The inhibition of a tumour surface protease in vivo and its re-activation by oxidation

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Summary Colonic tumour cells possess a cell surface protease capable of binding 9-aminooacridine to its active centre, thus locating cells when viewed under a fluorescence microscope. In vivo and in frozen sections, the enzyme is masked by a protein inhibitor. This inhibitor can be displaced by formaldehyde fixation of the tissue and then replaced by adding a fresh extract of colon or lung tissue. The inhibitor is modified by oxidation; provided by air, oxidized glutathione or potassium permanganate, resulting in a change in conformation in the inhibitor and this then results in the enzyme binding the fluorescent probe. The effect of oxidation can be reversed by dithiothreitol. It is proposed that these changes are brought about by a disulphide exchange acting on the inhibitor which indirectly controls the activity of the cell surface enzyme in vivo.

The steps described above can be conveniently followed on sections of tissue mounted on a microscope slide; this has the advantage that the same cells can be monitored during a sequence of reactions. It is believed that these techniques could well be applied to other enzyme systems than the tumour protease described in this study.

This paper concerns a protease which is associated with cells capable of migration and in particular tumour cells (Steven et al., 1985, 1986a,b, 1987; Johnson et al., 1986). The enzyme is referred to as guanidinobenzoatase (Steven & Al-Ahmad, 1983), has been purified by affinity chromatography (Steven et al., 1986b) and has been shown to be a protease, cleaving the arginyl bond in the peptide GlyArgGlyAsp (Steven et al., 1986c) considered to be the link region for attachment of cells to fibronectin (Piersbacher & Ruoslathi, 1984). Previous studies have been concerned with the kinetics of inhibition of guanidinobenzoatase (Steven et al., 1985; Steven & Al-Ahmad, 1983) in solution and the application of these data to the selection of fluorescent competitive inhibitors which may be used to locate cells possessing this enzyme (Steven et al., 1985, 1986a) in formaldehyde fixed, wax embedded sections and resin embedded sections (Steven et al., 1986b). We now present evidence from a study of fresh frozen sections demonstrating the presence of inhibitors of guanidinobenzoatase in vivo which control the activity of this cell surface enzyme. These inhibitors combine with the enzyme to form an inactive latent form of guanidinobenzoatase which is incapable of binding the fluorescent probes used to detect this enzyme. To illustrate this situation, we have chosen to employ frozen sections of human colonic tumours as an experimental system. The evidence is presented in the form of fluorescent micrographs which have been obtained after treating the sections with 9-aminooacridine, a successful probe for the active centre of guanidinobenzoatase (Steven et al., 1985, 1986a). Tumour cells with active enzyme fluoresce yellow (Steven et al., 1985, 1986c) whilst tumour cells with inhibited enzyme appear green-blue and do not fluoresce. The frozen tumour section thus replaces the reaction vessel in monitoring for the presence or absence of an inhibitor in situ on the cell surface protease. This test system has the advantage that the same cells can be repeatedly examined after a sequence of reactions involving the activation or inhibition of a defined enzyme, in this case guanidinobenzoatase. The same principle can be applied to other enzymes, for example alkaline phosphatase (Steven & Burby, 1988), using a suitable fluorescent probe. It would appear that the techniques described in this paper could be applied to cell surface receptors or to any specific molecule with an affinity for low molecular weight ligands.

The results of this study provide evidence for the presence of inhibitors of guanidinobenzoatase in vivo which can be modified by disulphide exchange reactions leading to reversible control of enzymic activity. In this case, the disulphide exchange has been shown to directly affect the structure of the inhibitor so that it can no longer react with the guanidinobenzoatase. Earlier studies (Steven & Podrakzy, 1978a,b, 1979) demonstrated that disulphide exchange had a direct effect on trypsin-like enzymes. The later extensive studies on the role of disulphide exchange in collagenase inhibition confirmed and greatly extended these observations (Tchesche & Macartney, 1981; Macartney & Tchesche, 1983a,b,c,d).

Materials and methods

Fresh frozen sections of human colonic tumours were obtained immediately after surgery. In all we examined 190 frozen sections obtained from colonic tissue taken from 6 patients. Material for wax embedding was fixed in 10% w/v formaldehyde in isotonic saline, embedded, sectioned and dewaxed by conventional histopathological techniques. Formaldehyde treatment of frozen sections was carried out for 18h in 10% w/v formaldehyde in isotonic saline. Homogenates of human lung, liver, heart and colon were prepared by employing an Ultra-Turrax homogeniser on chopped tissue suspended in isotonic saline. The cell debris was removed by centrifugation and the supernatant extract (~1 mg protein ml⁻¹) was used in the inhibition exchange experiments.

