THE INHIBITOR REACTING WITH A TUMOUR CELL SURFACE PROTEASE CAN BE EXCHANGED WITH PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1)

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Tumour cells possess the cell surface protease guanidinobenzoatase (GB) which can be located by the fluorescent probe 9-amino acridine (9-AA). Frozen sections and formaldehyde fixed sections of tumour tissue were used to demonstrate the interactions between GB, 9-AA and two protein inhibitors of GB. A cytoplasmic extract from the tumour tissue, and a purified inhibitor of plasminogen activator (PAI-1) were shown to be exchangeable components of the enzyme-inhibitor complex on the fixed tumour cell surfaces. The evidence suggests that GB is functionally very similar to plasminogen activator and that this enzyme can be regulated by protein inhibitors *in vivo* and also by changes in the redox potential at the cell surface.

KEY WORDS: Guanidinobenzoatase, Plasminogen activator, Tumour cell.

INTRODUCTION

Tumour cells possess a cell surface protease[1] referred to as guanidinobenzotase (GB).[2] GB has the ability to cleave 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) as a substrate,[2] rather than as an active site titrant.[3] A similar reactivity towards MUGB has recently been reported for tissue plasminogen activator (t-PA),[4] suggesting that GB and t-PA may be similar enzymes. T-PA is one of two immunologically-distinct mammalian enzymes that activate the zymogen plasminogen to yield plasmin.[5] The other, urokinase (u-PA), differs from t-PA in its action on MUGB, which can therefore be used as an active site titrant for u-PA.[4] Cell surface GB is recognised by the protein inhibitors extracted from frozen sections of cells containing GB[6] resulting in an enzyme-inhibitor complex formation which can be dissociated by treatment with 4% w/v formaldehyde solution. This process of inhibition[6] can be followed by the binding of the fluorescent probe 9-amino acridine (9-AA). When the cell surface GB is active 9-AA binds and the cells fluoresce yellow, whilst when the enzyme-inhibitor complex is formed, no binding of 9-AA can take place and the cells fail to bind 9-AA.

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The purpose of the present study is to illustrate two types of molecular recognition between the cell surface GB and, (a) the naturally occurring cytoplasmic inhibitors in frozen sections of tumour tissue, (b) the plasminogen activator inhibitor (PAI-1), the major physiological inhibitor of both t-PA and u-PA,[7] which occurs in plasma, platelets and in a variety of cultured cells.[8]

Both these protein inhibitors recognise the cell surface GB of colonic tumour cells and one type of inhibitor can be replaced by the other. We also draw attention to the effect of redox reactions on the stability of the naturally occurring cytoplasmic inhibitor.

We have used as our model system the GB on the surface of human colonic tumour cells presented in the form of fresh frozen sections. The status of the GB can be followed by fluorescent microscopy after exposure to the fluorescent probe 9-AA which binds to the active centre of GB.[6] The fact that the protein inhibitors in the cytosol of cut tumour cells are readily extractable in isotonic saline requires that the volume of liquid on the surface of the section be strictly controlled. If the volume is kept to a minimum (e.g. $10\,\mu l$) then the inhibitor will make contact with the cell surface enzyme and interact. If the volume of NaCl is large (e.g. 200 ml) the inhibition will not be observed, since the inhibitor diffuses into the large volume and makes little contact with the GB of the cell surface.

MATERIALS

Human colonic tissue containing easily recognisable carcinoma cells was generously provided by the Pathology Department of St. Mark's Hospital, London. Frozen sections ($5\,\mu m$) were freshly prepared in the Department of Rheumatology, University of Manchester. Formaldehyde fixed sections were prepared from the frozen sections by placing them in 4% formaldehyde dissolved in isotonic saline for 1 h, followed by washing in isotonic saline.

Dithiothreitol, potassium permanganate and 9-amino acridine (9-AA) were purchased from Sigma Chemical Co., St. Louis, Mo, USA. The protein inhibitor of tissue plasminogen activator, PAI-1, was purified from human umbilical vein endothelial cell culture supernatant.[9] The purified PAI-1 was activated by treatment with 4 M guanidine,[10] as described previously,[9] to a specific activity of 290,000 U/mg PAI-1.

