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OBSERVATIONS ON THE CONFORMATIONAL CHANGES IN THE STRUCTURE OF A CELL SURFACE PROTEASE, FOLLOWED BY ITS ABILITY TO BE RECOGNISED BY COMPETITIVE AND NON-COMPETITIVE INHIBITORS

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Lung tumour cells possess a cell surface protease which can be inhibited by a cytoplasmic protein inhibitor extracted from these cells. The dissociation of this enzyme-inhibitor complex on the surface of tumour cells in sections treated with 10^{-4} M sodium dodecyl sulphate has been studied. The dissociation of the inhibitor and regain of enzymic activity was followed by the use of a fluorescent probe which binds to the active centre of the cell surface enzyme in a competitive manner.

KEY WORDS: Cell surface protease, conformational changes, inhibition.

INTRODUCTION

It has been observed that sodium dodecyl sulphate (SDS) at 10^{-4} M caused the activation of tissue plasminogen activator (t-PA) in the presence of serum inhibitors of t-PA.[1] This activation of t-PA was not observed with the other plasminogen activator, referred to as urokinase (u-PA).[2] Tumour cells have long been known to be associated with increased plasminogen activator activity[3] and a tumour cell surface protease first referred to as guanidinobenzoatase (GB) has now been shown to be closely similar in function to t-PA.[4] Active GB can be demonstrated on the surface of carcinoma cells by the fluorescent probe, 9-amino acridine (9AA) which binds to the active centre of GB.[4] It has recently been shown that 10^{-4} M SDS caused the release of a cytoplasmic derived protein inhibitor (I) from the GB-I complex on the surface of colonic carcinoma cells in frozen sections, with the subsequent regain in GB activity.[5] It is well known that SDS binds to peptide bonds[6] and this property has been extensively employed in reduced SDS polyacrylamide gel analysis of protein molecular weight.[7] The SDS causes an extended rod-like polypeptide to be produced from globular proteins by inducing a massive negative charge and consequently causing extensive conformational changes in the protein which was originally globular in shape.

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Abbreviations: 9-Amino acridine, 9AA; sodium dodecyl sulphate, SDS; guanidinobenzoatase, GB; SDS modified GB, GB*.

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In this study of SDS dissociation of GB-I on tumour cell surfaces, it could be asked whether the SDS has an effect on the conformation of the GB, the inhibitor, or on both of them. The observed fact remains that if the GB was altered it could be refolded to the active form of cell surface GB. We thought that a study of this enzyme on cell surfaces in the presence of SDS might be of interest in both frozen and formaldehyde fixed wax embedded sections, since it might provide information concerning possible conformational changes taking place in membrane bound enzymes, particularly in fixed embedded sections.

We studied the ability of 9AA to bind to GB during and after exposure of the frozen sections to SDS. This equilibrium was also approached from the other side, namely the effect of SDS on GB already loaded with 9AA. These observations were extended to formaldehyde fixed wax embedded tumour sections. In each series of experiments, it was possible to reuse the slides in a sequence of reactions which ensure that molecular interactions were being observed on the same group of tumour cells. An equilibrium was demonstrated in which the GB could be reversibly modified with loss of ability to bind 9AA. This also had the advantage that the sequence of reactions could be confirmed by repeating the whole process on the same slide.

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MATERIALS

9-Amino acridine (9AA) and Sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co. Ltd, St Louis, MO, USA.

Frozen sections of colonic carcinoma, breast carcinoma and squamous cell carcinoma of the lung were generously provided by local Pathology Departments. Wax embedded formaldehyde fixed blocks of lung and colonic carcinoma were also provided. The preparation of the protein inhibitor (I) derived from colonic carcinoma cells has already been described.[5]

METHODS

9AA Staining of Frozen Sections

Three methods were employed in this study:

(*i*) *Direct 9AA Staining* The sections were placed in a tank (200 ml) containing 9AA 10^{-3} M dissolved in isotonic saline for 2 min. Excess stain was drained from the slides and the slides were washed in a tank of isotonic saline for 30 s.

(*ii*) *Half Strength 9AA Staining* This used 0.5×10^{-3} M in place of 10^{-3} M 9AA for 2 min.

(*iii*) *SDS/9AA Staining* The sections were exposed to 0.5×10^{-4} M SDS in isotonic saline containing 0.5×10^{-3} M 9AA. This is the same as half strength staining except that SDS is included with the 9AA; both at half strength. The excess reagent was washed from the slide by a quick dip in a tank of fresh saline or by immersion in a tank of saline for 30 s, as above, depending on the needs of the experiment (see text).

