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TEMPORARY COMPETITIVE INHIBITION OF A TUMOUR CELL SURFACE PROTEASE AS A PROTECTIVE MECHANISM IN THE PREPARATION OF THE MEMBRANE BOUND NATIVE ENZYME IN THE PRESENCE OF EXCESS CYTOPLASMIC INHIBITORS

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Tumour cells possess a cell surface protease which is recognised and inhibited by a cytoplasmic protein extractable from frozen sections of tumour cells. In order to prepare sections with tumour cells carrying cell surface-bound native protease in the absence of this internal inhibitor we have used a reversible competitive inhibition step as a temporary measure to protect the active centre of GB whilst the cytoplasmic inhibitor is extracted from the frozen sections. These sections are described as *protected* in the sense that the enzyme is native and fully functional now that potential inhibitors have been extracted. The *protected* cell surface protease immobilised in the cell surface of squamous cell carcinoma cells has been used as the target for inhibition studies and displacement studies. The ability to follow these inhibition and exchange reactions concerning the cell surface protease has been made possible by virtue of the fluorescent probe, 9-amino acridine, which locates the active centre of the protease. Cells with active protease bind 9-amino acridine and fluoresce yellow; cells lacking this protease or having inhibited protease fail to bind 9-amino acridine and do not fluoresce.

KEY WORDS: Tumour, protease isoenzymes, inhibition, serum, plasminogen activator.

INTRODUCTION

Tumour cells possess a cell surface protease[1], referred to as guanidinobenzoatase (GB)[2], as well as intracellular soluble protein inhibitors which are capable of recognising the cell surface GB specifically[3]. Interaction of GB with such protein inhibitors leads to the formation of latent GB (or the enzyme-inhibitor complex) which can be artificially dissociated by treatment with 4% formaldehyde resulting in a regain of function at the active centre of GB[3]. In the past, we have been able to study these interactions using formaldehyde pretreated sections in which the GB remained active

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and onto which freshly extracted inhibitor protein could be added[1]. Such studies have been successful in demonstrating such phenomena as isoinhibitors and isoenzymes of GB[4], fluorescent protein inhibitors of GB[1] and the targeting of suitably liganded drugs to the surface of cells possessing active GB[5]. It would be an asset to be able to direct biological and pharmacological molecules to native GB attached to cell surfaces in the total absence of complications arising from the presence of these naturally occurring soluble cytoplasmic inhibitors of GB. The present paper describes a technique employing a temporary inhibition step, using a reversible competitive inhibitor, to block the active centre of GB, during the removal of cytoplasmic protein inhibitors. The competitive inhibitor is then washed from the section and leaves the intact, functional GB still attached to the tumour cell surface. To demonstrate this *protective* inhibition technique, we have employed frozen sections of a well defined oral tumour, squamous cell carcinoma, which contains both cell surface GB and potential inhibitors in the form of cytoplasmic proteins extracted from these frozen sections. In order to locate the active centre of GB we have used the fluorescent yellow probe 9-amino acridine (9-AA), which binds a competitive inhibitor to GB[6] and reveals those cells which possess active GB in the sections when subsequently examined by fluorescence microscopy. Since the binding of 9-AA to the active centre of GB is reversible and concentration dependant, this 9-AA acts as a convenient temporary protection for the active centre of GB when exposed to the cytoplasmic inhibitors. Thus, in the presence of 10^{-3} M 9-AA, the cytoplasmic inhibitors of GB can be extracted into a large volume of isotonic saline, whilst the immobilised GB remains *protected* with 9-AA. Removal of the 9-AA by extensive washing in isotonic saline results in tumour cells within the frozen section possessing cell surface GB in a native form, which can then be studied in the presence of subsequently added specific protein inhibitors of GB or chemically designed active site directed irreversible inhibitors e.g. 1,5-dansyl glutamyl glycyl arginine chloromethyl ketone (DNSGGACK) a molecule with high affinity for the active centre of plasminogen activator[7,8] (t-PA). In this study, native immobilised GB (which has been claimed to be very similar to plasminogen activator previously) has now been shown to be irreversibly inhibited by DNSGGACK and displaced from the cell surface by exposure to fibrin fibrils, which have a high affinity for plasminogen activator[9].

