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INHIBITION OF A CELL SURFACE PROTEASE AFTER CISPLATIN CHEMOTHERAPY

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The epithelial cells in squamous carcinoma and leukoplakia of the oral cavity possess the cell surface protease, guanidinobenzoatase (GB), in an active form. GB is closely similar to plasminogen activator, a protease associated with both transformed cells and tumour cells. The active centre of GB binds the fluorescent probe 9-aminoacridine (9-AA) enabling cells containing active GB to be visualised by fluorescent microscopy. It was observed that chemotherapy with cisplatin resulted in a marked decrease in cell surface GB activity and this decrease was due to the formation of an enzyme-inhibitor complex. One of the results of chemotherapy was shown to be the suppression of a cell surface protease which is known to be associated with migration and malignancy of cells *in vivo*.

KEY WORDS: Cell surface protease, inhibition, plasminogen activator, guanidinobenzoatase, chemotherapy.

INTRODUCTION

This study has been concerned with an enzyme associated with tumour cells and leukoplakia cells of the oral cavity. Leukoplakia is a precancerous form of oral epithelial cells. The effect of cisplatin chemotherapy on the activity of this enzyme has been studied by employing a fluorescent probe which binds to the active enzyme but not to the inhibited enzyme.[1] Fresh frozen sections of normal tongue and oral mucosa were used as well as defined regions of leukoplakia and sqamous cell carcinoma.

The enzyme that we are concerned with has been described as guanidinobenzoatase

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(GB),[2] due to its ability to cleave the active site titrants, 4-methylumbelliferyl-p-guanidinobenzoate (MUGB[3]) and 4-nitrophenyl-*p*-guanidinobenzoate[4] as true substrates. Single chain tissue type plasminogen activator has recently been reported to cleave MUGB in a continuous manner[5] and the plasminogen activator inhibitor described[6] as PAI-I has now been shown to inhibit cell surface GB, suggesting that GB and plasminogen activator are closely related in structure and function.[7]

GB has been shown to be competitively inhibited by 9-amino acridine (9-AA),[8] which binds to the active centre of GB only when the enzyme is fully functional.[1] Since 9-AA has fluorescent properties, this reagent can be used to locate cells possessing active GB (immobilised on the external surface of the cell membrane)[9] employing fluorescence microscopy. Cell surface GB can be reversibly inhibited by soluble proteins extracted from the cytoplasm of cells which have been cut in frozen sections.[1]

In this study our observations are presented in the form of colour prints in which cells possessing active GB combine with 9-AA to produce a yellow fluorescence, whilst cells which lack this yellow fluorescence either lack GB entirely or may possess latent (inhibited) GB.[1] In the latter case, displacement of the inhibitor from the enzyme inhibitor complex resulted in regain in the enzyme's ability to bind 9-AA and a regain of yellow fluorescence.[1]

MATERIALS AND METHODS

9-Amino acridine hydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo, USA. Oral smears were obtained by collecting cells form the oral cavity on a plastic spatula and spreading these cells on clean glass microscope slides. Frozen sections were obtained from a collection of forty code numbered small pieces of deep frozen tissue, obtained at the time of surgery. This collection contained tissues from normal epithelial surfaces, leukoplakia, squamous cell carcinoma and combinations of these tissues which had been taken both prior to and after chemotherapy with cisplatin. The code linking these numbered sections with their medical history was not known to the biochemist carrying out the study but only to the surgeons and pathologist involved in this collaborative study. This arrangement allowed unbiased observations to be made concerning the status of the epithelial cell surface enzyme (GB) which could later be correlated with the clinical and pathological data already available for these tissues.

9-AA Staining of Smears and Sections

9-AA (10^{-3} M) dissolved in isotonic saline (with pH adjusted to 7.2 with $NaHCO_3$ when necessary) was employed to stain those cells possessing GB. The smears, or sections, were placed in a tank (250 ml) of 9-AA solution for 2 min, drained of excess 9-AA and then washed in isotonic saline as follows: the sections were washed for 1 min in saline and then covered with a glass coverslip prior to microscopy. The smears required rather longer washing in isotonic saline (75 s) since many of the cell surfaces were folded and these tended to trap 9-AA and required further washing than did the frozen sections.

Formaldehyde Treatment of Smears and Sections

Formaldehyde (10% v/v or 4% w/v in isotonic saline) displaces protein inhibitors from latent cell surface GB.[1] All the oral smears were placed in formaldehyde (4%) for 1 h prior to staining with 9-AA. Selected frozen sections of normal epithelial surfaces, leukoplakia and squamous cell carcinoma, before and after chemotherapy, were treated with formaldehyde to displace inhibitor and regain GB which was able to bind 9-AA.