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Treatment of the Sections with SDS

Sections were covered with either 10 μl or 10^{-4} M SDS in isotonic saline alone or with the half strength 9AA/SDS stain for 10 min, followed by a quick rinse in isotonic saline to remove excess reagents. Alternatively, the GB on sections was preloaded with 9AA, by placing the sections in the direct 9AA stain for 10 min and then rinsing in saline, prior to SDS treatment and 9AA staining.

Preparation of Affinity Purified Protein Inhibitor of GB derived from Cultured Colonic Carcinoma Cells

This preparation has already been described.[5] Essentially it requires the extraction of the cytoplasm of cultured colonic carcinoma cells, transferring this to an immobilised GB affinity system, which binds the appropriate inhibitor, followed by the recovery of inhibitor protein (I).

Challenge of Active GB on Colonic Carcinoma Cells with Inhibitor

The inhibitor protein recognizes the carcinoma GB, forming GB-I and inactivates the enzyme.[5] This (I) was used to challenge GB in colonic carcinoma cells which had been exposed to SDS, with the result that the GB had become modified by binding SDS to form GB*. The SDS treated sections were quickly rinsed, dried and exposed to (I) (10 μl) for 15 min. They were then rinsed again and stained directly with 9AA (Figure 4). As a second step, these sections were washed for 10 min in isotonic saline, followed by a second direct staining with 9AA (Figure 5).

After the positive binding of 9AA to the above section (Figure 5), the 9AA was washed from the GB by leaving in a tank of isotonic saline for 20 min. The sections were then shown to lack bound 9AA. They were submitted to a second challenge with (I) (10 μl) for 15 min, followed by direct 9AA staining to determine whether the (I) had recognised the cell surface GB and blocked the binding of 9AA.

Wax Embedded Sections

We employed wax embedded sections of carcinoma of the colon and squamous cell carcinoma of the lung for this study. The sections were dewaxed and equilibrated with isotonic saline before being used and stained as described for the frozen sections.

Microscopy

The sections were immersed in isotonic saline and covered with a glass coverslip prior to microscopy. A Leitz Diaplan fluorescence microscope was employed, fitted with filter cube [G] (Leitz catalogue no. 513602) and barrier filter K490 in place. The new photomicrographic system, Leitz M46 photoautomat, was used for automatic exposure and recording of our results. All photomicrographs were taken using Kodak Ektachrome colour slide film, ASA 400.

RESULTS AND DISCUSSION

In this study, three types of tumour in frozen sections were examined: colonic carcinoma (illustrated below), squamous cell carcinoma of the lung and breast carcinoma. In each case, the GB behaved as described for colonic carcinoma.

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Direct 9AA staining of the frozen colonic sections resulted in fluorescent labelling of the surface of the carcinoma cells (Figure 1). These cells possess active GB, bind 9AA and fluoresce yellow under appropriate microscopic conditions. When these sections were stained with half strength 9AA and the section rinsed before microscopic analysis, the carcinoma cells exhibited intense yellow fluoresence (similar to Figure 1). Treatment of the sections with SDS/9AA for 10 min followed by a quick rinse and microscopic analysis resulted in the complete failure of 9AA to bind to the carcinoma cell surfaces and these cells failed to fluoresce (Figure 2). It could be argued that the presence of SDS caused quenching of 9AA fluorescence; this can be rejected, since washing sections in a bath of isotonic saline for 30 s failed to lead to a regain in yellow fluorescence on the cell surfaces (data similar to Figure 2).

Although the GB on the carcinoma cells failed to bind 9AA in the presence of SDS, the enzyme was still present on the cell surface and could be refolded by washing in a tank of isotonic saline for 2 min, followed by direct 9AA staining (Figure 3). It can be seen that the GB binds 9AA equally well in Figure 1 and Figure 3, but fails to do so in the presence of SDS (Figure 2). The data indicate a reversible conformational change induced in cell surface GB by the action of SDS. We have previously presented data showing a reversible binding of a carcinoma derived protein inhibitor of GB in the presence of SDS.[5] This protein recognises part of the secondary structure of GB as well as the active site. It was therefore of interest to challenge

