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RETINOIC ACID INHIBITION OF A TUMOUR PROTEASE IMMOBILISED ON CELL SURFACES AND IN FREE SOLUTION

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Retinoids are inhibitors of tumour cell proliferation in culture and have been shown to suppress carcinogenesis and decrease the levels of proteases. The present study has demonstrated that retinoic acid is a potential non-competitive inhibitor of a protease (GB) immobilised on the surfaces of tumour cells in thin sections and free GB in solution. The enzymic status of GB on the cell surfaces in sections has been determined by challenging the retinoic acid-treated cells with a second fluorescent inhibitor (9-AA), followed by fluorescence microscopic analysis. The inhibition of cell surface GB by retinoic acid was demonstrated to be reversible. The activity of soluble GB has been measured by the MUGB assay in the presence and absence of retinoic acid. It is suggested that retinoic acid acts on GB by interacting with a binding site, different from the active site, and causes major conformational changes, resulting in enzyme inhibition. It is possible that the modulation of GB activity by retinoic acid may play a role in the control of cell migration and metastasis.

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

INTRODUCTION

Retinoic acid and its precursor retinol are essential growth factors and play important roles in normal growth, night blindness and epithelial differentiation[1,2] and they also possess anti-carcinogenic and anti-tumour activities.[3,4] Retinoids deficiency enhances the susceptibility of many tissues to carcinogenesis.[3] Retinods are also inhibitors of tumour cell proliferation in culture,[5] and have been shown to suppress carcinogenesis and promote the differentiation of several types of tumour cells.[4,6] Selected compounds from this group have been used and are under evaluation in the prevention and treatment of different cancers.

Specific binding proteins for retinol and retinoic acid have been isolated and purified from rat testes.[7] The cells of several malignant tumours of lung and breast contained a retinoic acid-binding protein,[8] but this protein was not detected in the normal tissues of the same patient. The presence of this binding protein suggest that it might mediate the action of retinoic acid in malignant tumours.[8] Furthermore, different sublines of A375 melanoma exhibited a dose-dependent retinoic acid (10^{-8} M – 10^{-5} M)

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Abbreviations: Guanidinobenzoatase (GB); 4-methylumbelliferyl-*p*-guanidinobenoate (MUGB); 4-methylumbelliferone (MU); 9-aminoacridine (9-AA); Tissue plasminogen activator (t-PA); H-D-Ile-Pro-Arg-pNA (S-2288); H-D-Val-Leu-Lys-pNA (S-2251); 4-methylumbelliferone (MU).

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inhibition. Retinoic acid treatment resulted in morphological changes, inhibition of tumour cell invasion of reconstituted extracellular matrix, decreased adhesiveness and decreased level of protease secretions, particularly collagenase and tissue plasminogen activator.[9]

Tumour cell surfaces possess a protease referred to as guanidinobenzoatase (GB),[10] and now known to be similar to tissue plasminogen activator.[11] The cell surface GB can be located by the yellow fluorescent active site directed competitive inhibitor 9-aminoacridine (9-AA). Cells which possess active GB bind 9AA and fluoresce yellow under appropriate microscopic conditions.[12] If a compound interacts with GB in such a manner that the active centre of GB is modified, then 9AA is unlikely to bind to the GB and these cells will no longer exhibit yellow fluorescence. In the present study, evidence has been presented for the inhibition of GB located on the surfaces of lung squamous cell carcinoma in wax embedded sections, which had previously been treated with retinoic acid (10^{-5} M). Kinetic studies have shown that the retinoic acid was also an inhibitor of soluble GB isolated from lung tumours. The activity of GB was measured by fluorogenic substrate 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB), which was continuously hydrolysed by GB.[10]

MATERIALS AND METHODS

Deep frozen blocks of lung tumours, which had been defined as squamous cell carcinoma, were kindly provided by the Department of Pathology, Wythenshawe Hospital Manchester. Frozen sections and wax embedded sections were cut in the Histology Department, University of Manchester.

