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INHIBITION OF A PROTEASE ASSOCIATED WITH TUMOUR CELLS AND THE PROBABLE MECHANISM OF REGULATION OF THIS INTERACTION *IN VIVO*

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The regulation of activity of a protease on the surface of human colonic tumour cells implanted in nude mice has been studied. A synthetic fluorescent probe was used to locate cells possessing active guanidinobenzoatase in frozen sections of tumour bearing tissues. These studies demonstrated the presence of intracellular protein inhibitors of guanidinobenzoatase which could be extracted in isotonic saline and transferred to the outer surface of the tumour cells. This study showed that the inhibition of guanidinobenzoatase was pH dependent and that the tumour cell may regulate the activity of its surface guanidinobenzoatase by exporting lactic acid.

KEY WORDS: Guanidinobenzoatase, protease, inhibition, regulation, tumour.

INTRODUCTION

We have chosen a model system to investigate the possible role of protein inhibitors which are capable of recognizing and reversibly inhibiting the protease guanidinobenzoatase[1] (GB) associated with human colonic tumour cells grown under the skin of nude mice. We have employed frozen sections in which the main types of easily recognizable cells were clumps of tumour cells surrounded by inflammatory cells. Previous studies[2,3] have shown that GB can be located by fluorescent microscopy using 9-aminoacridine (9-AA) as a fluorescent probe for the active centre of GB. Inhibition of GB with irreversible inhibitors (e.g. BZAR)[4] or by reversible protein inhibitors[5] results in subsequent failure of 9-AA binding and consequent loss of fluorescence. In this study the protein inhibitors were extracted in isotonic sodium chloride from the cut cells in the frozen sections and these potential inhibitors of GB were allowed to react with the membrane-bound GB of the tumour cells within the same section. In this way we were able to determine whether the tumour cells and the surrounding cells of the host tissue could provide sufficient inhibitor molecules to block the binding of 9-AA to the GB on the surface of the tumour cells. We also

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examined the status of GB on the colonic tumour cells grown in cell culture. In this study we employed a membrane bound enzyme (GB) in cells attached to a glass slide as the test system to demonstrate the interaction of this enzyme with a soluble protein inhibitor. This technique greatly accelerates the analysis and enables the conditions of interaction to be quickly elucidated.

MATERIALS AND METHODS

Tumour cells and solid tumours

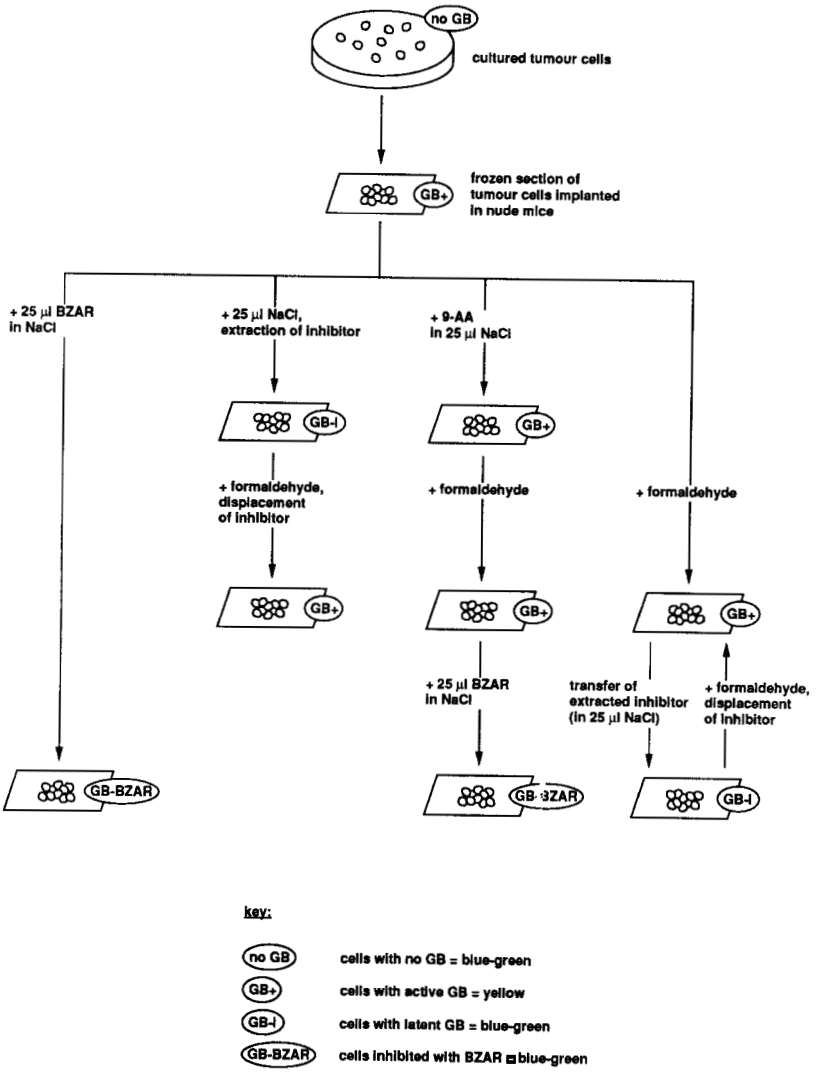
All the tumour cells and solid tumours used in this study were very kindly provided by Professor Gerhard Sauer and his colleagues at the Deutsches Krebsforshungszentrum, Heidelberg. Female athymic mice (nu/nu) average weight 20 g were kept under aseptic conditions. Human colorectal carcinoma cells (SW707, provided by Dr. M. Herlyn, the Wister Institute, Philadelphia Pa, USA) were innoculated subcutaneously on both flanks of the animals (5×10^6 cells suspended in PBS). After 10–14 days the tumours had reached a palpable size of approximately 8–10 mm diameter. The tumours were excised and prepared as frozen sections. The SW707 cells were kept in tissue culture (BME, 10% foetal bovine serum and 1% streptomycin and penicillin). The SW707 were attached to glass cover slips for convenience of handling and microscopic analysis.

