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INTERACTION OF TISSUE PLASMINOGEN ACTIVATOR INHIBITOR WITH CELL SURFACE GUANIDINOBENZOATASE AND UROKINASE PLASMINOGEN ACTIVATOR

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This study employs fluorescent inhibitor molecules to detect both cell surface proteases and their receptor sites on colonic carcinoma cells. Present studies are concerned with the interactions of the tumour associated proteases, guanidinobenzoatase (GB) and plasminogen activators (PAs) with PAs inhibitor type 1 (PAI-1). The active enzymes on the cell surfaces in frozen sections of human colonic carcinoma tissue were located by staining with two active site directed fluorescent inhibitors, 9-aminoacridine (9-AA) and Rhodamine labelled PAI-1 (Rh-PAI-1), followed by fluorescence microscopy. Fibrin treated sections, which now lack GB but have receptor proteins for GB, fail to bind 9-AA and Rh-PAI-1. When these fibrin-treated sections were incubated with purified colonic carcinoma GB and u-PA, both enzymes were bound to the tumour cells in these sections and subsequent challenging with fluorescent probes for GB resulted in bright fluorescence under appropriate microscopic conditions. On the other hand when fibrin treated sections were incubated with t-PA, followed by challenging with Rh-PAI-1, no red fluorescence was observed. It is suggested that the GB and u-PA have similar specific binding sites which can recognise and bind to the receptors on tumour cells in fibrin-treated sections, but t-PA has no such binding site and fails to recognise the cell surface receptors for GB. These GB-receptors may have a possible role in the regulation of GB and u-PA activity during tumour cell invasion and metastasis.

KEY WORDS: Colonic carcinoma, protease inhibition

INTRODUCTION

Tumour cell surfaces possess proteases; the levels of these proteolytic enzymes are significantly enhanced during tumour invasion and metastasis. PAs are serine proteases that convert the proenzyme, plasminogen into active plasmin. Two types of PAs are known, t-PA is a key enzyme in thrombosis and u-PA participates in the breakdown of extracellular matrix proteins involved in cell migration and tissue degradation.[1] The u-PA pathway of plasminogen activation is regulated by PAs inhibitors[2] and cell surface receptors.[3]

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Abbreviations: Guanidinobenzoatase (GB); Plasminogen activators (PAs); Tissue plasminogen activator (t-PA); Urokinase Plasminogen activator (u-PA); Plasminogen activator inhibitor (PAI-1); Rhodamine labelled PAI type 1 (Rh-PAI-1); 9-aminoacridine (9-AA).

GB is a tumour associated protease,[4] now known to be similar to tissue plasminogen activator,[5,12] but not identical to t-PA.[6] Previous studies have shown that the cell surface GB is bound to receptor proteins and can be dissociated by the fibrin-overlay technique from lung squamous cell carcinoma sections.[7] In the present studies evidence has been presented that affinity purified GB extracted from colonic carcinoma or lung carcinoma tissues and commercially available u-PA can bind to fibrin-treated sections which have GB-receptors, but t-PA failed to recognise these cell surface receptors.

MATERIALS AND METHODS

Colonic carcinoma and normal colon tissues were provided by Dr. I.C. Talbot of the ICRF colorectal unit, St. Mark's Hospital, London. Frozen sections were cut in the Histology Department, University of Manchester. These colonic tumour tissues were also used for the extraction and purification of GB.

PD-10 disposable columns were purchased from Pharmacia/LKB, Uppsella, Sweden. 9-AA, and Rhodamine-isothiocyanate B was purchased from Sigma Chemical Co. Ltd; St. Louis, Mo, USA. PAI-1 was kindly provided by Dr Nuala Booth, Department of Biochemistry, the University of Aberdeen. GB was purified from colonic carcinoma tissues as described earlier.[9]

Direct 9-AA staining

Direct 9-AA staining was carried out by placing the slides in 300 ml isotonic saline, containing 10^{-3} M 9-AA for 2 min followed by washing the excess stain from the slides in fresh isotonic saline[10] for 20 s. Protected frozen sections were prepared as previously described[11] to provide cells lacking cytoplasmic inhibitors, but with active GB attached to the cell surfaces.