Oxidized glutathione, 4-methylumbelliferyl-p-guanidinobenzoate, N-ethylmaleimide, dithiothreitol, potassium permanganate, oxalic acid and 9-aminooacridine were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Aprotinin (trasylo) was a generous gift from Bayer Chemical Company.

Methods

The fluorescent staining technique employing 9-aminooacridine has been fully described (Steven et al., 1985). Essentially, cells which posses guanidinobenzoatase in a form

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which can bind the 9-aminoacridine fluoresce a bright yellow on a blue background.

The displacement of the inhibitor from the latent enzyme was achieved by treatment with formaldehyde (see above) or by treatment for 2 h at 16°C with oxidized glutathione (10⁻² M), dissolved in isotonic saline, followed by 30 min washing in isotonic saline. Treatment with dithiothreitol (10⁻² M) dissolved in isotonic saline was for 2 h followed by a 2 min wash in isotonic saline. Alternatively, oxidation was carried out by placing the slides in 0.1 M potassium permanganate for 2 min followed by bleaching in 1% aqueous oxalic acid for 2 min and raising the pH to 7.4 with isotonic saline containing 0.1 M NaHCO₃ for 2 min. The replacement of inhibitor was achieved by covering the section on the slide with tissue extract and leaving for 18 h at 16°C in a wet box, followed by washing off the excess tissue extract with isotonic saline. After each of these reactions the sections were monitored by staining with 9-aminoacridine.

Simple kinetic experiments were designed to demonstrate inhibition of mouse guanidinobenzoatase (Steven & Al-Ahmad, 1983) as previously described (Steven et al., 1986a) employing 4-methylumbelliferyl-p-guanidinobenzoate as substrate. The object of these experiments was to define whether the inhibition was competitive or non-competitive. In the plot presented in Figure 4 the change in fluorescence due to the production of methylumbelliferone was used rather than the molarity of the product in the values of (I/v).

Results and discussion

Very few fluorescent cells were observed (Figure 1) when fresh frozen sections of colonic tumour were stained directly with 9-aminoacridine and examined by fluorescent microscopy. Yet, when the same or identical sections were pretreated with formaldehyde prior to the staining with 9-aminoacridine, the tumour cells were clearly stained (Figure 2). Similar results to those shown in Figure 2 were obtained when formaldehyde-fixed tissues in wax embedded sections were stained with 9-aminoacridine. It was previously shown that a similar situation was found in pancreatic acinar cells (Steven et al., 1986c) and it was demonstrated that formaldehyde displaced an inhibitor from the guanidinobenzoatase. Affinity systems were used to isolate this protein...
Figure 5 Frozen section exposed to air for 3 weeks at −20°C. Tumour cells exhibit cell surface staining of guanidinobenzoatase. (×250).

Figure 6 Fresh frozen section treated with oxidized glutathione (10⁻² M). The tumour cells now stain strongly. (×500).

Figure 8 Fresh frozen section treated with potassium permanganate 10⁻¹ M. Tumour cells now stain strongly. (×250).

Frozen Section (Fig. 1)

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\begin{align*}
\text{SH} & \quad \text{KmnO}_4 \\
E & \quad \text{GSSG} \\
\text{SH} & \quad \text{O}_2 \\
\hline
\text{DTT} & \quad \text{E} + \text{I} \quad \text{S} \\
\text{E} & \quad \text{Oxidized Enzyme-Inhibitor complex} \\
\text{I} & \quad \text{Binding of probe} \\
\text{SH} & \quad \text{HCHO} \quad \text{Treatment} \\
\text{SH} & \quad \text{HCHO} \quad \text{Formalin-treated Section (Fig. 2)} \\
\text{SH} & \quad \text{WASH} \quad \text{SH} \\
\text{SH} & \quad \text{S} \quad \text{Oxidized Frozen Section (Figs. 5,6)} \\
\text{SH} & \quad \text{E} \quad \text{Loss of I} \\
\text{SH} & \quad \text{E} \quad \text{Tumour Cell Migration} \\
\text{SH} & \quad \text{E} \quad \text{Active Protease} \\
\text{SH} & \quad \text{E} \quad \text{No Inhibitor} \\
\text{SH} & \quad \text{E} \quad \text{Binding of probe}
\end{align*}
\]

Figure 7 Schematic representation of the reversible disulphide exchange mechanism for control of tumour cell surface guanidinobenzoatase. The inhibitor can only bind to the enzyme when in the reduced state.

Inhibitor from pancreatic and liver tissue; the purified inhibitor was then exchanged with the cell surface bound inhibitor (Steven et al., 1986c).

The evidence presented in this study clearly shows that the colonic tumour cells in vivo are unable to bind 9-aminoacridine due to the presence of an inhibitor which can be displaced by formaldehyde without interfering with the enzyme's ability to bind 9-aminoacridine (Figure 2). We believe the formaldehyde either cross-links amino groups leading to an inactive conformation of the inhibitor or modifies thiol groups in the inhibitor with consequent loss of inhibitory action.