Microscopic location of cells possessing active guanidinobenzoatase (GB) and inhibited GB

The coverslip containing the cultured cells or the slide with the attached frozen section was placed in a tank (300 ml) of isotonic saline containing 10^{-3} M 9-aminoacridine (9-AA) for 2 min, the excess reagents were quickly washed from the slide in isotonic saline and the slide placed in a final tank of isotonic saline for 1 min. The cells were then examined by the Leitz Diaplan fluorescent microscope employing filter cube G. Under these conditions the cells possessing active GB bound 9-AA and fluoresced yellow[2,4]. Cells lacking the ability to bind 9-AA appeared blue-green. Latent GB, or inhibited GB was revealed by formaldehyde displacement of the inhibitor followed by 9-AA location of the reactivated GB[5].

Inhibition of GB on tumour cell surfaces

We employed the selective inhibitor of GB, referred to as BZAR[4,6] and the naturally occuring protein inhibitor of GB found in the tumour sections[5], to block the binding of 9-AA to the active centre of GB. Pretreatment of the sections with these reagents varied in different experimental regimes, each of which is defined in the appropriate section of the results. After the inhibition step the section was treated with 9-AA, examined by fluorescent microscopy and if a latent enzyme was suspected this was tested for by formaldehyde treatment and a second exposure to 9-AA. The experimental results have been grouped into numbered sections in order to keep the different inhibition pathways separate, these have been outlined in the flow diagram presented in scheme 1. Scheme 1 correlates all the data concerning the status of GB and the potential for inhibition of GB within the test systems. The latter part of this study



SCHEME 1

concerns the regulation of enzyme-inhibitor interaction which may be artificially brought about by changing the pH.

Regulation of GB-inhibitor interaction by pH

It was necessary to generate a range of pH values from pH 7 to pH 5 in isotonic saline buffered with acetic acid or lactic acid. This was achieved by taking 200 ml of 10^{-2} M $NaHCO_3$ in isotonic saline and adding incremental amounts of these carboxylic acids to adjust the pH by 0.1 pH unit increments; 25 µl of each buffer solution was then placed over the surface of a frozen section of the mouse tumour-bearing tissue. After

1 h this buffer was removed and the slides placed for 2 min in a tank (200 ml) containing the same stock buffer with pH adjusted to 7.0. The sections were then treated with 9-AA and the activity of the tumour cell surface GB examined over the whole pH range. Those slides from pH values which resulted in inhibition of the tumour cell GB were treated with formaldehyde and the regain of GB activity subsequently checked as described above.