MATERIALS

Frozen sections (3 μm) of squamous cell carcinoma tissue were prepared by Mrs. Angelika Bönisch and generously supplied by Dr. A. Born of the Pathologisches Institut, Im Neuenheimer Feld 220, 6900 Heidelberg - 1, Germany. 9-Amino acridine (9-AA), fibrinogen and thrombin were purchased from Sigma Chemical Co., St. Louis, Mo, USA and the plasminogen activator inhibitor DNSGGACK was obtained from Novabiochem UK, Nottingham NG7 2QJ. Normal human serum was provided by FSS and MMG.

METHODS

Direct staining of Cell Surface GB with 9-AA

Frozen sections were placed in isotonic saline containing 10^{-3} M 9-AA for 2 min; the

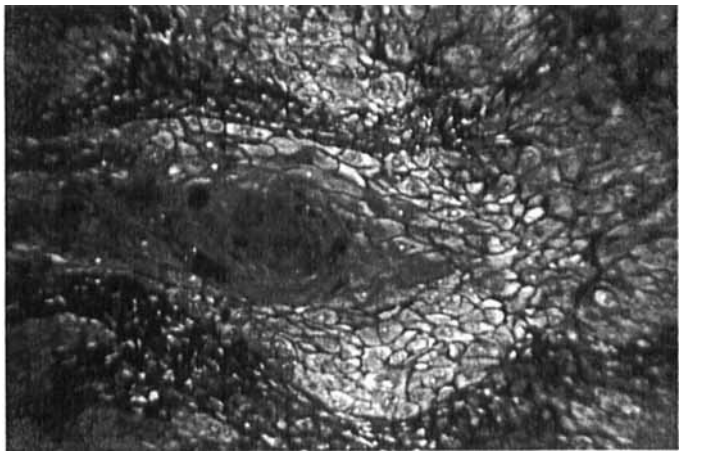


FIGURE 1 Frozen section of squamous cell carcinoma stained with 9-AA directly. Cells possessing active GB bind 9-AA and fluoresce yellow. The central "pearl" consists of keratinised epithelial cells, lacks active GB and fails to bind 9-AA, appearing blue-green. Undifferentiated squamous cell carcinoma cells possess active GB and fluoresce yellow. Magnification × 250. (See colour plate at back of issue).

excess reagent was drained from the slide and the slide washed in a tank of isotonic saline for 1 min (Figure 1).

Inibition with Cytoplasmic Protein Inhibitors

Fresh frozen sections were exposed to 0-15 μl isotonic saline for 1 h, then washed in isotonic saline prior to direct staining with 9-AA as above (Figure 2).

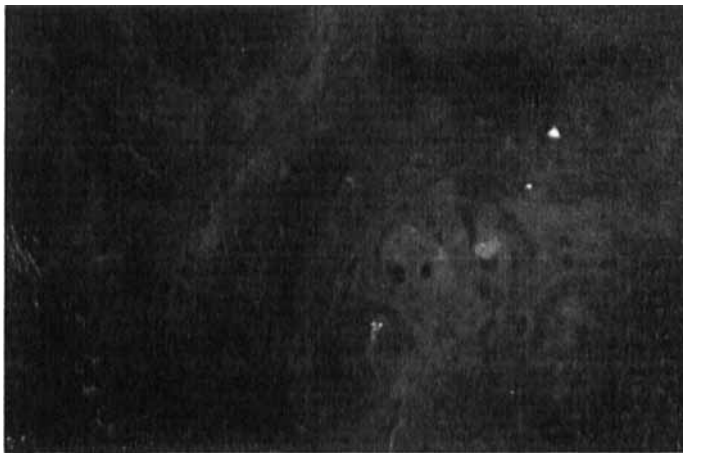


FIGURE 2 Frozen section of squamous cell carcinoma exposed to 10 μl isotonic saline prior to staining with 9-AA. The tumour cells do not bind 9-AA, indicating the absence of active GB due to the formation of latent GB by interaction of the cell surface GB with cytoplasmic inhibitor of GB extracted in isotonic saline. This can be reversed by formaldehyde treatment. Magnification × 250. (See colour plate at back of issue).