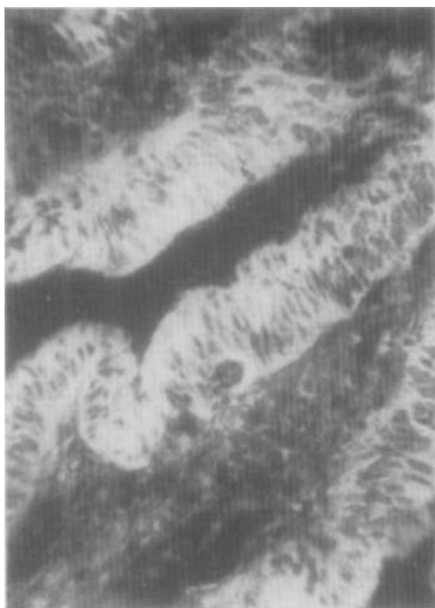

Fig. 1

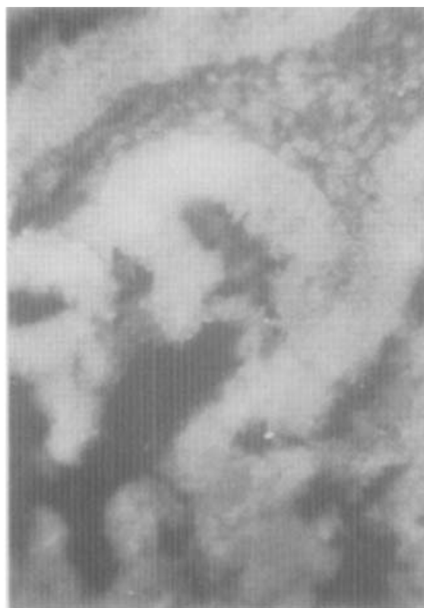

Fig. 2

FIGURE 1 *Frozen section of colonic carcinoma, stained directly with 9AA.* The cell surface GB on the carcinoma cells binds 9AA and the cells exhibit yellow surface fluorescence. Magnification ×250.

FIGURE 2 *Frozen section of colonic carcinoma treated with SDS/9AA for 10 min.* After a quick rinse in isotonic saline, the cell surface GB exhibits no yellow fluorescence. Magnification ×250.

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SDS treated frozen sections of colonic carcinoma (from which the SDS had not been washed out) with this protein inhibitor of GB. In order to do this it was necessary to prepare sections in which the GB was first loaded with 9AA, exposed to SDS, rinsed free of available SDS (similar to Figure 2). This section was then allowed to react with 10 μl of the inhibitor protein for 15 min. At the end of this period, the section was stained with 9AA and observed to be virtually nonfluorescent (Figure 4). This could be interpreted as either (a) inhibition by (I), or (b) a conformational change, preventing both (I) and 9AA binding.

After 10 minutes washing in a tank of isotonic saline, the section was again directly stained with 9AA and the carcinoma exhibited intense yellow surface fluorescence (Figure 5). Clearly, the GB on the SDS treated section was not recognised by (I) immediately after SDS treatment.

When this section was again washed to remove all the bound 9AA and subsequently challenged again with (I), the GB was recognised and 9AA staining failed to cause cell surface fluorescence (data similar to Figures 2 and 4). This demonstrates the binding of (I) to GB, which is not reversed by washing or by 9AA competition. The results obtained with the carcinoma cytoplasmic (I) demonstrate that SDS causes a