The fibrin-overlay technique

GB was removed from the colonic carcinoma cell surfaces by the fibrin overlay technique as described earlier.[7] In brief, protected carcinoma sections were overlaid with fibrin for 2 h. Then, the fibrin clot was removed from the cell surfaces. Dissociation of GB from cell surface receptors was checked by 9-AA staining, followed by fluorescence microscopy. These carcinoma cells now lacked GB but retained the cell surface receptor for GB.

Location of plasminogen activators

PAs were located by staining with Rh-PAI-1. Before staining with Rh-PAI-1 the nuclei of the cells in frozen sections of colonic carcinoma tissues were blocked by staining with haematoxylin for 1 min. Rh-PAI-1 staining was carried out by covering each section on slide with 10 μl of fluorescent inhibitor (30 μg/ml) for 30 min, followed by washing the excess stain from the slides in fresh isotonic saline for 5 min.

Coupling of PAI-1 with rhodamine-isothiocyanate

Excess rhodamine isothicyanate B (20 μg/ml) was reacted with the uncharged amino terminal groups of PAI-1 inhibitor (30 μg/ml) in sodium bicarbonate buffer at pH 8.0, for 1 h.[11] The excess rhodamine isothiocyanate was used to ensure the complete conjugation of rhodamine with inhibitor molecules. Dialysis was carried out against isotonic saline (500 ml), for 4–6 h with repeated changes. The residual un-reacted rhodamine isothiocyanate was removed by gel filtration through Sephadex G-25 (commercially available PD-10 disposable column). The high molecular weight rhodamine labelled inhibitor was eluted first, followed by low molecular weight rhodamine isothiocyanate.

Fluorescence microscopy and photography

Sections stained with 9-AA were examined in the Leitz fluorescence microscope with cube G (Leitz catalogue No 513602). The microscope was fitted with an automatic camera and Kodak ALSA 400 colour film was used to record the data. Under these conditions cells with active GB exhibited yellow fluorescence and the cells lacking active GB appeared green. Sections stained with Rh-PAI-1 were examined with cube N (Leitz catalogue No. 513608), and the cells with either active GB or u-PA fluoresced red.

RESULTS AND DISCUSSION

The tumour cells in protected colonic carcinoma sections which possess active GB bind 9-AA and fluoresce yellow (Figure 1). When such sections were treated with the fibrin overlay technique and subsequently challenged with 9-AA, the tumour cells failed to bind the fluorescent probe 9-AA and do not fluoresce yellow under the fluorescent microscope (Figure 2). Clearly the GB on these cell surfaces was dissociated from the GB receptor proteins. It was important to determine whether these GB receptors would recognise and bind different isoenzymic forms of GB, when challenged with these purified proteins. Fibrin treated colonic carcinoma sections were challenged with purified GB isoenzymes (10 μg/ml), isolated from both lung and colonic carcinoma tissues. Binding of GB took place with consequent regain in ability of these cell surfaces to bind 9-AA and fluoresce yellow (Figure 3).

Rh-PAI-1 is thought to be specific for plasminogen activators, but it can also recognise and bind to the colonic carcinoma GB isoenzymes.[8,13] Rh-PAI-1 binds to cell bound PAs and GB and makes these cell surfaces fluoresce red under appropriate microscopic conditions (Figure 4). Similarly, fibrin-treated sections after challenging with Rh-PAI-1, virtually failed to bind Rh-PAI-1 and lacked red fluorescence (Figure 5).

When similar fibrin-treated sections were challenged with two chain u-PA, these sections failed to bind 9-AA (data similar to Figure 2) since 9-AA does not recognize two chain u-PA. However, when these u-PA containing cells were challenged with a second red fluorescent probe, Rh-PAI-1, the tumour cells did recognise this fluorescent inhibitor and consequently these cells now exhibit red cell surface fluorescence (Figure 6). It was of interest that genetically engineered two chain t-PA, incubated with fibrin treated sections followed by challenging with Rh-PAI-1 failed to fluoresce red (data similar to Figure 5).

