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DANSYL FLUORIDE, A FLUORESCENT INHIBITOR FOR THE LOCATION OF TUMOUR CELLS IN HUMAN TISSUES

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Dansyl fluoride (Dan-F), an active site directed fluorescent inhibitor of guanidinobenzoatase (GB), has been used for the location of tumour cells in frozen sections of human lung squamous cell carcinoma and colonic carcinoma tissues. The tumour cell surfaces having active GB bind Dan-F and fluoresce blue. The surrounding normal epithelial lung cell surfaces fail to bind Dan-F and hence lack fluorescence, whilst the normal colon cell surfaces have another isoenzymic form of GB, bind Dan-F and fluoresce blue. Kinetic studies have shown that Dan-F is an irreversible inhibitor of GB, and Dan-GB complexes are not dissociated with SDS and high salt concentration. However hydroxylamine (1 M) can dissociate Dan-GB complexes in the presence of 0.1% SDS, both on membrane-bound and in free solution. These studies suggest that Dan-F is a potent inhibitor of GB, and in very low concentration (3×10^{-8} M) can be used as a novel fluorescent probe for the location of tumour cells in histological sections of human tissues.

KEY WORDS: Lung carcinoma, colon carcinoma, cell surface, protease, guanidinobenzoatase

INTRODUCTION

Guanidinobenzoatase (GB) is a tumour associated protease,[1] now known to be similar to tissue plasminogen activator,[2] but not identical to it.[3] Previous studies have shown that active GB can be localised on cell surfaces with an active site directed fluorescent probe 9-aminoacridine (9-AA), a competitive inhibitor of GB.[4]

Dan-F is an active site directed fluorescent inhibitor of serine proteases, which binds to the active centre of these enzymes and inactivates them by sulfonating the serine group, in a highly selective manner.[5] Presently, the interaction of Dan-F with cell surface GB in

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Abbreviations: Guanidinobenzoatase (GB); Plasminogen activator (PAs); 9-aminoacridine (9-AA); Dansyl fluoride (Dan-F); Sodium dodecyl sulphate (SDS); 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB).

sections and free GB in solution has been studied. We have demonstrated that Dan-F is an active site directed fluorescent inhibitor of GB isoenzymes associated with lung squamous cell carcinoma and colon cell surfaces. Dan-F binds to the active centre of GB and make the cell surfaces fluoresce blue, under appropriate fluorescent microscopic conditions. The surrounding normal epithelial cells of lung tissue fail to bind with Dan-F and hence lack fluorescence, whilst the normal colon epithelial cell surfaces having a different isoenzymic form of GB[6] bind Dan-F and fluoresce blue. The normal colon GB isoenzyme can be differentially inhibited by a serum inhibitor and subsequently Dan-F fails to recognise normal colon cells.

Literature studies have shown that Dan-F has never been used for the location of membrane-bound proteases on tumour cells in thin sections. From these studies it is concluded that Dan-F is a potent inhibitor of GB at very low concentrations and can be used as a novel probe for the location of tumour cells in thin sections of lung squamous cell carcinoma and colonic carcinoma tissues.

MATERIALS AND METHODS

Lung squamous cell carcinoma tissues were provided by the Department of Pathology, Wythenshawe Hospital, Manchester. Colonic carcinoma and normal colonic tissues were provided by Dr. I.C. Talbot of the ICRF Colorectal Unit St. Marks Hospital, London. Frozen sections were cut in the Histology Department, University of Manchester. These tissues were also used for the extraction of GB.

PD-10 disposable columns were purchased from Pharmacia/LKB, Uppsella, Sweden. 9-Aminoacridine (9-AA), hydroxylamine, SDS and dansyl fluoride were purchased from Sigma Chemical Co. Ltd, St. Louis, USA. GB was purified from lung carcinoma as described earlier.[10]

Direct Dan-F Staining

Direct Dan-F staining was carried out by placing the slides in 300 ml isotonic saline, containing 3×10^{-8} M, Dan-F for 10 min, followed by washing the excess stain from the slides in fresh isotonic saline for 5 min, with slight modification as described by Steven *et al.*[4] Protected frozen sections were prepared as previously described,[11] to provide cells lacking cytoplasmic inhibitors but with active GB attached to the cell surfaces. Trypsin-like enzymes on the cell surfaces also binds Dan-F, which were completely inhibited by pretreatment of 1 mM MUGB, an active site titrant for trypsin-like enzymes.[13]

Assay of GB by MUGB

GB was assayed with MUGB as substrate and the fluorescent product, methylumbelliferone (MU), was measured by an Aminco-Bowman fluorescence Spectrophotometer.[4] Cleavage of the substrate was monitored at excitation wavelength 323 nm and emission wavelength 446 nm. Inhibition experiments were carried out by preincubating purified GB (10 μg/ml) with variable amounts of inhibitor ($0 - 3 \times 10^{-8}$ M) for 10 min at 37°C, prior to adding the 2×10^{-4} M substrate (final concentration).