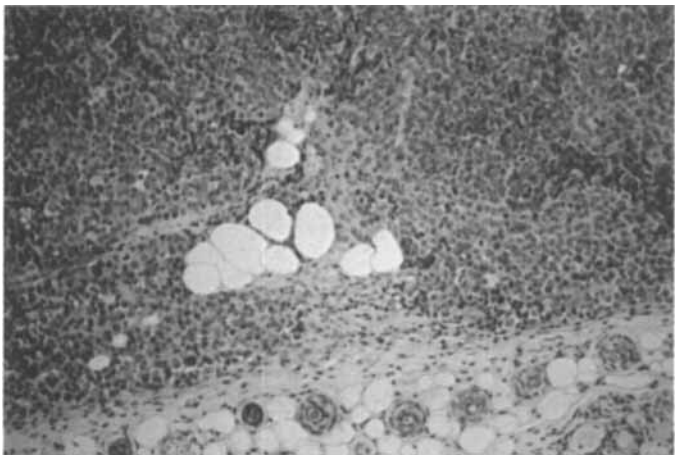


FIGURE 1 Wax embedded sections of colonic tumour cells grown in nude mouse, stained with haematoxylin-eosin. The clumps of tumour cells appear purple due to their nuclear staining. Magnification × 500. (See colour plate at back of issue.)

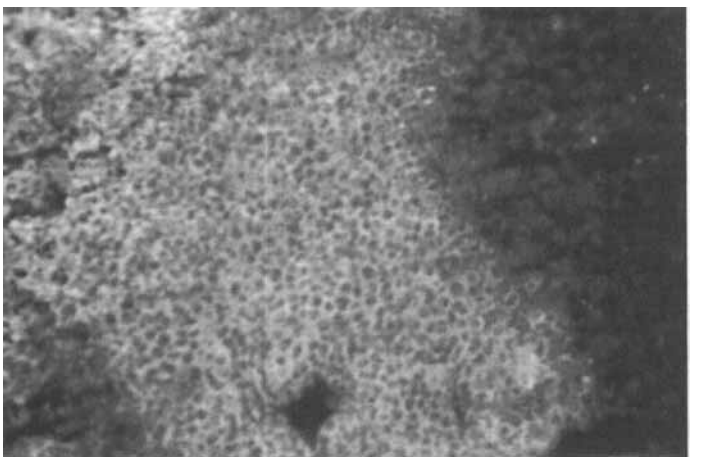


FIGURE 2 Frozen section of human colonic tumour cells grown in nude mouse, treated with 9-AA. The colonic tumour cells possess active GB, bind 9-AA and fluoresce yellow. The staining is extra nuclear and appears as a fluorescent yellow ring on the surface of each cell. The surrounding inflammatory cells of the host do not have GB and do not fluoresce. Magnification × 530. (See colour plate at back of issue.)

RESULTS

1. Conventional staining of frozen sections with haemaroxylin-eosin

Clumps of tumour cells can be seen invading the host tissue, surrounded by inflammatory cells (Figure 1).

2. Direct 9-AA-labelling of GB in frozen sections

We examined unfixed frozen sections from four nude mice implanted with human colonic tumour cells. In each case we examined sections which had been stored for 10 min, 4 h, 1 d, 2 d, 3 d and 4 d. In every case the sections contained islands of tumour cells surrounded by host cells. The tumour cells expressed active GB and this was located as extranuclear yellow fluorescent ring staining under the microscopic conditions used in this study (Figures 2 and 3). The host cells did not bind 9-AA and appeared blue-green.

3. Inhibition of GB in frozen sections with BZAR

Exposure of the surface of the sections to 25 μl BZAR (10^{-4} M) in isotonic saline for 2 min, 5 min, and 10 min prior to 9-AA staining led to complete inhibition of GB on tumour cells. This is shown by the lack of fluorescence on the tumour cells in Figure 4.

4. Reversible inhibition of GB in frozen sections by proteins extracted from the same sections

Exposure of the surface of the sections to 25 μl of isotonic saline for 1 h prior to 9-AA

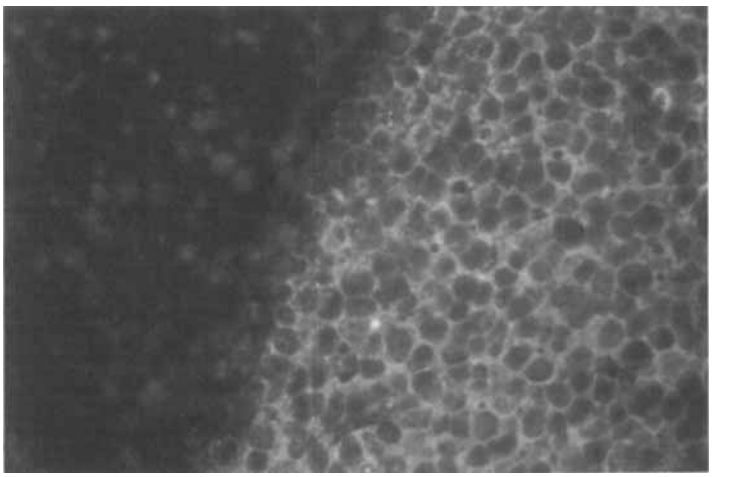


FIGURE 3 Frozen section of human colonic tumour cells grown in nude mouse, treated with 9-AA. Higher magnification of section shown in Figure 2. Note the ring staining of the tumour cells to provide yellow fluorescence whilst the host cells lack yellow fluorescence. Magnification × 1100. (See colour plate at back of issue.)