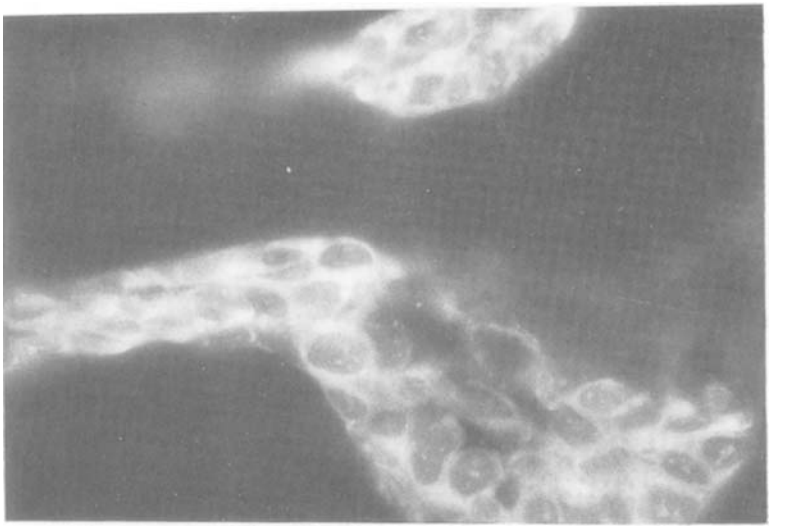

FIGURE 1 Protected frozen section of colonic carcinoma tissue stained directly with 9-AA. The surfaces of the tumour cells which possess active GB, binds 9-AA and fluoresce yellow. The surrounding cells lack this cell surface GB and hence lack fluorescence. Magnification × 250. See Colour Plate I.

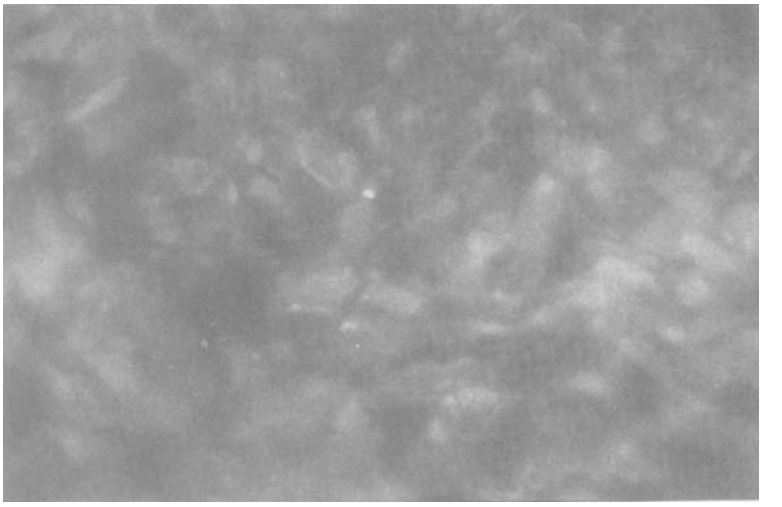

FIGURE 2 Fibrin treated colonic carcinoma sections fail to bind 9-AA and do not fluoresce yellow. Fibrin has removed GB from the cell surfaces and consequently the cells did not bind 9-AA and failed to fluoresce. Magnification × 250. See Colour Plate II.