Protection of Cell Surface GB by Temporary Competitive Inhibition

Frozen sections of squamous cell carcinoma were placed in a tank containing 300 ml fresh 9-AA (10^{-3} M) in isotonic saline for 1 h. The excess 9-AA was thoroughly washed from the section by placing in 300 ml fresh isotonic saline for 30 min. At this stage, the sections were viewed by fluorescence microscopy and the absence of yellow fluorescence (due to 9-AA) was checked. The sections were then allowed to dry and

FIGURE 3 Frozen section of squamous cell carcinoma after protection by 9-AA. The 9-AA *protects* the GB from the cytoplasmic inhibitor which is extracted into the large volume of 9-AA staining fluid. This section can now be washed free of 9-AA and be ready for studies on the native GB bound to the tumour cell surface in the absence of cytoplasmic inhibitors. Magnification × 250. (See colour plate at back of issue).

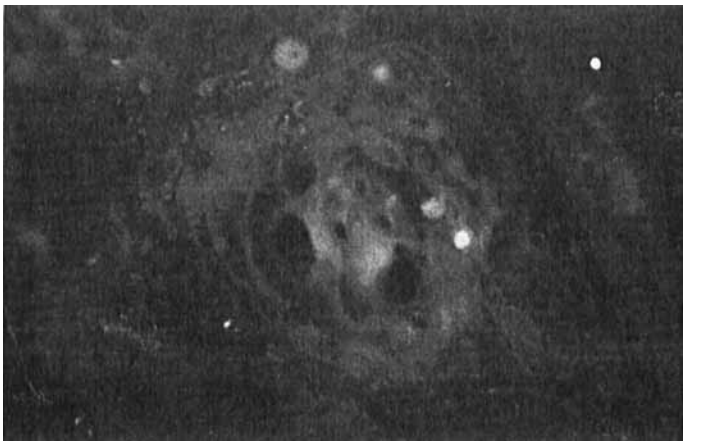


FIGURE 4 Protected GB on squamous cell carcinoma section exposed to transferred cytoplasmic inhibitor and then stained with 9-AA. The GB on the *protected* slide is recognised by the inhibitor extracted from frozen sections of squamous cell carcinoma and the enzyme-inhibitor complex prevents the binding of 9-AA with consequent lack of yellow fluorescence. This can be reversed by formaldehyde treatment. Magnification × 250. (See colour plate at back of issue).

were ready for the study of native GB embedded in the cell membranes of tumour cells and also superficial epithelial cells. We describe such sections as having *protected* GB, in the sense that the GB is native and there is no local protein inhibitor associated with the section. These *protected* slides were stained with 9-AA (Figure 3) and treated with extracted inhibitor (Figure 4) transferred from fresh frozen sections.

Transfer of Inhibitor between Sections

Three fresh frozen sections were each exposed to 10 μl isotonic saline for 1 h, and the extractable inhibitor collected in the combined isotonic saline extracts was transferred by microsyringe to the surface of the *protected* GB section of squamous cell carcinoma tissue. After 30 min the section was washed in isotonic saline and stained with 9-AA (Figure 4). The presence of latent GB was tested for by treatment with 4% formaldehyde for 18 h followed by 9-AA staining.

Inhibition of GB with DNSGGACK

The GB on *protected* sections was exposed to 10^{-7} μl DNSGGACK (10^{-7} M in phosphate buffered saline) for 1 h; the excess reagents were washed from the slide with isotonic saline and stained with 9-AA (Figure 5). The effect of a competitive inhibitor (9-AA 10^{-3} M) included in the 10^{-7} M DNSGGACK was also investigated to see whether the 9-AA protected the GB from the inhibition caused by the DNSGGACK.

Displacement of the Native GB with a Fibrin Clot

Fibrils of fibrin have a high affinity for plasminogen activator[9] and might be used to displace GB from the GB-receptor protein complex found on the cell surface. We therefore exposed these *protected* slides to fibrin fibrils and subsequently stained with 9-AA to determine whether or not the cell surface GB had been removed.