D-H-Ile-Pro-Arg-*p*-nitranilide (S-2288) and D-H-Val-Leu-Lys-*p*-nitranilide (S-2251) were purchased from Kabi, Sweden. 4-Methylumbelliferyl-*p*-guanidiobenzoate (MUGB), *p*-nitrophenyl phosphate, plasmin, alkaline phosphatase, 9-aminoacridine (9-AA) and retinoic acid were purchased from Sigma Chemical Co. Ltd; St. Louis, Mo, USA. Genetically engineered t-PA was provided by Dr. Neil Bulleid, Department of Biochemistry and Molecular Biology, University of Manchester.

WAX EMBEDDED SECTIONS

We employed wax embedded sections of squamous cell carcinoma of lung for this study. The sections were dewaxed and equilibrated with isotonic saline before being used and directly stained with 9-aminoacridine (9AA).

DIRECT 9AA STAINING

Direct 9AA 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[12] the excess stain from the slides in fresh isotonic saline for 20 s. Formaldehyde treated wax embedded sections, were prepared as previously described,[13] to provide cells lacking cytoplasmic inhibitors, but with active GB attached to the cell surfaces.

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Assay of GB and its Inhibition

GB was assayed with MUGB as substrate and the fluorescent product, methylumbelliferone (MU) was measured by an Aminco-Bowman fluorescence spectrophotometer.[10] Inhibition experiments were carried out by pre-incubating freeze dried GB (20 μg/ml) with the potential inhibitor for 10 min at 37°C prior to adding the substrate (10^{-4}M final concentration). Lineweaver-Burk plots were used to define the type of inhibition.

Assay of t-PA

Tissue plasminogen activator (10 μg/ml) was assayed with 10^{-4} M substrate S-2288 in 50 mM Tris-HCl, pH 7.4 (final concentration) and the yellow product (*p*-nitraniline) was quantified spectrophotometerically at 405 nm. Substrate MUGB was also used to determined the t-PA activity.

Assay of Plasmin

The activity of plasmin was evaluated with the chromogenic substrate S-2251 at 37°C, and pH 7.4 in a buffer consisting of 50 mM Tris-HCl/100 mM NaCl. Substrate hydrolysis was monitored at 405 nm. The final enzyme concentration was 100 mU/ml.

Assay of Alkaline Phosphatase

Alkaline phosphatase activity (17 mU/ml) was measured by using the 10^{-4} M chromogenic substrate, *p*-nitrophenyl phosphate and the product (yellow nitrophenate ions) was measured at a wavelength of 405 nm, pH 9.5 in 0.5 M sodium hydrogen carbonate/sodium carbonate buffer for 15 min.

Isolation of GB by Affinity Column

GB was isolated and purified by agmatine-sepharose affinity column as previously described,[12] with slight modification. Hypertonic saline (9%) was used to displaced the GB from agmatine-sepharose column. Agmatine, the decorboxylation product of arginine was coupled with activated Sepharose-4B by its α-amino group according to the procedure recommended by the manufacturers. Lung tumours were weighed and chopped with scissors in order to disrupt the tumour cells. After centrifugation the pellet was re-suspended in hypertonic saline (9% w/v) for 10 min and centrifuged. The supernatant fluid containing GB activity was applied to the agmatine-sepharose column and the unbound proteins were eluted with isotonic saline and water. The agmatine-bound GB was eluted with hypertonic saline, dialysed and freeze dried for further inhibition studies.

Fluorescence Microscopy and Photography

Sections stained with 9-AA were examined in the Leitz fluorescence microscope with cube G (Leitz cataloge No 513602). The microscope was fitted with an automatic camera attached and Kodak ASA 400 colour film was used to record the data. Under these conditions cells with active GB exhibited yellow surface fluorescence.

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RESULTS AND DISCUSSION

Formaldehyde treated wax embedded sections, which lack local inhibitors but have active GB, were directly stained with 9-AA. The cells possessing active GB bind 9-AA and fluoresce yellow (Figure 1). When sections were treated with retinoic acid (10^{-5} M) for 30 min , followed by 9AA staining, the surfaces of the tumour cells failed to fluoresce yellow (Figure 2), indicating that GB was inhibited by interacting with retinoic acid and the active centre of GB could not bind 9-AA. The sections were then treated with SDS (10^{-4} M) for 1 h and after washing with iso-tonic saline, re-stained with 9-AA. The SDS solution was used to extract retinoic acid from the GB. The cell surface GB regained its ability to bind 9-AA and fluoresced yellow (Figure 3).