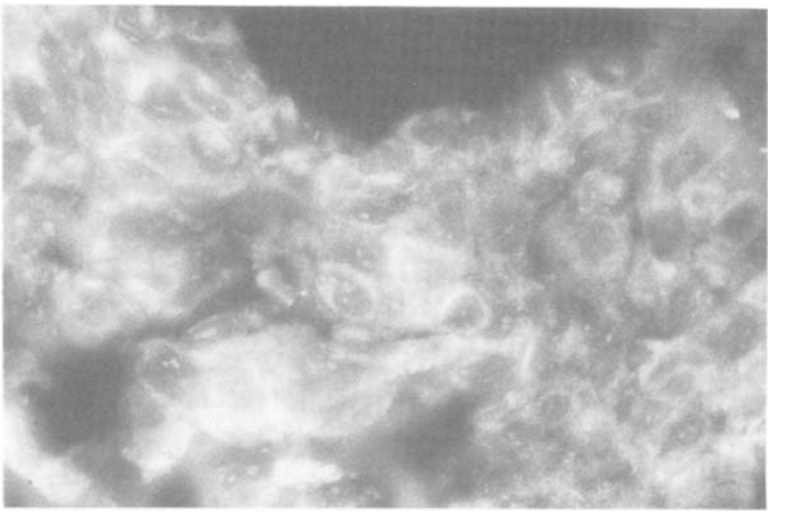

FIGURE 3 Fibrin treated section of colonic carcinoma after incubating with purified lung carcinoma GB isoenzyme for 2 h , followed by 9-AA staining. The cell surfaces in the section now bind 9-AA and fluoresce yellow, indicating that added GB has recognised the receptors and after binding 9-AA, the cell surfaces fluoresce yellow. Magnification × 250. See Colour Plate III.

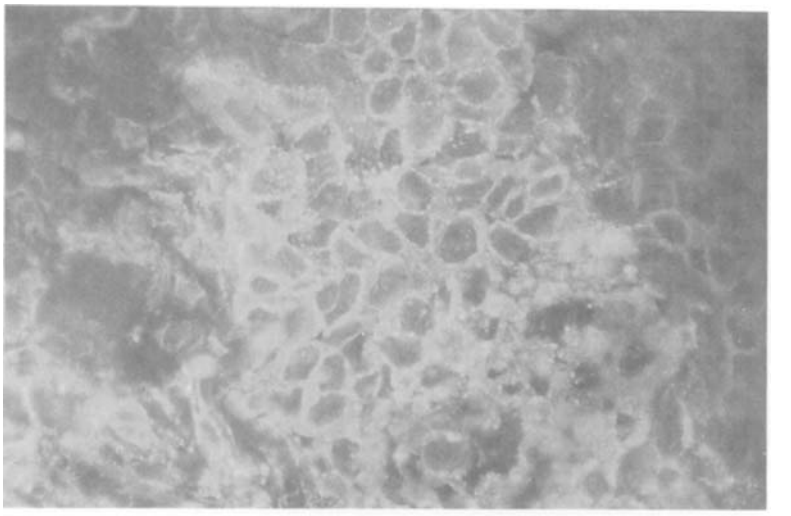

FIGURE 4 Protected frozen section of colonic carcinoma, after staining with Rh-PA-I for 30 min. The tumour cell surfaces of the colonic carcinoma cells having either active PAs or GB, after binding Rh-PAI-1 fluoresce red. The fluorescent probe can locate both enzymes. Magnification × 250. See Colour Plate IV.

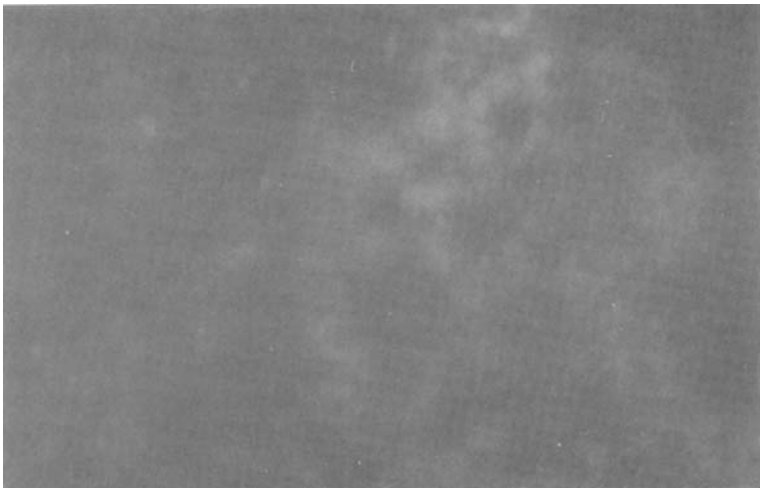

FIGURE 5 Fibrin treated colonic carcinoma section stained wih Rh-PAI-1 for 30 min. The tumour cells surfaces fail to recognise and bind the Rh-PAI-1 and virtually failed to show red fluorescence. Magnification × 250. See Colour Plate V.