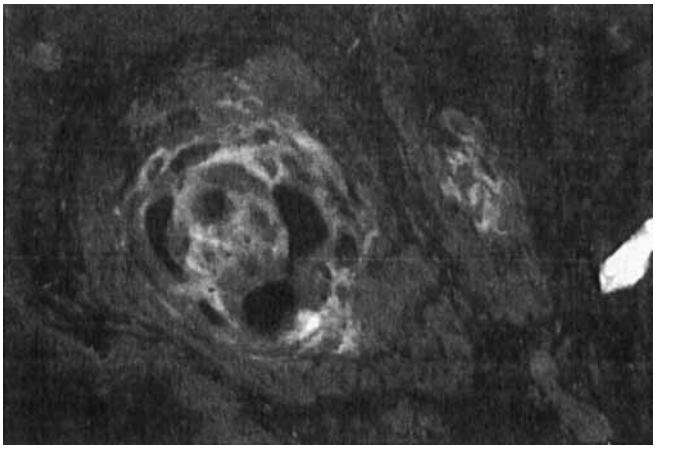


FIGURE 5 Protected GB on squamous cell carcinoma section exposed to 10^{-7} M DNSGGACK followed by 9-AA staining. The DNSGGACK binds to the active centre of the *protected* GB and prevents the subsequent binding of 9-AA with consequent lack of yellow fluorescence. This is not reversed by formaldehyde treatment. Magnification × 250. (See colour plate at back of issue).

A solution of fibrinogen (7 mg/ml) was made up in 0.1 M Tris/HCl buffer pH 7.2. An aliquot (100 μl) of this fibrinogen solution was applied to the surface of the fresh frozen section and 5 μl thrombin solution (10 μg/ml buffer) was very slowly and gently added. Fibrin fibrils formed within 10 min and were left on the section for at least 2 h at room temperature in a wet box. The fibrin clot was then carefully removed under saline and the section was ready for 9-AA staining.

Exposure of Protected Sections to Normal Serum followed by 9-AA Staining

It was considered that any inhibitor in the blood which recognised an isoenzyme of GB would be fast acting since the circulation time of a red cell in the blood is very rapid. Air dried *protected* sections were treated with 10 μl normal human serum for 3 min, washed with saline and then stained with 9-AA for 2 min followed by a wash in isotonic saline for 1 min.

Photographic Recording of Experimental Results

A Leitz Diaplan fluorescence microscope with filter cube [G] (Leitz catalogue no. 513602) was employed and the yellow barrier filter K490 to view the sections after 9-AA staining. We employed Kodak ASA 400 colour film in an Olympus OM-2_N camera fitted with automatic exposure to record our results.

Cells which possessed active GB, bound 9-AA and fluoresced yellow under these conditions. Cells lacking GB or possessing inactive, latent GB lacked yellow fluorescence and appeared pale green but were often recorded as very pale yellow due to the prolonged exposure time necessary to film the low levels of light associated with the minimal amount of fluorescence produced by sections in which GB was absent.

RESULTS AND DISCUSSION

The typical appearance of the differentiated squamous cell carcinoma cells treated with 9-AA in these frozen sections is presented in Figure 1. The central region of keratinised cells (the 'pearl') appears weakly stained with 9-AA, whilst the peripheral cells bind 9-AA and fluoresce yellow. Undifferentiated squamous cell carcinoma cells, which lack keratinisation, bind 9-AA and fluoresce bright yellow (see Figure 3). In the fresh frozen section, the inhibitors of tumour cell surface GB can be extracted in 10 μl isotonic saline, with the consequent inhibition of GB (Figure 2) as judged by the failure of GB in these sections to bind 9-AA. The inhibitor can be displaced from the GB by formaldehyde treatment[3] with a regain in the ability of the cell surface GB to bind 9-AA.

Consideration of the information provided in Figures 1 and 2 led to the concept of competitive temporary inhibition of GB whilst removing the soluble protein inhibitors present in the cytoplasm of frozen sections of tumour tissue. Basically, Figure 1 shows that the GB binds 9-AA rapidly (2 min) whilst in Figure 2 the protein inhibitor takes 30–60 min in the absence of 9-AA to react with cell surface GB.