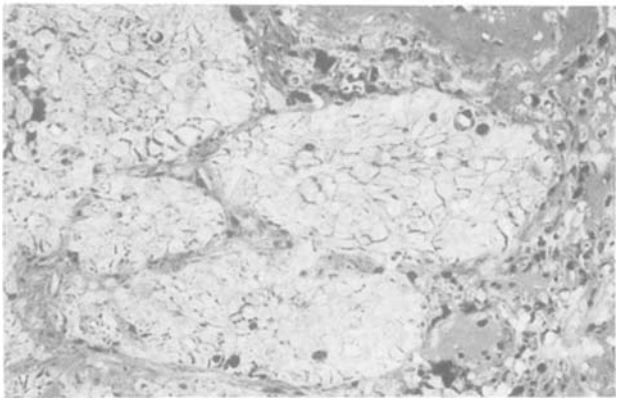

FIGURE 1 Formaldehyde fixed wax embedded section of lung squamous cell carcinoma tissue directly stained with 9-AA. The surfaces of the tumour cells possess active GB, bind 9-AA and fluoresce yellow. The surrounding cells lack this cell surface GB and hence lack fluorescence. Magnification × 250.

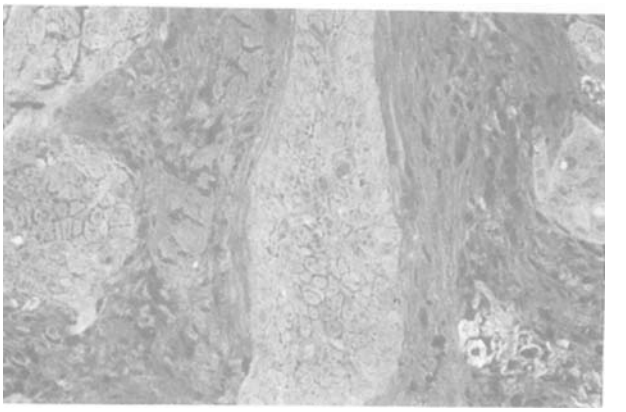

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FIGURE 2 Formaldehyde fixed wax embedded section, after treatment of retinoic acid for 30 min, followed by 9-AA staining. The cell surfaces of the lung carcinoma cells fail to fluoresced yellow, due to the change of conformation of GB on the surfaces of cells, and 9-AA did not bind this section. Magnification × 250.

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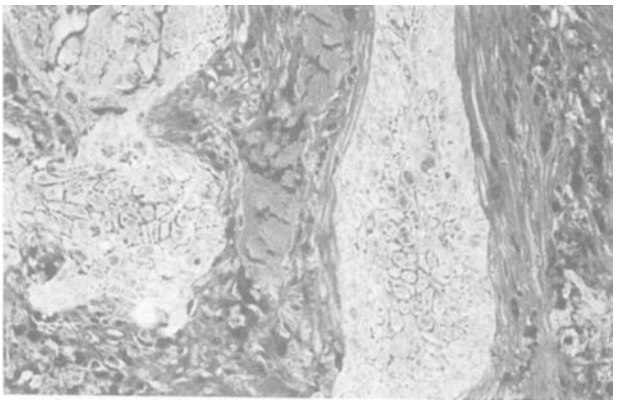

FIGURE 3 Section shown in Figure 2, after displacement of retinoic acid with SDS (10^{-4}M), followed by 9-AA staining. The GB on the lung squamous cell carcinoma in this section, after displacement of inhibitor from the regulatory site with SDS, regained its ability to bind 9-AA, and now fluoresced yellow, similar to Figure 1. Magnification × 250.