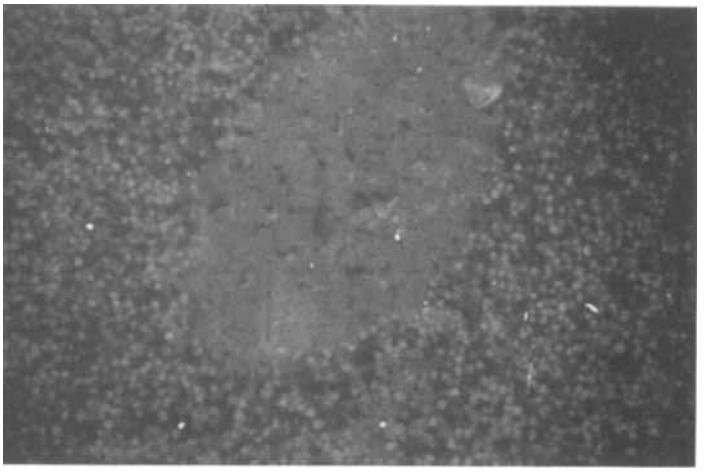


FIGURE 4 Frozen sections of human colonic tumour cells grown in nude mouse, treated with BZAR then with 9-AA. Prior treatment of the section with 10^{-4} M BZAR for 2 min prior to 9-AA staining resulted in complete inhibition of 9-AA uptake by the clump of tumour cells seen in the centre of this photograph. Magnification × 530. (See colour plate at back of issue.)

staining resulted in complete loss of tumour cell GB activity as demonstrated by the lack of ability to bind the fluorescent probe (Figures 5 and 6). The colour prints obtained from sections with inhibited GB appear much yellower than the inhibited cells in the section. This is due to the prolonged time of exposure needed to take these photographs and the fact that all photographs were taken using the K490 yellow filter to ensure that the conditions were strictly comparable. This inhibition could be

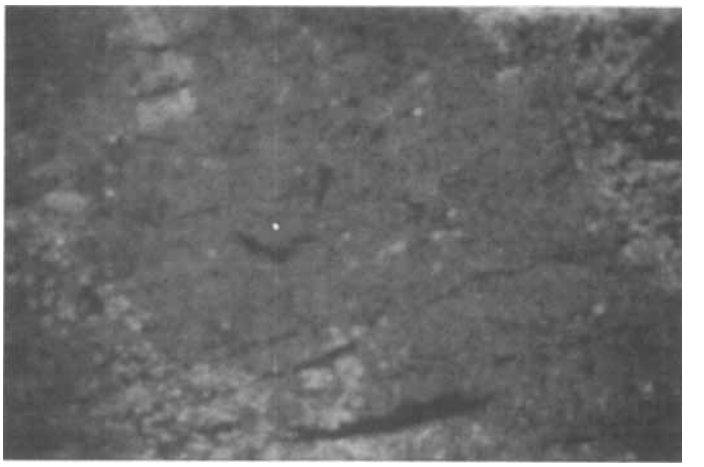


FIGURE 5 Frozen section of human colonic tumour cells grown in nude mouse, exposed to local inhibitor and then treated with 9-AA. In the presence of 25 μl NaCl, local inhibitors are solubilised and react with the tumour cell's surface GB, preventing the uptake of 9-AA. The tumour cell clump is located in the central region of the photograph. Magnification × 530. (See colour plate at back of issue.)

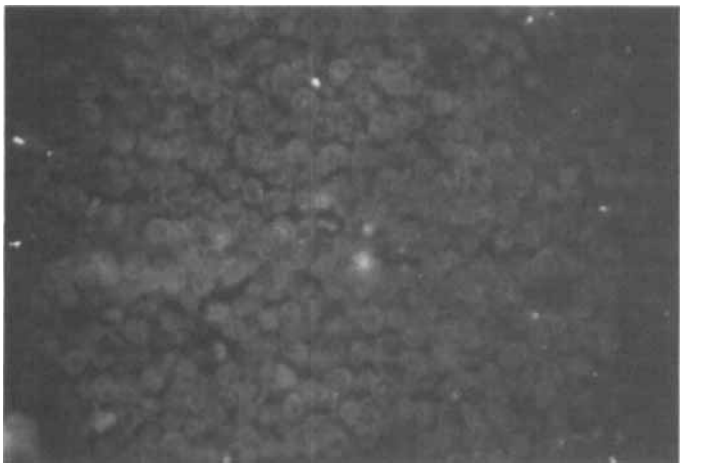


FIGURE 6 Frozen sections of human colonic tumour cells grown in nude mouse, exposed to local inhibitors and then treated with 9-AA. Higher magnification of the section shown in Figure 5. Note the total absence of cell surface yellow ring staining on the tumour cells. The nuclei appear pale green. Magnification × 1060. (See colour plate at back of issue.)