Fluorescence Microscopy and Photography

Sections stained with Dan-F were examined with a Leitz fluorescence microscope, fitted with an automatic camera and Kodak ASA 400 colour film was used to record the data. Under these conditions cells with active GB exhibited blue surface fluorescence.

RESULTS AND DISCUSSION

Dansyl fluoride (Dan-F) is an active site directed fluorescent inhibitor of GB. It binds to the cell surfaces in frozen sections of lung squamous cell carcinoma and make them fluoresce blue whilst the surrounding normal epithelial cells which lack GB fail to bind Dan-F and do not fluoresce blue (Figure 1a). The colonic carcinoma and normal colon cell surface GB isoenzymes also behave similarly to the lung squamous cell carcinoma GB; both bind Dan-F and fluoresce blue (Figures 2a, b). If the normal and the colonic carcinoma GB isoenzymes are treated with serum inhibitors for 3 minutes, followed by challenging with Dan-F, the normal colon GB isoenzyme on cell surfaces fail to bind Dan-F and these cells lack fluorescence (Figure 2c), whilst the colonic carcinoma GB isoenzyme remains fully active and after binding Dan-F carcinoma cells fluoresce blue (data similar to Figure 2a).

The above data confirms the earlier claim that normal colonic epithelium cells have an isoenzymic form of GB[6] which is recognised by serum inhibitors after 3 minutes, resulting in the formation of an enzyme-inhibitor complex (GB-I). On the other hand the colonic carcinoma has a different GB isoenzyme which was not recognised and inhibited by serum. The GB-I complex formed by the recognition of the serum inhibitor on the normal colon epithelial cell surfaces was dissociated with 0.3% SDS and the cell surfaces regained their ability to bind Dan-F and fluoresced blue (data similar to Figure 2b).

Previous studies have shown that 9-AA is active site directed fluorescent competitive inhibitor of GB and binds to tumour cell surfaces so that they fluoresce yellow.[4,10] In this study we have observed that Dan-GB complexes are not competitively dissociated by 9-AA and the cell surfaces fail to fluoresce yellow under appropriate conditions (Figure 1b), after placing up to 2 hours in a tank of 9-AA. Furthermore, when these Dan-GB complexes are treated with 0.3% SDS for 1 hour,[7] they are not dissociated and the cell surfaces, after challenging with 9-AA, fail to fluoresce yellow (data similar to Figure 1b). The interactions between colonic carcinoma GB isoenzyme and Dan-F were irreversible and similar to that of GB isoenzyme and plasminogen activator inhibitor PAI-1.[15] However, hydroxylamine in the presence of SDS dissociated both these complexes, and the cell surfaces after challenging with 9-AA fluoresce yellow (Figure 1c).

These results suggest that Dan-F occupies the same binding site on the GB as the 9-AA, and the binding of Dan-F is not reversed by competition with 9-AA or SDS treatment except in the presence of hydroxylamine. This leads to the conclusion that Dan-F is an irreversible inhibitor of GB and forms SDS-stable complexes (unlike GB-serum inhibitor complex, which is dissociated with SDS). These findings are in agreement with the previous observation of Vaz and Schoellmann.[5] They demonstrated that Dan-F sulfonates the active site serine residue of soluble proteases in a highly selective manner. Subsequently, Schoellman[12] showed that human low and high molecular weight forms of u-PA can be covalently labelled with active site directed Dan-F.