These results suggest that like SDS,[14] retinoic acid changed the conformation of GB by binding to the hydrophobic part of the polypeptide chain, causing conformational changes. Consequently, GB failed to bind 9-AA and the cell surfaces lacked yellow fluorescence (Figure 2). When these sections were put in a large tank of 9-AA for 2 h, retinoic acid was slowly washed out and, after refolding, the cell surface GB regained its ability to bind 9AA and fluoresced yellow (data similar to Figure 3). Unlike SDS,[14] retinoic acid could not be washed out immediately in isotonic saline, due to its insolubility in water. Thus the effect of retinoic acid on the conformation of GB was longer lasting than SDS which can be washed out in 30 s in isotonic saline. In the above experiment, we used SDS after retinoic treatment, in order to facilitate the exchange of retinoic acid from cell bound-GB to the aqueous phase, in which the retinoic acid is rather insoluble in the absence of a detergent such as SDS.

Kinetic studies have shown that GB isolated from lung carcinoma tissue was capable of cleavage of MUGB, an active site titrant for trypsin like enzymes,[15] with a consequent release of the fluorescent product, methylumbelliferone (MU), as shown in Figure 4. The production of MU was linear with the duration of the experiment. The effect of retinoic acid suggests that it caused a dose dependent inhibition (Figure 5).

Hendrix *et al.*[9] demonstrated that retinoic acid (10^{-5} M) decreased the secretion of t-PA and collagenases in different human melanoma cell lines *in vitro*. Separate experiments here have shown that t-PA, plasmin and alkaline phosphate are not inhibited by retinoic acids (Table 1) but that GB is inhibited by incremental addition of retinoic acid (Figures 5 and 6). In cultured cell lines retinoic acid might stop the secretion of t-PA and collegenases by acting on the binding sites located on the surfaces of these cells, suggesting that the hydrophobic part of GB has a binding site for retinoic acid, whilst t-PA, plasmin and alkaline phosphate have no such binding site for retinoic acid.

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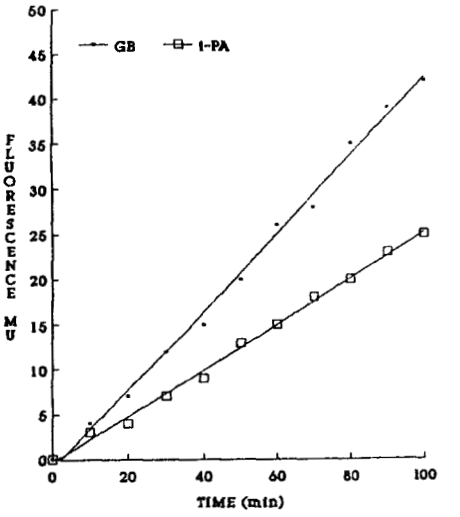

FIGURE 4 MUGB assay of guanidinobenzoatase and t-PA as a function of time. Freeze dried GB (20 μg/ml) and purified t-PA (10 μg/ml) was incubated with the fluorogenic substrate 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) at a final concentration of 10^{-4}M. MUGB was continuously hydrolysed by GB and t-PA and a linear relationship was obtained. The fluorescent product (MU) was measured by an Aminco-Bowman fluorescence spectrophotometer. Cleavage of the substrate was monitored at excitation wavelength 323 nm and emission wavelength 446 nm.

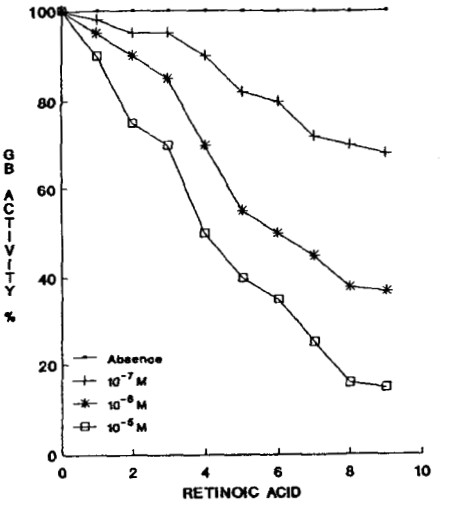

FIGURE 5 Effect of retinoic acid on GB in solution, when various concentration of retinoic acid (10^{-7} – 10^{-5}M) were incubated with GB. 100% activity of 20 μg freeze dried GB was equal to 2 nmol of MU produced per min. The experiment was repeated 4 times with essentially the similar results, but only one experiment is shown here.