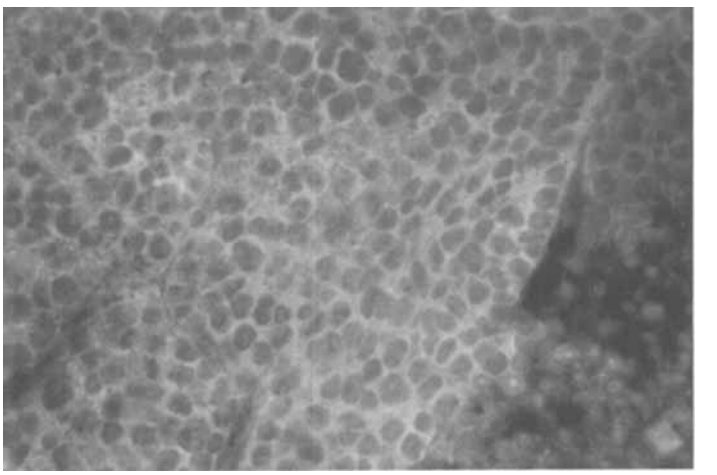


FIGURE 7 Frozen section of human colonic tumour cells grown in nude mouse, exposed to local inhibitors and then to formaldehyde prior to 9-AA treatment. The inhibitor on the cell surface GB (see Figure 6) has been displaced with formaldehyde and the reactivated GB now binds 9-AA. The tumour cells exhibit yellow fluorescent ring staining similar to Figure 3. Magnification × 1100. (See colour plate at back of issue.)

reversed by subsequent treatment of the sections with formaldehyde followed by a second 9-AA staining step (Figure 7).

5. Competitive inhibition of GB in frozen sections

When frozen sections were exposed to either (a) 25 μl isotonic saline or (b) 25 μl isotonic saline containing 10^{-3} M 9-AA for 1 h, it was possible to compare non-

competitive and competitive binding of inhibitors to the tumour cell GB. The non-competitive experiments (a) resulted in no 9-AA binding to the tumour cells (data similar to Figures 5 and 6); in this case the protein inhibitors were unopposed in their action on tumour cell GB. This resulted in latent enzyme formation from which the GB could be reactivated by formaldehyde treatment (Figure 7).

The competitive experiments (b) resulted in the failure of the tissue inhibitor to displace 9-AA from the active centre of GB on the tumour cells, consequently these cells fluoresce yellow. The latter result is comparable to that obtained in the direct staining of frozen sections (Figures 2 and 3).

6. *Transfer of extracted inhibitor to formaldehyde fixed sections*

The inhibitors extracted in 25 μl isotonic saline during 1 h from a frozen section were transferred to a second section which had been pretreated with formaldehyde in order to destroy all the tissue inhibitor activity against GB. After exposure to these inhibitors for 1 h the second section was stained with 9-AA (Figure 8). The tumour cells in the second section were unable to bind the fluorescent probe indicating the presence of latent GB. The nuclei of formaldehyde treated cells bind 9-AA weakly. This latent GB on the tumour cells was subsequently reactivated by formaldehyde treatment with the re-appearance of the typical ability to bind the fluorescent probe.

7. *Cultured human colonic tumour cells*

Direct staining with 9-AA on glass cover slips resulted in the appearance of fine yellow stippling on the surface of some of these cells (Figure 9). This was thought to be an artefact since only some of the cells show this. It is quite distinct from the typical