In the competitive temporary inhibition technique the GB is *protected* from the cytoplasmic inhibitors by placing the section in a tank containing 300 ml 9-AA (10^{-3} M) for 1 h and allowing the inhibitors to diffuse away from the cells into the surrounding large volume of isotonic saline. The protected GB on these cells is still able to bind

9-AA (Figure 3) and should be in the native form rather than an unnatural form such as that formed after formaldehyde treatment. This native form of GB is also recognised by the cytoplasmic inhibitor, extracted in 10 μl saline from fresh frozen sections of squamous cell carcinoma cells, (Figure 4). The presence of latent GB in this section was confirmed by formaldehyde treatment and the consequent regain of ability to stain GB on these cells with 9-AA (data similar to Figures 1 and 3).

This native GB in these *protected* sections was inhibited by exposure to the plasminogen activator inhibiting peptide DNSGGACK at 10^{-7} M (Figure 5) as shown by these cells, subsequent failure to bind 9-AA. The ability of DNSGGACK to block the binding of 9-AA was not reversed by prolonged exposure to 9-AA (18 h). *Protected* sections exposed to 9-AA (10^{-7} M) in the presence of DNSGGACK (10^{-7} M) resulted in *protection* of the active centre of GB from the inhibiting effect of the DNSGGACK (data similar to Figure 3). It would thus seem that DNSGGACK reacts with GB at the same locus as 9-AA, *viz.* the active centre of GB. This would seem to be a second example of protection of the active centre of GB by 9-AA (in a temporary, reversible manner) from the inhibition by DNSGGACK.

The preparation of frozen sections with protected native GB on the surface of tumour cells allows us to extend our studies to the interaction of the GB with the cell surface receptor protein responsible for binding GB to the external surface of the tumour cell. The first step in this direction is to demonstrate the dissociation of the cell surface GB from the receptor by providing a normally encountered physiological system which has a high affinity for GB. We reasoned that if GB and t-PA are similar, or even identical proteases, then GB might have a high affinity for fibrin fibrils, since it is known that t-PA binds avidly to these fibrils with a consequent increase in plasminogen activator activity[9]. We exposed our *protected* GB on these frozen sections to fibrin fibrils and then re-examined the ability of 9-AA to bind to cells in the section (Figure 6). Clearly all the ability to bind 9-AA to the tumour cells in these

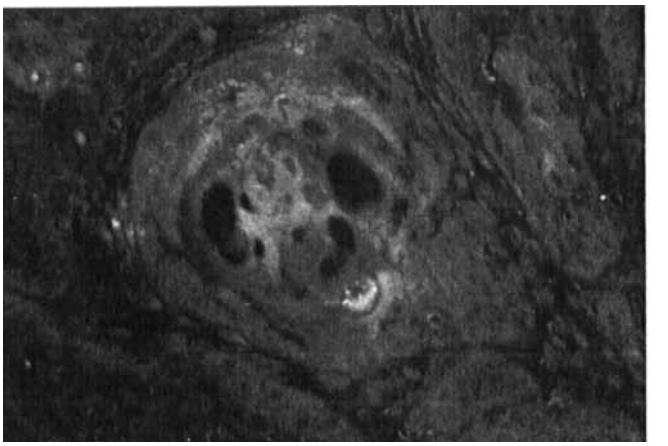
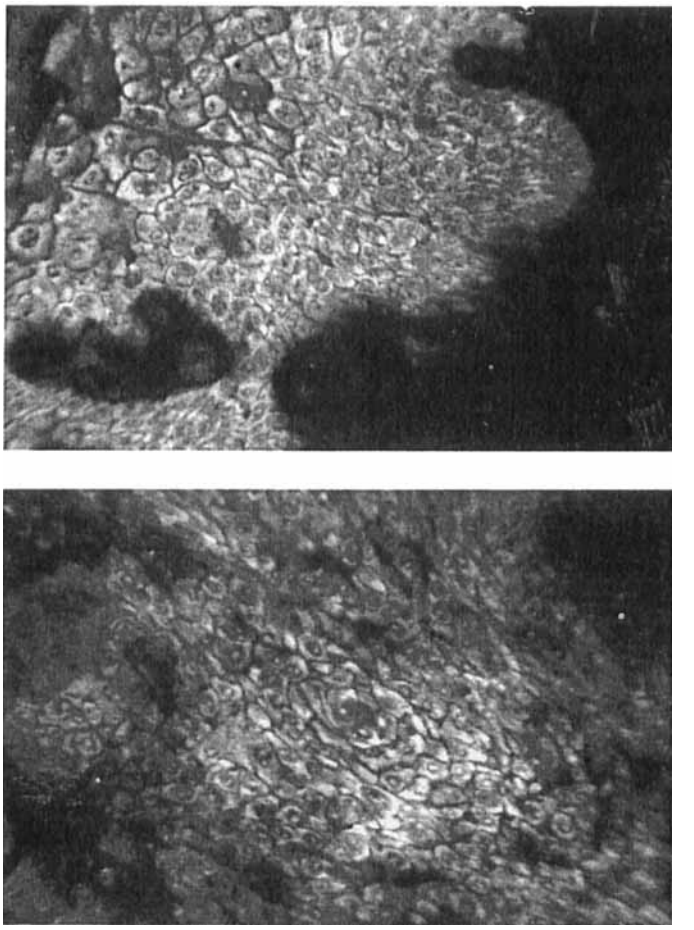