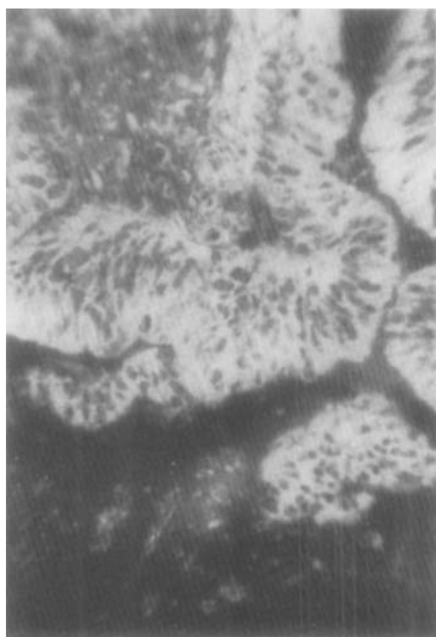

Fig. 3

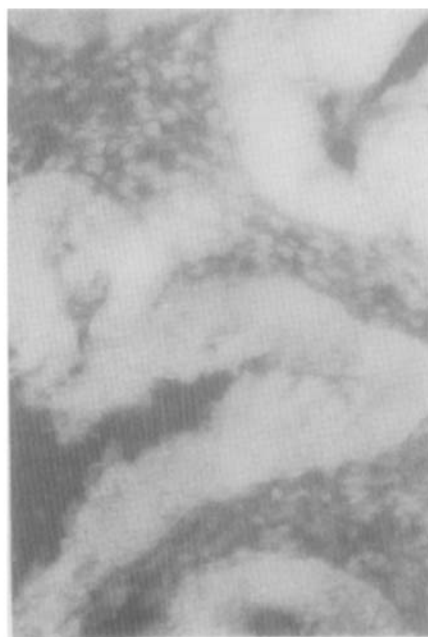

Fig. 4

FIGURE 3 *Frozen section of colonic carcinoma treated with SDS/9AA for 10 min, washed in isotonic saline and stained directly with 9AA.* When the protein-bound SDS is washed out of the slide, the GB refolds to the active conformation and can then bind 9AA again and consequently the surfaces of the cells fluoresce yellow. Magnification $\times 250$.

FIGURE 4. *Frozen section of colonic carcinoma, treated with SDS prior to being challenged with (I) and later stained with 9AA.* When the protein inhibitor, (I), is used to challenge the SDS treated GB, no binding of 9AA is observed. For explanation see Figure 5. Magnification $\times 250$.

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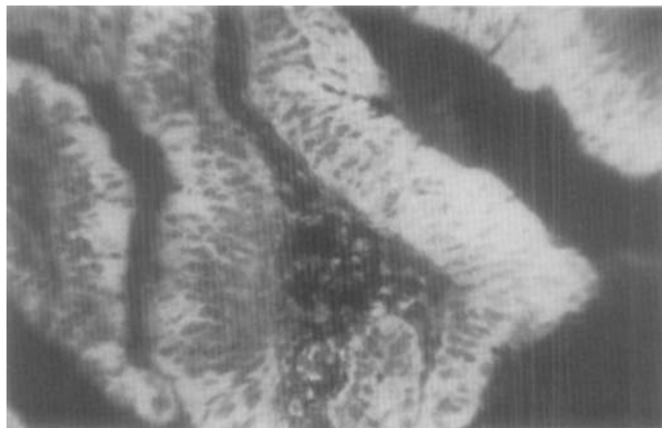

FIGURE 5 *Frozen section of colonic carcinoma used in Figure 4, washed for 10 min in saline and then stained directly with 9AA*. The carcinoma cells now contain GB in an active form which binds 9AA. The washing of the slide used in Figure 4 has resulted in the removal of protein bound SDS and refolding of GB on the tumour cells. If (I) had recognised the secondary structure of GB, then washing would not have resulted in recovery of GB activity. The lack of binding of 9AA in Figure 4 is therefore entirely due to a conformational change in the GB induced by SDS. Magnification ×250.

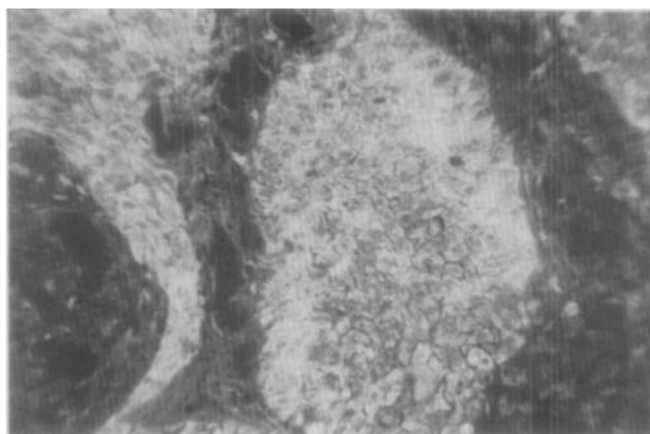

FIGURE 6 *Wax embedded section of lung squamous cell carcinoma stained directly with 9AA*. Squamous cell carcinoma cells have active GB and bind 9AA causing cell surface yellow fluorescence. Magnification ×250.

conformational change in cell surface GB in frozen sections which prevents the binding of both 9AA and (I); unless the SDS is washed out of the system prior to testing with 9AA or (I).