METHODS

9-AA Staining of GB

Frozen sections and formaldehyde fixed sections were placed in a tank of 200 ml 9-AA (10^{-3} M in isotonic saline) for 2 min. The slides were drained of excess reagent and then washed in a tank of isotonic saline for 1 min. A glass coverslip was placed on the surface of the slide prior to examination with a Leitz Diaplan fluorescent microscope, employing barrier filter K490 and filter cube [G] (catalogue No. 513602). Cells with GB, binding 9-AA, fluoresced yellow under these conditions. We recorded our observations using an Olympus OM-2_N camera with automatic exposure, using Kodak ASA 400 colour film. A selection of these photographs is presented in the results section as colour prints.

Inhibition of GB with protein inhibitors extracted from frozen sections

Fresh frozen sections were covered with 10–15 μl isotonic saline and placed in a wet box for 1 h prior to 9-AA staining. The naturally occurring inhibitors of GB were displaced from the enzyme-inhibitor complex by formaldehyde treatment as described above. Oxidation of the inhibitor was allowed to take place by first exposing frozen sections to isotonic saline (10–15 μl) and then by drying them in air for 2 days or by using $KMnO_4$ (10^{-4}M) in the isotonic saline for 2–4 min. Reduction of the test system was carried out in 10^{-4}M dithiothreitol dissolved in isotonic saline (10–15 μl) for 1 h.

Inhibition of GB with PAI-1

Formaldehyde fixed sections were treated with 10 μl PAI-1 (34.5 μg/ml) for 1 h, washed with isotonic saline and then stained with 9-AA.

RESULTS AND DISCUSSION

In frozen sections the tumour cells possess active GB, bind 9-AA and fluoresce yellow (Figure 1). When frozen sections of tumour tissue were placed in isotonic saline, 200 ml, for 1 h and then stained with 9-AA the tumour cells retained GB activity and fluoresced yellow (data similar to Figure 1). On the other hand, if the frozen sections were covered with a limited volume of isotonic saline, 10–15 μl, for 1 h prior to 9-AA staining, the GB activity was abolished and the tumour cells failed to exhibit yellow fluorescence (Figure 2). Treatment of similar sections to the one shown in Figure 2 with formaldehyde resulted in the recovery of GB activity and the regain of the ability of the GB to bind 9-AA (Figure 3). The results presented above indicate that an inhibitor of GB can be extracted in isotonic saline from the sections and this blocks the binding of 9-AA to the active centre of GB in the enzyme-inhibitor complex. The inhibitor has little chance of reacting with the cell surface GB if the frozen section is equilibrated in 200 ml isotonic saline. The demonstration of inhibition requires the volume of extracting saline to be just enough to wet the surface of the section. Inhibition is clearly competitive with respect to 9-AA binding since if the fresh frozen
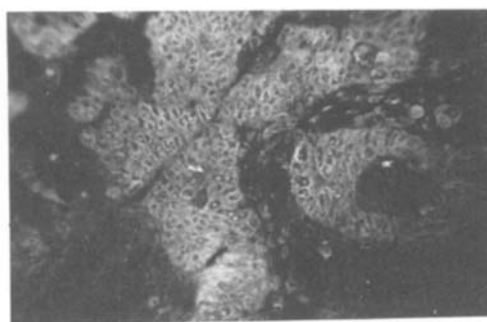

FIGURE 1   Frozen section, direct staining with 9-AA. The tumour cells possess active GB and fluoresce yellow; note the extranuclear ring staining of the GB on these cells. (X250) (See colour plate at back of issue.)
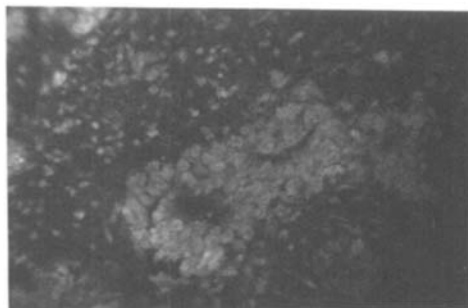
FIGURE 2   Frozen section, inhibition by cytoplasmic inhibitors of GB. The tumour cells lack active GB and cannot bind 9-AA and consequently do not fluoresce yellow. The prolonged exposure needed for photographing these cells accounts for the pale yellowish green colour (due to the use of the yellow barrier filter K490) (X250). (See colour plate at back of issue.)
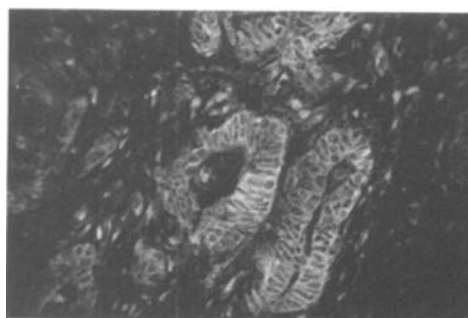