FIGURE 6 Protected GB on squamous cell carcinoma section exposed to fibrin clot followed by 9-AA, this was not reversed by formaldehyde treatment and could not be due to inhibition by a cytoplasmic protein (see Figures 2 and 4). We believe this is due to the displacement of the GB from the tumour cell surface receptor and binding of GB to fibrin fibrils. Magnification × 250. (See colour plate at back of issue).

fibrin treated sections has been lost and this was not regained after formaldehyde treatment. This loss of cell surface GB is believed to be due to the transfer of the GB from the cell surface receptor to the fibrin fibrils.

The use of the competitive inhibitor 9-AA in this temporary protection mechanism has allowed us to exclude the naturally occurring protein inhibitor from forming a complex with cell surface GB and as a consequence has led to these studies of inhibition and displacement of the native enzyme GB still embedded in the cell surface.

The preparation of *protected* GB in the native state still bound to tumour cell



FIGURES 7 AND 8 Protected GB on squamous cell carcinoma section exposed to normal human serum for 3 min followed by 9-AA staining. The normal epithelial cells and infilrating lymphocytes failed to bind 9-AA and do *not* fluoresce yellow. The tumour associated isoenzymic form of GB failed to be recognised by the serum inhibitors and consequently bound 9-AA and fluoresced yellow. In Figure 7, the squamous cell carcinoma cells are shown at their origin (i.e. the basal layers of cells of the epithelium) whilst in Figure 8, the carcinoma cells have penetrated the subdermal layers and are more differentiated, showing the beginnings of a "pearl". Magnification × 250. (See colour plate at back of issue).

surface should provide a good test system for studying the membrane bound enzyme's function.

We believe that this *protected* form of native GB bound to the tunour cell surfaces may also provide us with a mechanism of preparing tumour cells with native receptor proteins ready to receive genetically engineered preparations of t-PA. If these suggestions are correct, the implication is that GB on the cell surface may have a different conformation and function from the GB which has interacted with fibrin fibrils since considerable conformational change must be expected in such a transfer from a receptor site to a fibrin clot.

Normal human serum contains at least seven protein inhibitors of trypsin-like enzymes[10]. Previous studies have demonstrated the presence of isoenzymes of GB on cell surfaces which may be selectively recognised by cytoplasmic protein inhibitors of GB[4]. It is therefore of particular interest that inhibitors present in normal human serum recognise the isoenzymes of GB on normal epithelial cells and on infiltrating lymphocytes but fail to recognise the isoenzymic form of GB associated with squamous cell carcinoma cells in *protected* frozen sections (Figures 7 and 8). The data in Figures 7 and 8 indicate that the use of *protected* frozen sections could possibly be used as a diagnostic method for deciding whether the GB isoenzyme associated with a particular cell type is a normal isoenzyme (recognisable by serum inhibitors) or whether the isoenzyme is an abnormal form of GB associated with neoplasia. This possibility will be examined in more detail in future work.

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