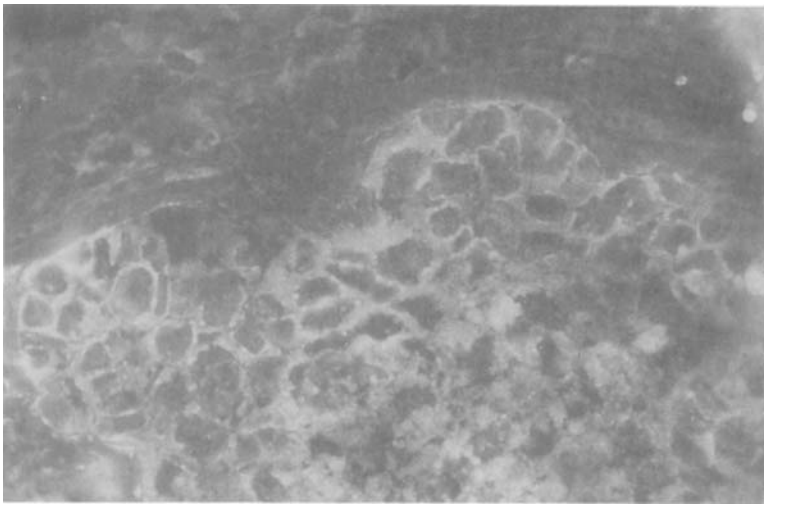

FIGURE 6 Fibrin treated section after incubating with two chain u-PA for 2 h, followed by Rh-PAI-1 staining. The cell surfaces in this section bind Rh-PAI and fluoresce red which indicates that u-PA has recognised the GB receptors and after binding Rh-PAI-1, the cell surfaces fluoresce red. Magnification × 250. See Colour Plate VI.

Thus the selectivity of these two fluorescent inhihitors has led to the location of active GB and u-PA associated with the cell surfaces of colonic carcinoma tissues.

These results were in agreement with the previous observation of Appella *et al.*[14] on PAs. They demonstrated that u-PA has specific binding regions (near to the cysteine-rich part of the growth factor domain) for u-PA receptors. These binding regions can recognise and bind to the cell surface receptor proteins. On the other hand t-PA has no binding regions for the receptors, which may account for the lack of t-PA binding to the tumour cell surfaces described above. Clearly the binding sites of u-PA are similar to those on GB and both enzymes have the ability to bind to the receptor for GB, located on the colonic carcinoma cells.

Previously it has been demonstrated that rhodamine labelled t-PA can recognise GB receptors on the cell surfaces of fibrin-treated normal colon sections.[13] The present results confirmed the previous observations of cell surface receptors on the fibrin-treated sections using colonic tumours, but these results differ in the lack of recognition and binding of two chain t-PA. This discrepancy might be due to the use of a different technique and the single chain t-PA in the previous work, which may have a binding site for GB receptors on cell surfaces.

From the above results, it is concluded that the interactions of two enzymes (GB and u-PA) with the cell surface GB receptors can be demonstrated with yellow and red fluorescent inhibitors which are selectively directed to the GB and u-PA. However, Rh-PAI-1 can also recognise the isoenzymic form of GB associated with lung and colonic carcinoma tissues.[8] GB receptors can recognise and bind both GB and u-PA, which suggests that these receptors may have a role in the regulation of both enzymes during tumour invasion and metastasis. The above data also indicate that the type of GB isoenzyme associated with tumour cell surfaces may be artificially modified by exchange reactions mediated by the fibrin-overlay technique.

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