FIGURE 3   Frozen section, inhibited by cytoplasmic inhibitors of GB followed by reactivation of the GB by formaldehyde displacement of the inhibitor. When stained with 9-AA after the cytoplasmic inhibitor step, the cells in the section were negative and lacked active GB (similar to Figure 2). Subsequent treatment with formaldehyde reactivated the GB which now binds 9-AA and the cells again exhibit yellow fluorescence similar to Figure 1. (X250) (See colour plate at back of issue.)

section is wetted with $10-15\,\mu l$ 9-AA (10^{-3} M dissolved in isotonic saline) for 1 h, then the tumour cell GB binds 9-AA preferentially to the inhibitor (data similar to Figure 1).

Previous experiments[6] have shown that it is possible to transfer inhibitors from one slide to another and study their effect on cell surface GB using the 9-AA probe to monitor the enzymic status of GB. When frozen sections of colonic tumour were first exposed to $10-15\,\mu l$ isotonic saline in order to inhibit the GB (Figure 2) followed by displacement of inhibitor (Figure 3) and then exposed to fresh inhibitor obtained from similar fresh sections, total inhibition of the GB resulted (Figure 4). This type of experiment clearly showed that the inhibitors of tumour cell surface GB could be displaced with formaldehyde and replaced by fresh inhibitors on the same cell surface GB. The cytoplasmic inhibitors of GB were reversibly exchangeable; the process being monitored by the yellow fluorescence of 9-AA which locates active GB but not inhibited GB on these cells.

The formation of this GB-inhibitor complex enabled us to study its stability under redox conditions. When the fully inhibited GB (e.g. Figure 2) was exposed to air for

FIGURE 4   Frozen section, inhibited by cytoplasmic inhibitors then reactivation of the GB by for-maldehyde displacement of the inhibitor followed by a second inhibition of GB with transferred cytoplas-mic inhibitors. This section followed the path of the section filmed in Figure 3, except that the reactivated GB was exposed to the NaCl extract of a fresh frozen section. This last step provided fresh inhibitor to block the binding of 9-AA to the active centre of GB due to enzyme-inhibitor complex formation. (X250) (See colour plate at back of issue.)
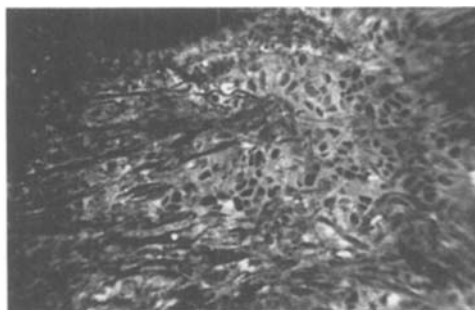

FIGURE 5   Reactivation of inhibited GB by exposure to air. The frozen section was first exposed to inhibitors extracted from the cells[1] cytoplasm in 10–15 μl aCl. The cells in the section failed to bind 9-AA at this stage (data similar to Figure 2). After exposure to air for 2 days, the section was retreated with 9-AA and the tumour cell GB was found to be reactivated, binding 9-AA and exhibiting yellow fluorescence. (X250) (See colour plate at back of issue.)

2 days at room temperature, prior to restaining with 9-AA, it was observed that the GB had regained activity (Figure 5). This evidence suggested that oxidation resulted in dissociation of enzyme-inhibitor complex with loss of inhibitor activity. If this interpretation is correct then the inactive inhibitor should still be present on the dry surface of the section. If the surface of such an air dried section is wetted with 10–15 μl isotonic saline for 1 h prior to 9-AA staining, no inhibition was observed, i.e. the cells bound 9-AA similar to data in Figure 5. Addition of a reducing agent, e.g. 10^{-4} M dithiothreitol, to the 10–15 μl isotonic saline resulted in reactivation of the inhibitor and complete inhibition of the cell surface GB (data similar to Figure 2). Clearly the presence of the reducing thiol reactivated the inhibitor which had previously been oxidised during the 2 day exposure to air.