We examined the possibility of exchanging inhibitors on the colonic tumour cells. We observed that extracts of human heart and liver did not provide suitable inhibitors to block the arrival of 9-aminoacridine at the active centre of guanidinobenzoatase; the results were similar to those in

Figure 2. On the other hand, extracts of lung and colonic tissue did inhibit the binding of 9-aminoacridine to the surface of tumour cells (results similar to Figure 1). Independent studies showed that the heart and liver extracts were able to block the binding of 9-aminoacridine to other types of tumour (e.g. those found in liver) which indicated the presence of functional inhibitors in these extracts. We were able to demonstrate by kinetic analysis, employing purified guanidinobenzoatase in solution, that the heart and liver extracts contained an inhibitor(s) of this enzyme. It is also possible that variations in the thiol/disulphide concentrations in these tissue extracts could contribute to the observed effect of these extracts on cell surface guanidinobenzoatase activity. We infer from these observations that there are probably iso-enzymes of guanidinobenzoatase, each having the ability to bind 9-aminoacridine but having slightly different secondary and tertiary structures which may be recognised by inhibitors associated with the cells of different tissues. It is hoped to develop this aspect of the work elsewhere. It is sufficient at this stage to state that the inhibition of colonic tumour cell surface guanidinobenzoatase is reversed by formaldehyde and can be regained by addition of fresh inhibitor in the form of the tissue extract. The observation that the natural inhibitor of colonic tumour cell surface guanidinobenzoatase could be replaced by a protein from the lung extract was confirmed by kinetic analysis (Figures 3, 4) of the non-competitive inhibition of guanidinobenzoatase in solution. It might be suggested that
the lung inhibitor was really aprotinin, a protein with a wide range of inhibitory action against proteases. Commercially available aprotinin (trasylo) failed to inhibit guanidino-
benzoatase, either in free solution or on the surface of colorectal tumour cells. Aprotinin could therefore be excluded from the role of the inhibitor present in the lung extract.

The conclusion that the colorectal tumour cell surface guanidinobenzoatase is inhibited in vivo, based on the evidence presented above (Figures 1, 2), raises a number of questions as to how the tumour cell benefits from a latent protease and what chemical mechanism might be exercised by the cell to activate guanidinobenzoatase. Clearly, this protease is not a zymogen activation since the action of formaldehyde is not a known zymogen-activation mechanism and the inhibition has been demonstrated to be reversible by addition of the tissue extracts. We consider it most likely that the observed latency of guanidinobenzoatase could be a form of migratory control mechanism which might be altered by cell metabolism. A similar mechanism involving the pentose phosphate pathway has been described for the control of collagenase inhibition (Tschesc & Macartney, 1981). In the present case, we consider that the extracellular ratio of oxidised to reduced glutathione might provide the conditions for disulphide exchange reactions (Steven & Podraska, 1987a, b, 1979) which could affect the conformation of either the cell surface protease (Macartney & Tschesc, 1982a, b, c, d) or its inhibitor, leading to a change in enzymic potential. In our sections, we could measure changes in enzyme potential by the ability of the cell to bind 9-aminoacridine.