It could be argued that the negative charge on SDS would attract the positive charge on 9AA and that this interferes with the reaction between GB and 9AA. Our results indicate that this is not the case in the present study. As shown above, not only the 9AA but also the (I) fails to recognise the GB in the SDS treated section, unless the section is washed for 2 min in isotonic saline.

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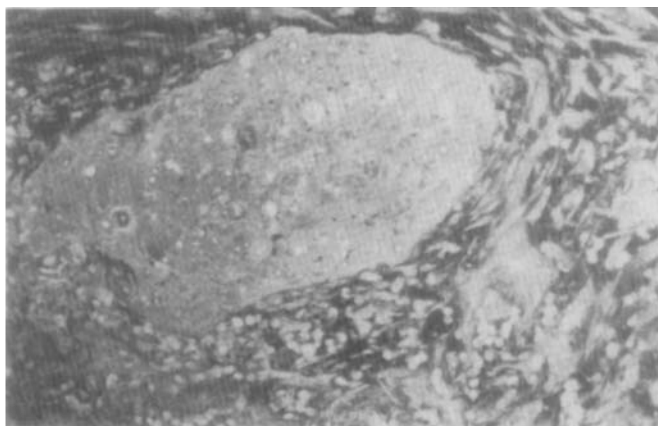

FIGURE 7 *Wax embedded section of lung squamous cell carcinoma treated with SDS/9AA.* The GB on the cell surfaces cannot bind 9AA and the cell surfaces lack yellow fluorescence. Magnification ×250.

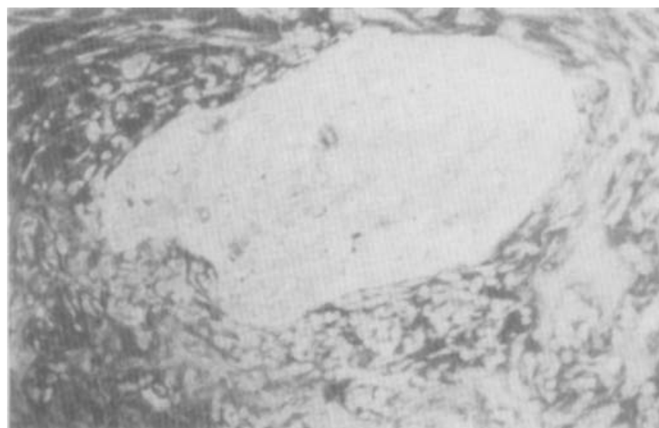

FIGURE 8 *Wax embedded section of lung squamous cell carcinoma, treated with SDS/9AA, washed for 2 min and restained directly with 9AA.* The section used for Figure 7, after washing out the SDS and restaining with 9AA, now exhibited yellow fluorescence and active GB on the surface of the squamous cell carcinoma cells and also on the surrounding unidentified cells. Magnification ×250.

The unfolding of globular proteins by SDS is due to the combined effects of the long hydrocarbon chain and the ionisation of the terminal sulphate group, sodium salts of eicosapentaenoic acid (Smith, Steven and Tisdale unpublished data) and azelaic acid (Steven, unpublished data) at 10^{-3} M have a similar effect to that produced by SDS on the active centre of GB in frozen sections.

Wax embedded sections of lung squamous cell carcinoma behaved in the same way as did the frozen sections, when treated with SDS. Direct 9AA staining of the lung sections located the GB on the surface of the squamous cell carcinoma cells

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(Figure 6) and also on other unidentified activated cells present in the lungs of these patients. Pretreatment with SDS/9AA for 10 min resulted in no fluorescent staining of the carcinoma cells (Figure 7) due to the modification of GB by the SDS. When these sections had been equilibrated with isotonic saline for 2 min followed by direct 9AA staining, the squamous cell carcinoma cells now fluoresced yellow, indicating the presence of reactivated GB (Figure 8). This whole process could be reversed by further treatments with SDS, as was found with the frozen sections.

The data presented above show that SDS can cause conformational changes in cell surface GB, and that these changes can be quickly reversed even in wax embedded sections. This study is of interest since it has shown how much flexibility in the conformation of proteins is still possible in 'fixed sections'.

The use of two inhibitors of GB, one a protein which was nonfluorescent and the other a competitive inhibitor of GB which is fluorescent (i.e. 9AA) enabled these conformational changes to be observed on the surface of cells in thin sections of tissue.

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