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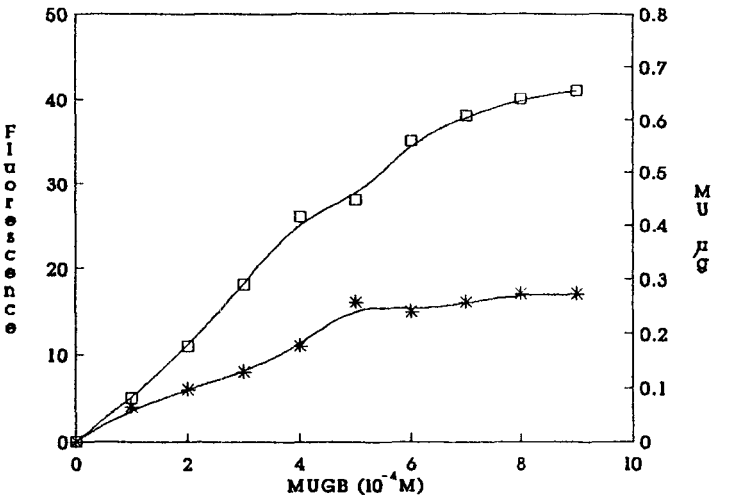

FIGURE 6 GB activity is presented as a function of increasing concentration of MUGB (10^{-4}M), in the absence (—) or presence (*—*) of 6.6 $\times$ 10^{-5}M retinoic acid. The experiment was repeated 3 times with essentially the similar results, but only one representative experiment is shown here.

In the absence of retinoic acid, and at a saturating substrate concentration the activity of GB was 5.52 $\pm$ 1.87 mU/mg protein. One mU can be defined as amount of GB required to release 1 μM of MU per min. Addition of retinoic acid to a final concentration of (10^{-5} M), caused a marked reduction in MU production (Figure 6). When the inhibition of GB was presented in the form of a Lineweaver-Burk plot (Figure 7), it was concluded that retinoic acid is a non-competitive inhibitor of GB, with a calculated apparent K_i0.41 $\pm$0.05 μM. On the other hand when the effect of retinoic acid was studied on t-PA, plasmin and alkaline phosphatase, concentrations of retinoic acid up to 10^{-4} M did not effect the activity of these enzymes (Table 1).

TABLE 1
Effects of retinoic acid (9 $\times$ 10^{-5} M) on different enzymes.

Enzyme	concentration (μg/ml)	substrate	residual activity (%)
None	–	–	100
t-PA	10	S-2288	97
t-PA	10	MUGB	98
Plasmin	9.5	S-2251	99
Alkaline phosphatase	7.5	p-nitrophenyl phosphate	98
GB	20	MUGB	15

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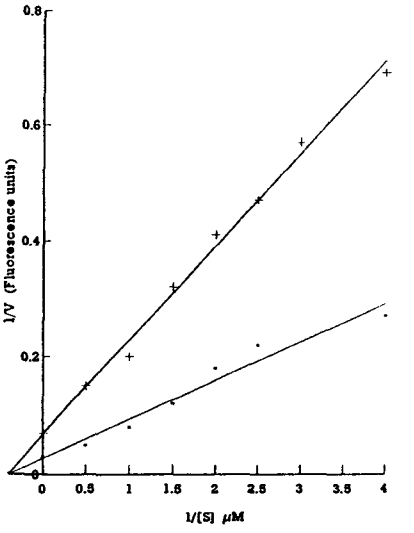

FIGURE 7 Lineweaver-Burk plot of the inhibition of GB by retinoic acid (6.6×10^{-5} M) with fluorogenic substrate MUGB. In the presence of retinoic acid (+—+) and in the absence of retinioc acid (—), with a calculated apparent $K_i 0.41 \mu$M.

From the above observations with GB on tumour cell surfaces and in solution, it is suggested that retinoic acid is a reversible, non-competitive inhibitor of GB. Retinoic acid possibly binds to a hydrophobic region in GB, resulting in major conformational changes and inhibition of GB activity. It is possible that the inhibitory effect of retinoic acid on the cell surface GB may play a role in the control of cell migration and metastasis.

Acknowledgement:

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