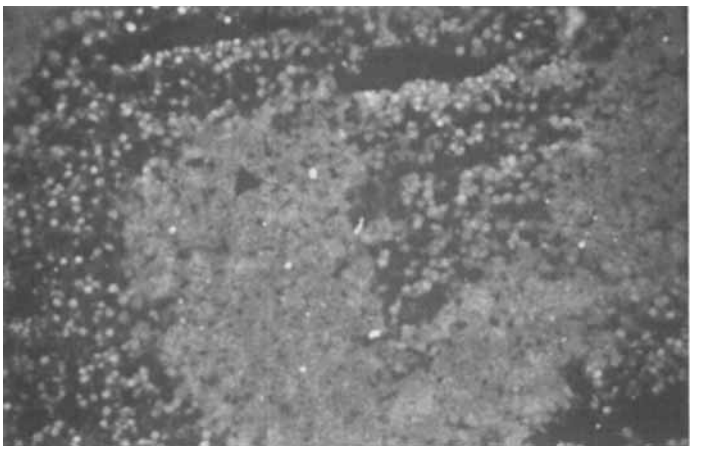


FIGURE 8 Frozen section of human colonic tumour cells grown in nude mouse, exposed to formaldehyde, then to inhibitor extracted into 25 μl sodium chloride on a frozen section, then to 9-AA. The formaldehyde treated tumour cells originally possessed active GB. The addition of the 25 μl extract provided inhibitors of GB and the subsequent treatment with 9-AA failed to cause ring staining with yellow fluorescence. The nuclei of some of the host cells (treated with formaldehyde) bind 9-AA. Magnification $\times$ 530. (See colour plate at back of issue.)

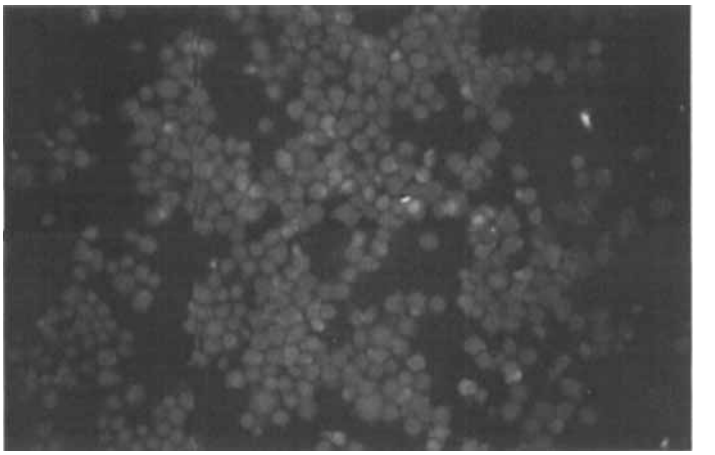


FIGURE 9 Cultured SW707 colonic tumour cells treated with 9-AA. These cells lack the ability to bind 9-AA and lack active GB; there is no yellow ring staining. Magnification × 530. (See colour plate at back of issue.)

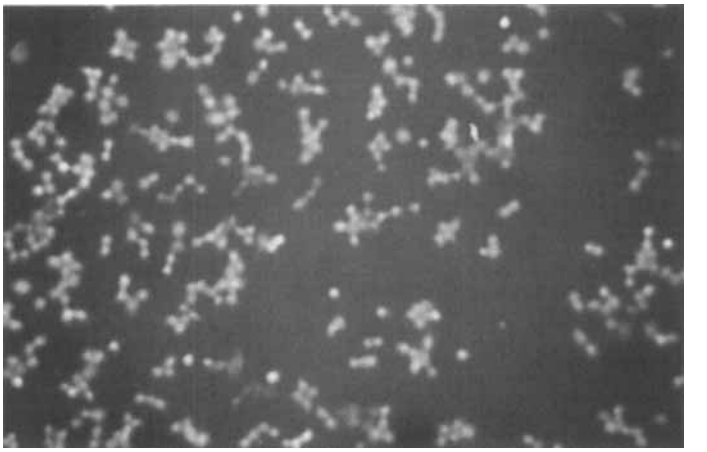


FIGURE 10 Cultured SW707 colonic tumour cells treated with formaldehyde prior to 9-AA. These cells appear faintly yellow, as does the background due to the prolonged exposure used to obtain this photograph with yellow filter K490 in the microscope. The cell surface ring staining is absent, whilst a small yellow dot (probably the nucleolus) in the nucleus of most cells can be seen. Magnification × 530. (See colour plate at back of issue.)

yellow ring staining of GB seen in the frozen sections (Figures 2 and 3). These cultured cells lack active GB. Formaldehyde fixation of these cultured cells followed by 9-AA staining (Figure 10) confirmed the absence of latent GB on these cultured cells surfaces; a weak nuclear staining was observed which was probably confined to the nucleolus.