It was possible to speed up the process of oxidation by employing a solution of $KMnO_4$, 10^{-4} M in isotonic saline. We used fresh frozen sections and exposed these to 10–15 μl of isotonic saline to inhibit the GB, prior to exposure to the sections to
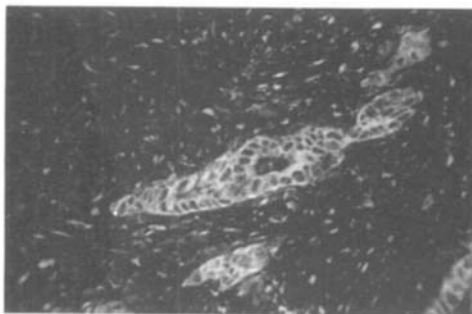
FIGURE 6   Reactivation of inhibited GB by exposure to oxidising agents. The frozen section was first exposed to inhibitors extracted from the cell cytoplasm in 10–15 μl NaCl. The cells in the section failed to bind 9-AA at this stage (data similar to Figure 2). After a short exposure to KMnO₄ the section was retreated with 9-AA and the tumour cell GB was found to be reactivated, binding 9-AA and exhibiting yellow fluorescence. (X250) (See colour plate at back of issue.)

10–15 μl KMnO₄-saline for 2 min. The excess KMnO₄ was removed with an oxalic acid wash and the section treated with 9-AA. Clearly the oxidising agent destroyed the inhibitor and reactivated the cell surface GB (Figure 6). Oxidation had no effect on the ability of cell-bound GB to react with 9-AA but destroyed the inhibitor's ability to form an enzyme-inhibitor complex with GB. We suggest the oxidation of cysteine residues in the inhibitor leads to a conformational change which can be reversed by reduction with the dithiothreitol.

It can be concluded that the frozen sections of colonic tumour tissue contain tumour cell surface GB and an extractable protein inhibitor of GB.[6] This inhibitor can be reversibly displaced from the enzyme-inhibitor complex and replaced by free inhibitor. These reactions can be followed with 9-AA and are subject to regulation through changes in redox potential.

The recent evidence that t-PA cleaves MUGB as a substrate[4] suggested that GB and t-PA might be similar enzymes. We used a specific inhibitor of plasminogen ac-
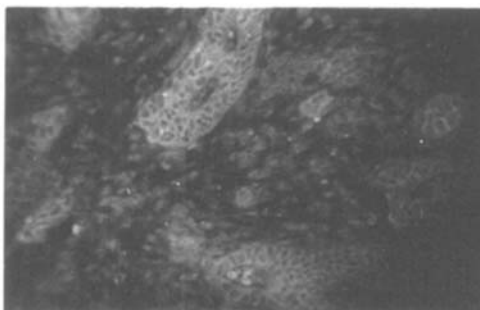

FIGURE 7   Inhibition of tumour cell GB with PAI-1. The frozen section was first treated with for-maldehyde to displace and destroy cytoplasmic inhibitors of GB. At this stage the cells possessed active GB (data similar to Figure 3). The section was then exposed to purified PAI-1 and restained with 9-AA; marked inhibition of GB and very little binding of 9-AA was observed. The prolonged exposure during photography using barrier filter K490 accounts for the yellowish green colour of the tumour cells. (X250) (See colour plate at back of issue.)

tivators, PAI-1, in experiments designed to show whether PAI-1 could replace the naturally occurring inhibitor of the tumour cell surface GB. It was first necessary to remove all the natural inhibitor of GB from the section by treatment with formaldehyde. The cell surface GB on these formaldehyde treated sections was inhibited by exposure to PAI-1 (Figure 7) as shown by the marked reduction in the cells' ability to bind 9-AA (compare Figure 7 with Figure 3). The evidence presented in Figure 7 indicates that PAI-1 recognises the cell surface enzyme GB and by blocking the binding of 9-AA causes loss of cell fluorescence. Since the GB on these cells could be recognised by the natural inhibitor extracted in isotonic saline from these sections, the GB is recognised by both classes of inhibitor, which can be exchanged under suitable laboratory conditions. The binding of PAI-1 to GB on the tumour cell surface also implies that GB is a closely similar cell surface protease to t-PA or u-PA.

Acknowledgements

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