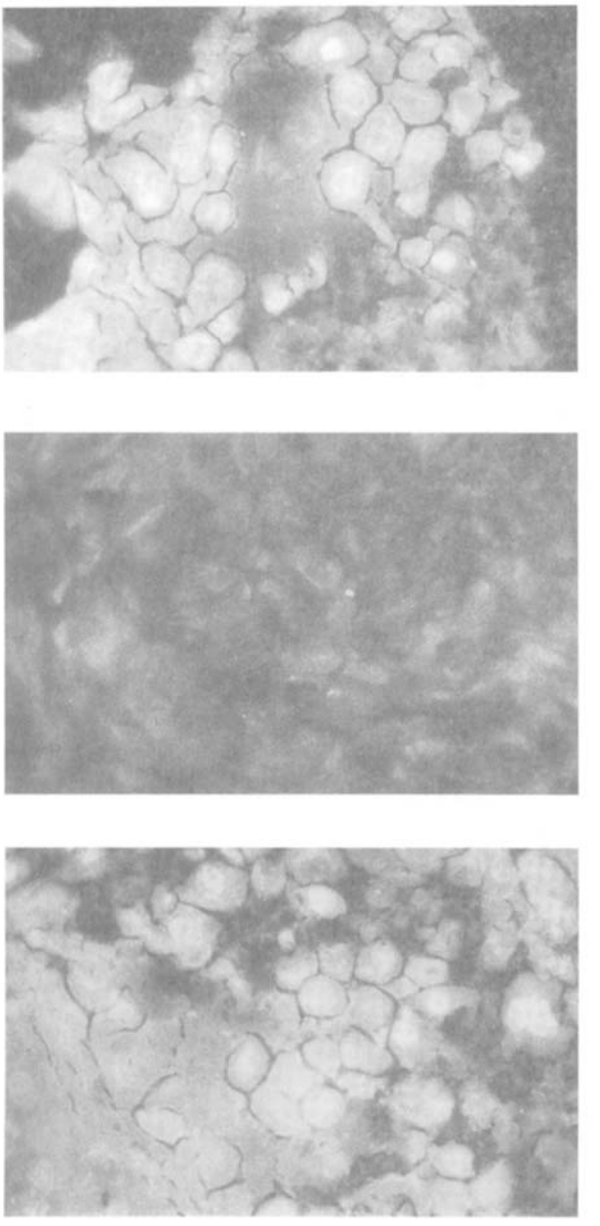

FIGURE 1 Frozen sections of lung squamous cell carcinoma tissue directly stained with Dan-F for 2 min, followed by 5 min wash in isotonic saline. (a) The cell surfaces of lung squamous cell carcinoma possess active GB which binds Dan-F and fluoresces blue. The surrounding normal lung epithelial cells fail to fluoresce blue due to the lack of GB on the cell surfaces. (b) The Dan-GB complex on the lung squamous cell carcinoma cells in this section is not dissociated competitively with 9-AA or SDS treatment and the cell surfaces do not fluoresce yellow. (c) Dan-GB complex dissociate after hydroxylamine and SDS treatment and the cell surfaces after binding 9-AA fluoresce yellow. The cells nuclei in these sections appeared pink due to the pre-staining with propidium iodide. ×750. See Colour Plate I.