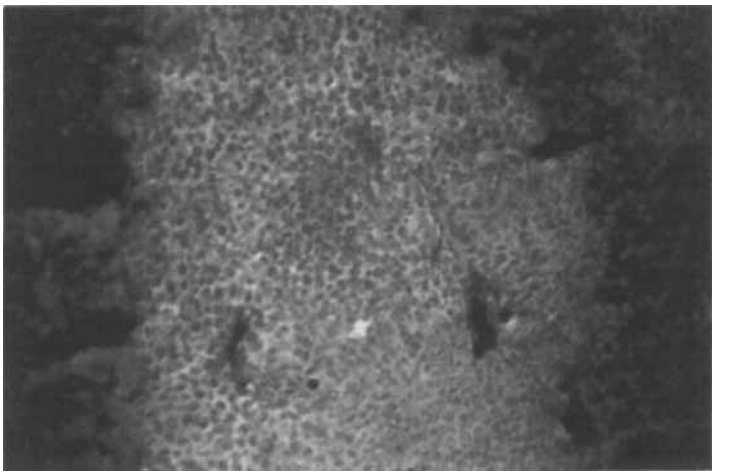


FIGURE 11 Frozen section of human colonic tumour cells grown in nude mouse, after formaldehyde treatment and exposure to the cell culture fluid, followed by 9-AA. The formaldehyde fixed cells have active GB which is not inhibited by subsequent exposure to the cell culture fluid and 9-AA binding to the GB takes place. Magnification × 530. (See colour plate at back of issue.)

8. Lack of inhibitor in the cell culture fluid

We considered the possibility that the culture fluid might contain inhibitors of GB. We therefore placed, (a) 100 μl of fresh culture fluid on formaldehyde fixed sections of tumour tissue and (b) 100 μl of the culture fluid used to culture the tumour cells on other formaldehyde fixed sections for 1 h. 9-AA staining clearly showed that neither of these culture fluids possessed inhibitors of the tumour cell GB (Figure 11).

9. Regulation of interaction of inhibitor with GB

We observed that at pH values above 6.0 complete inhibition by tumour cell extracts of tumour cell surface GB took place. On the other hand at pH values below 5.5 the GB on the tumour cells was protected from the action of inhibitors. These low pH values did not alter the ability of GB to bind 9-AA, the active centre remained functional with respect to binding of the fluorescent probe. When the fine control of pH was carried out it was observed that the critical pH was 5.8–5.7 with both the acetate and lactate buffers. Above pH 5.8 inhibition was complete (data similar to Figures 5 and 6), below pH 5.7 marked protection from inhibition was observed (data similar to Figures 2 and 3).

It was further shown that lowering the pH to 5.0, after inhibition had taken place, did not reverse this process, (data similar to Figures 5 and 6) although the inhibitor could be displaced with formaldehyde treatment (data similar to Figure 7).

DISCUSSION

In order to relate the fluorescent location of the tumour cells in these frozen sections we present a conventional HE-stained section in Figure 1. The seryl protease GB has

previously been shown to degrade fibronectin and in particular the link peptide, GlyArgGlyAsp[7,8]. Cells which are capable of migration have been shown to possess GB either in an active form or in a latent form. In this study we have used the fluorescent probe 9-AA (a competitive inhibitor of GB) to locate cells possessing active GB enabling these cells to exhibit typical fluorescent yellow ring staining. Cells lacking GB or possesing latent GB do not show this typical fluorescent staining. In Figures 2 and 3 the tumour cells possess active GB, whilst the surrounding inflammatory cells do not.

GB has previously been shown to be selectively inhibited[4] by the fluorogenic substrate for trypsin-like enzymes referred to as BZAR[6]. Frozen sections of tumours grown in the nude mouse were treated with BZAR in isotonic saline for 2 to 10 minutes and rapidly lost their ability to bind 9-AA (Figure 4). This result indicated inhibition of GB-activity by BZAR.

Latent GB consists of GB and an inhibitor of GB. The inhibitor may be displaced from the latent GB-complex by formaldehyde treatment without loss of the ability of the cell-bound GB to bind 9-AA. Thus it is possible to reveal the presence of latent GB on cell surfaces and to exchange inhibitors with cell-bound GB. The mechanism of inhibition has been shown to be by the binding of inhibitor to a part of GB distant from the active centre but resulting in the reversible modification of the active centre. Multiple forms of inhibitors of GB and corresponding multiple forms of GB have been demonstrated by such exchange techniques[5].

It was therefore of considerable interest to show that the frozen sections possessed inhibitor molecules which could be extracted into 25 μl of isotonic saline during 1 h at room temperature. These extracted inhibitors were then reacted with cell-bound GB and blocked the subsequent binding of the fluorescent probe to the tumour cells (Figures 5 and 6). This experiment demonstrated the conversion of the active GB on the tumour cell surfaces (Figures 2 and 3) to a latent form of GB (Figures 5 and 6). This latent GB or inhibited GB was dissociated by formaldehyde treatment (Figure 7) and regained the ability to bind the fluorescent probe.