We therefore allowed frozen sections of colorectal tumour to remain at −20°C for 3 weeks in the presence of air and re-examined these aged sections with 9-aminoacridine staining (Figure 5). The tumour cells in the aged sections were now able to bind 9-aminoacridine to their cell surface without needing to displace the inhibitor by formaldehyde treatment (cf. Figure 5 with Figures 1 & 2). Clearly the ageing (possibly by oxidation) had converted latent enzyme (initially unstained) to active enzyme now able to bind 9-
aminoacridine (Figure 5). The hypothesis that an oxidation reaction was involved in this conversion of latent to active enzyme was confirmed by the action of both oxidized glutathione and potassium permanganate. Fresh frozen sections of tumour, when placed for 2 h in oxidized glutathione (10−2 M) dissolved in saline, followed by washing for 30 min in isotonic saline, bound 9-aminoacridine (Figure 6). The tumour cells now behaved as though the tissue had been treated with formaldehyde (see Figure 2) or had been aged (Figure 5). We conclude that oxidized glutathione activated the latent enzyme by an oxidation reaction involving a disulphide exchange mechanism. The oxidized glutathione was shown to have no effect on the ability of formaldehyde-activated colorectal tumour cells to bind 9-aminoacridine (Figure 2). It therefore seemed likely that the oxidizing action of glutathione and air had been on the structure of the inhibitor leading to the inability of the inhibitor to bind to the cell surface enzyme. This suggestion was open to experimental investigation. We know that frozen tumour sections have little ability to bind 9-
aminoacridine and that after prolonged exposure of these sections to air (Figure 5) these tumour cells did bind the fluorescent probe. At an intermediate period (e.g. 5–7 days) the inhibitory moiety of the latent enzyme on the tumour cells is partially oxidized and some binding of 9-
aminoacridine could be observed. Treatment of these partially oxidized sections with oxidized glutathione led to complete oxidation and complete expression of the enzyme’s ability to bind 9-aminoacridine (results similar to Figures 2 & 5). On the other hand, treatment of fully oxidized sections with dithiothreitol (10−2 M) in isotonic saline for 2 h led to the complete suppression of the tumour cells’ ability to bind 9-aminoacridine (results similar to Figure 1). The presence of an excess of reducing thiol caused a reversal of the activation produced by an excess of disulphide or oxidizing agent. Clearly, the latency of tumour cell surface guanidinobenzoatase, as revealed by the ability to bind 9-aminoacridine, can be controlled by a disulphide exchange mechanism. Independent studies showed that this concentration of dithiothreitol had no inhibitory effect on the tumour cells’ ability to bind 9-aminoacridine in formaldehyde treated sections and we conclude that disulphide exchange did not directly alter the properties of the cell surface protease. In the case of latent tumour cell surface guanidinobenzoatase, the effect of disulphide exchange must be a modification of the structure of the inhibitor. This alteration has an indirect effect on the ability of the cells to bind 9-aminoacridine. Oxidation resulted in the inhibitor being unable to bind to the enzyme and could be reversed by the reducing agent, dithiothreitol. These events are diagrammatically represented in the scheme presented in Figure 7. Blocking of free thiols with N-ethylmaleimide did not result in activation of the cell surface latent enzyme which indicated that inhibition was not dependent on free thiols.

It could be argued that the activation of latent guanidino-
benzoatase on the surface of tumour cells by oxidized glutathione might be due to some property of the peptide other than its potential for oxidative disulphide exchange. In order to clarify this question we replaced oxidized glutathione by the oxidizing reagent 0.1 M potassium permanganate, for 2 min in contact with the frozen sections. Treatment of the frozen sections with potassium permanganate demonstrated (Figure 8) the role of oxidation in the conversion of the latent enzyme into the active form of guanidinobenzoatase. Likewise, the addition of the lung extract to the activated enzyme resulted in total inhibition of the ability to bind 9-aminoacridine (same as Figure 1) and this was reversed by treatment with formaldehyde. The observations that exposure to air, oxidized glutathione and potassium permanganate all resulted in cell surface protease activation, whilst none of these agents had any effect on the ability of formaldehyde treated sections to bind 9-
aminoacridine on the surface of tumour cells is presented as evidence that these agents oxidize the inhibitor rather than the enzyme. Kinetic experiments with the solubel enzyme, assayed with 4-methylumbelliferyl-p-guanidinobenzoate, demonstrated that oxidized glutathione and thiols had no direct effect on this enzyme.

Previous studies of colorectal tumour control have been concerned with a disulphide bond linking the inactive enzyme to the inhibitor (Steven & Podraska, 1978a, b, 1979; Macartney & Tschesc, 1983a, b, c, d). In these earlier reports, the active enzyme was directly inhibited by added thiols which mimicked the role of the naturally occurring inhibitors. The present study is novel in that the evidence points to the control of enzymic activity being mediated by a reversible disulphide exchange taking place only in the inhibitor molecule. This oxidative change results in the inability of the inhibitor to bind to the cell surface guanidinobenzoatase and the consequent binding of the fluorescent probe to the cell surface. Since guanidinobenzoatase has been shown to degrade fibronectin (Steven et al., 1986a), an increase in extra-cellular oxidizing potential (e.g. oxidized glutathione) could possibly promote reactivation of latent guanidino-
benzoatase in vivo and increase the potential of tumour cells to migrate. This may relate to the observation that tumour cell proliferation is often associated with a good blood supply.

We believe the observations presented are the first reported examples of the activation of a cell surface protease by means of a disulphide exchange reaction. We also believe that the first example of a disulphide exchange taking place in one molecule (the enzyme) had no inhibitory effect on a second molecule (the enzyme) leading to a control of a biological function of a tumour cell. Although it is not known whether extracellular oxidation reactions in vivo
activate latent tumour guanidinobenzoatase, we have demonstrated oxidized glutathione and molecular oxygen to be capable of such an activation in vitro. We believe that if such activation takes place in vivo, this would enable the protease to degrade fibronectin at the cell surface and thus enhance the cells' metastatic potential. The few positively staining cells observed in frozen tumour sections stained directly with 9-aminoacridine may be examples of cells with metastatic potential in vivo.

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