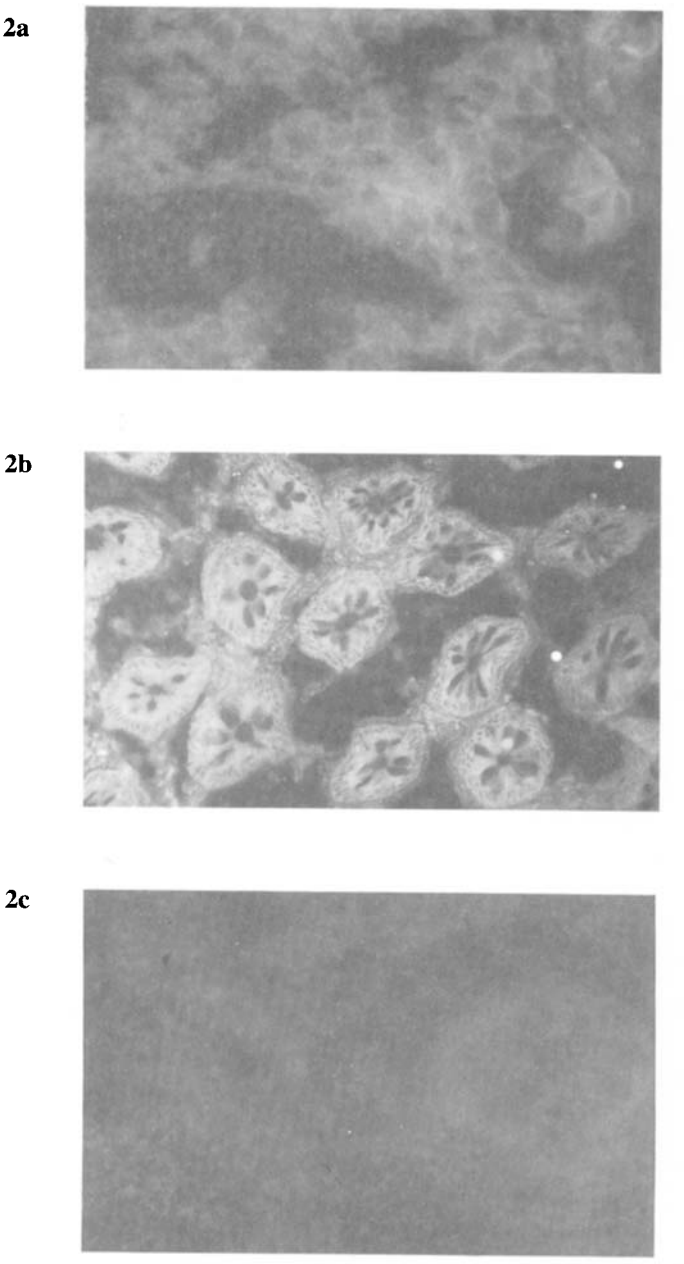

FIGURE 2 Frozen sections of carcinoma and normal colon tissues directly stained witn Dan-F for 2 min, followed by 5 min wash in isotonic saline. (a) The coloic carcinoma cells in section possess active GB which binds Dan-F and fluoresces blue. ×500. (b) The normal colon epithelial cells also bind to Dan-F and fluoresce blue, due to the presence of another isoenzymic form of GB on the cell surfaces. (c) The normal colon epithelial cells in section failed to bind Dan-F after prior treatment with serum inhibitors. ×750. See Colour Plate II.

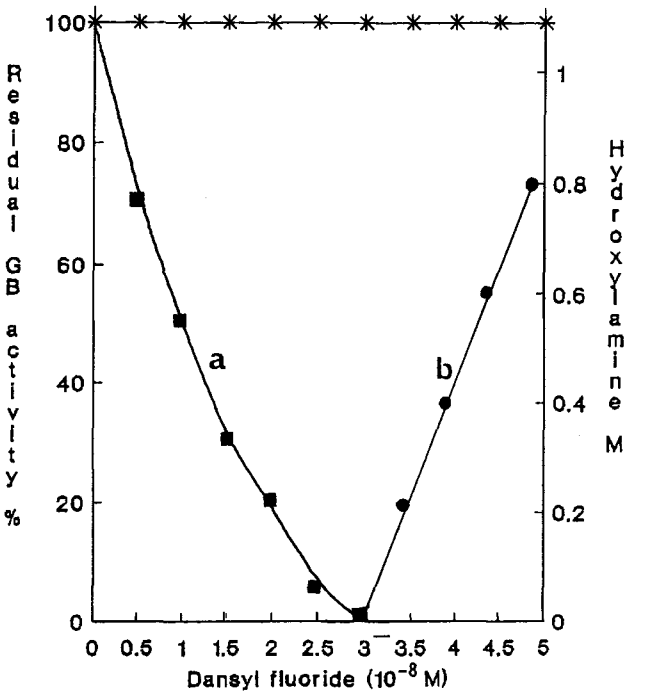

FIGURE 3 MUGB assay of guanidinobenzoatase (10 μg/ml) incubated with the fluorogenic substrate MUGB at a final concentration of 2×10^{-4} M. MUGB was continuously hydrolysed by GB in the absence of Dan-F (–∗–) and was significantly inhibited in the presence of (–■–) Dan-F (3×10^{-8} M). When Dan-GB complexes were incubated with 1 M hydroxylamine in the presence of SDS (0.1%), the enzyme was reactivated due to the dissociation of Dan-GB complexes (–●–).

GB isolated from lung carcinoma[10] continuously hydrolysed the fluorogenic substrate MUGB, an active site titrant for trypsin-like enzymes.[13] Kinetic studies confirmed that Dan-F is a potential inhibitor of GB and causes complete inactivation of GB at a very low concentration (3×10^{-8} M) when assayed with MUGB[1] as substrate (Figure 3a). SDS (0.3%) and NaCl (0.9%) fail to dissociate the Dan-GB complexes, but these complexes can be dissociated with 1 M hydroxylamine in the presence of 0.1% SDS. The GB activity was fully recovered after dialysis, when measured with the MUGB assay (Figure 3b). These finding were in line with those of Fish *et al.*[8] and Wiman *et al.*,[9] that hydroxylamine dissociated serine protease-inhibitor complexes coupled through SDS-stable bonds. Similar observations were made by Levin,[14] who dissociated PA-tPA complexes with hydroxylamine in the presence of 0.1% SDS, which results in reactivation of tPA activity.

From the above results it is concluded that Dan-F is an irreversible potent inhibitor of GB. Dan-F is diagnostically important for the location of tumour cells in frozen sections of lung squamous cell carcinoma and colonic carcinoma tissues.

Acknowledgements

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