is analogous to the use of fluorescent antibody techniques in that the cell surface GB is uniquely recognised by a fluorescent labelled inhibitor obtained from these cells. The cultured SW707 lack both cell surface GB activity and inhibition

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activity, yet in the nude mouse sections, both activities are present. This evidence suggests that both the cell surface GB and the inhibitor synthesis by the tumour cells in the mouse must have been induced after implantation of the SW707 cells. Recent studies[6] have shown that single chain tissue type plasminogen activator has the same molecular weight as GB (70,000) and that both the enzymes have the unusual property of degrading 4-methylumbelliferyl-p-guanidinobenzoatase as a substrate.[6] It is probable that these two surface proteases are very similar or even identical. The significance of studies on the inhibition and regulation of GB activity *in vivo* may be greatly enhanced if these two proteases are identical.

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