The observation that the frozen sections contained tumour cells with active GB in spite of the fact that the tissue contained cells capable of synthesizing an inhibitor of GB is significant. It indicates that the tumour cells in the mouse have a requirement for active GB and this may be important for their invasion into the surrounding host tissue. The same tumuor cells, when grown in culture do not possess either the active enzyme nor the latent enzyme. Clearly this enzyme has a function for the tumour cell in the mouse but not in cell culture. The appearance of this enzyme on the tumour cells in the mouse must be induced after implantation of the cultured cells. Kinetic studies have shown that tissue inhibitors of GB bind to a part of the enzyme molecule distant from the active centre and this binding modifies the active centre resulting in inhibition and failure to bind[8] 9-AA. In the present study it was possible to compare the action of the inhibitor extracted in isotonic saline with the inhibitor extracted in isotonic saline containing the fluorescent competitive inhibitor 9-AA. In the latter system, the fluorescent probe (at 10^{-3} M) is vastly in excess of the concentration of the protein inhibitor.

As a consequence, the 9-AA will fill the active centre of the enzyme with stacked molecules of this fluorescent probe; these molecules would have to be displaced by the protein inhibitor if these were to make successful interactions with tumour GB to form latent GB. In the experiment it was observed that in the absence of 9-AA the saline extracted inhibitor from the section and successfully converted the GB on the

tumour cells to latent GB (similar to Figures 5 and 6). In the competitive experiment the presence of 9-AA in the saline prevented the protein inhibitor forming latent GB (similar to Figiure 7). In each case the enzymic status of GB was determined by a subsequent staining with the fluorescent probe which cannot displace the protein inhibitor from the latent GB. It is clear that the presence of the competitive inhibitor 9-AA blocked the action of the inhibitor of GB extracted with saline.

We then went on to consider whether there was enough inhibitor extracted in the 25 μl isotonic saline to cause complete inhibition of all the GB in the tumour section. The evidence in Figures 5 and 6 suggested that there was sufficient inhibitor but we attempted to demonstrate an excess of this requirement as follows. We took frozen sections of tumour tissue and in each case inhibited the cell surface GB by exposing these sections to 25 μl isotonic saline for 1 h. This fact was confirmed by subsequent staining with 9-AA. We then transferred the excess fluid (20 μl) onto corresponding formaldehyde fixed sections. After 1 h these formaldehyde treated sections showed inhibition of tumour cell GB; latent GB was confirmed by subsequent formaldehyde treatment and positive 9-AA staining. We conclude from these transfer experiments that the mouse frozen sections contained more than enough extractable inhibitor to form latent GB on all the tumour cells in each section.

The fact remains that *in vivo* this potential for GB-inhibition has not affected the tumour GB-activity. This could be because the GB is on the cell surface and the potential inhibitor is retained within the cells. The significance of active GB on the tumour cells implanted in nude mice is emphasized when it is known that these same cells in culture lack active GB also lack latent GB. The implication is that the transfer of cultured tumour cells to the nude mouse must be accompanied by nuclear events which lead to the appearance of active GB on the transplanted tumour cells.

There remains another possible explanation for the tumour cell surface GB retaining its activity and ability to bind 9-AA in the presence of potential inhibitors. Tumour cells are known to reply mainly on glycolysis for energy production with the consequent obligatory export of lactic acid from these cells. We considered the possibility that the release of lactic acid at the tumour cell surface membrane could influence the binding of inhibitor protein to GB. The effect of a gradual drop in pH of the buffer system clearly showed that if the cell surface pH was maintained at 5.7 or below no interaction of inhibitor and GB took place. This same critical pH value was obtained whether lactic acid or acetic acid was used to modify the buffer pH. It was further shown that when inhibition of GB had taken place at pH values greater than 5.8, the latent GB could not be reactivated by simply lowering the pH to 5.0. Since the inhibitor is cytosolic in its synthesis and the GB is located on the external surface of the cell there seems little reason for the cells to make inhibitors of GB unless these are destined for export to the extracellular environment.

It is tempting to suggest that these inhibitors must be *exported* to fulfil that biological role. If this suggestion can be proved, the corollary is that tumour cells with active GB *in vivo* maintain this protease activity by exporting lactic acid in order to maintain a cell surface pH below 5.7.

The knowledge of enzyme-inhibitor interactions taking place at the tumour cell surface can be fairly quickly extended by this simple system of analysis employing cells fixed on a glass slide rather than soluble components in a conventional liquid system. The advantage of this system is obvious when it is realised that on every slide the host tissue's inflammatory cells lack GB and this acts as a sensitive internal control for the tumour cell's GB.

Acknowledgements

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