Inhibition of Cell Surface GB and Transfer of Inhibitors to Formaldehyde Treated Slides

Cells possessing active cell surface GB also possess[1] cytoplasmic inhibitors of GB which may be extracted in isotonic saline. In order to demonstrate the local inhibition of GB on leukoplakia and squamous cell carcinoma cell surfaces, we exposed frozen sections of these tissues to 10 μl isotonic saline for 1 h in a wet box. The sections were quickly washed in isotonic saline (2 s) and then stained with 9-AA (as described above) in order to demonstrate the presence of inhibitors of GB capable of preventing the subsequent binding of 9-AA to the surfaces of these cells (Figures 9 and 12). These sections were later washed to remove 9-AA and treated with formaldehyde to displace inhibitor, in order to regain cell surface GB activity. This latter step confirmed that throughout this inhibition process the enzyme, GB, remained attached to the cell and was still potentially functional. This step demonstrated that the temporary loss in enzymic function was due to the binding of an inhibitor to GB, extracted in the 10 μl NaCl from the frozen section.[1]

The transfer of inhibitor from one section to another posed technical problems, due to the presence of inhibitors in both the cells of the target tissue and those of the extracted donor tissue.[1] This problem can be overcome[1] by prior treatment of the target tissue with formaldehyde to remove all the local protein inhibitors of GB. Formaldehyde treated frozen sections were then exposed to the isotonic saline extracts[1] obtained from four fresh frozen sections of the donor tissue and placed in a wet box for 6 h prior to washing off excess potential inhibitor and staining the sections with 9-AA, as described above. It was necessary to increase the incubation period in these formaldehyde treated sections in order to allow time for the recognition of the formaldehyde treated GB with the native inhibitor molecules. When the analysis of these sections suggested that inhibition had taken place, this was confirmed by a subsequent treatment with formaldehyde and the observed regain of the cell's ability to bind 9-AA.

Fluorescence Microscopy

The 9-AA stained sections were examined using a Leitz Diaplan fluorescence microscope with barrier filter K490 and filter cube G (ref. No. 513602). The cells possessing active GB exhibited a bright yellow fluorescence. Cells which lacked GB appeared pale green. The microscope was used in conjunction with an Olympus OM-2_N camera, fitted with an automatic exposure meter. Kodak ASA 400 colour film was used to record our observations on the enzymic status of GB on the surface of these oral epithelial cells. Representative examples of these colour transparencies are presented as colour photographs, to illustrate our observations in the Results section.

RESULTS

Single Epithelial Cells in Oral Smears

Treatment of oral smears with formaldehyde followed by 9-AA staining demonstrated a marked difference between normal epithelial cells and cells taken from leukoplakia and tumour regions of the oral cavity. The epithelial cells from a normal subject lack GB activity even after formaldehyde treatment (Figure 1) whilst epithelial cells from regions of leukoplakia (Figure 2) and oral squamous cell carcinoma (Figure 3) fluoresced yellow after exposure to 9-AA and clearly possessed GB.

Normal Epithelial Layers of the Tongue and Oral Mucosa

In both the normal tongue and oral mucosa, surface epithelial cells were entirely without GB activity apart from the basal (immature) layers of cells (Figure 4). The basal layers of cells within the same section often exhibited regions where no GB activity was observed and other regions in which the basal cells were active and bound 9-AA. Treatment of these sections of normal tissue with formaldehyde failed to reveal mature epithelial cells possessing latent GB.

Leukoplakia

Frozen sections of leukoplakia typically contained greatly thickened epithelial layers

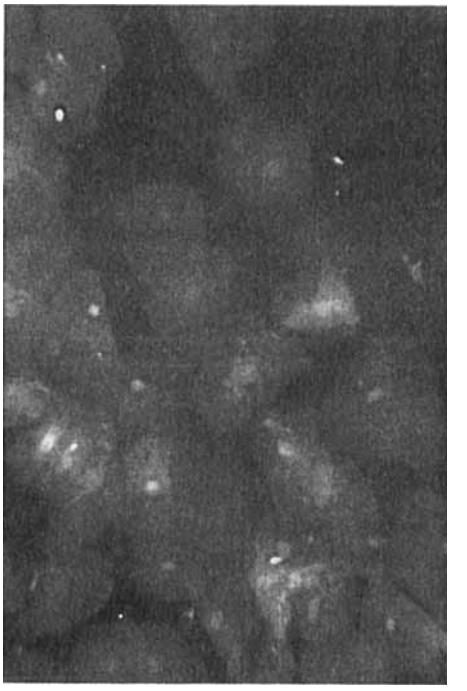

FIGURE 1 Formaldehyde treated normal oral mucosal smear; stained with 9-AA. The epithelial cells lack GB and do not exhibit cell surface yellow fluorescence. Magnification × 250 (see Colour Plate at rear).

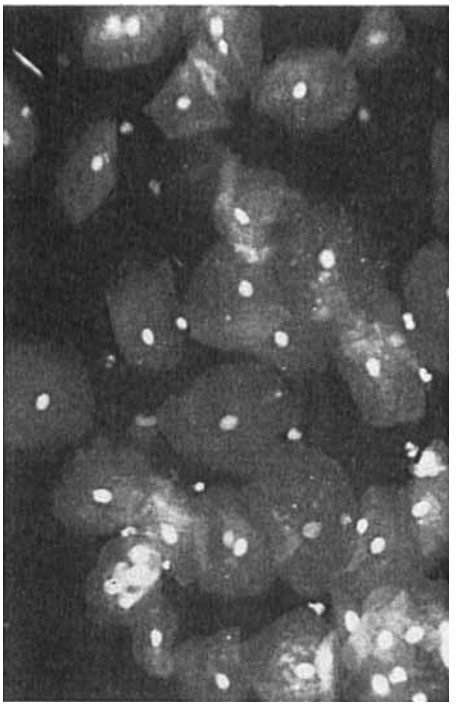

FIGURE 2 Formaldehyde treated leukoplakia oral smear; stained with 9-AA. Both normal and leukoplakia cells are present, the normal cells appear greenish, whilst the leukoplakia cells exhibit yellow surface fluorescence. Magnification × 250 (see Colour Plate at rear).

FIGURE 3 Formaldehyde treated squamous cell carcinoma smear; stained with 9-AA. Normal cells appear greenish, whilst the carcinoma cells appear bright yellow. A small clump of carcinoma cells (slightly out of focus) is also present. Magnification × 250 (see Colour Plate at rear).

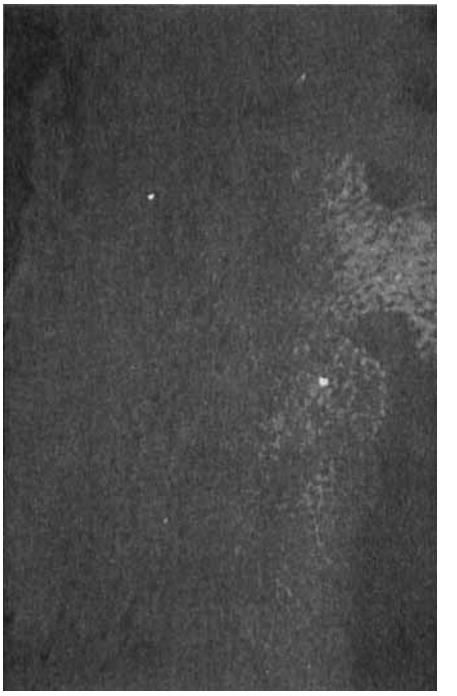

FIGURE 4 Frozen section of normal oral epithelial surface; stained with 9-AA. The superficial epithelial cells lack GB and do not exhibit yellow fluorescence. The basal cells show weak yellow fluorescence although in some regions this basal layer can exhibit yellow fluorescence due to the presence of active GB. Magnification × 250 (see Colour Plate at rear).

which contained active GB throughout the full thickness, with the possible exception of the superficial few layers of keratinised cells. The binding of 9-AA and consequent yellow fluorescence of the surface of these epithelial cells in leukoplakia is indicative of active GB throughout these epithelial cells (Figure 5). The ability of these leukoplakia epithelial cells to bind 9-AA was totally destroyed by exposure of the frozen section to 10 μl isotonic saline for 30 min (Figure 6). This loss of GB activity was due to the combination of the cell surface enzyme with an inhibitor present within the cytoplasm of the cells in this section; since the inhibitor was displaced with formaldehyde and the GB regained its ability to bind 9-AA (data similar to Figure 5).

After chemotherapy, the GB activity of the cells in the epithelial layer was greatly diminished, with a consequent lack of ability to bind 9-AA and lack of yellow fluorescence (Figure 7). This lack of ability to bind 9-AA in the leukoplakia cells after chemotherapy was caused by the presence of latent GB (Figure 8) since enzymic activity was regained after formaldehyde treatment. This regain in enzymic activity could be artifically reversed by adding inhibitor containing extract from fresh sections of leukoplakia tissue, followed by loss of cell surface yellow fluorescence (Figure 9) and subsequent regain of 9-AA binding after a further treatment with formaldehyde (data similar to Figure 8).

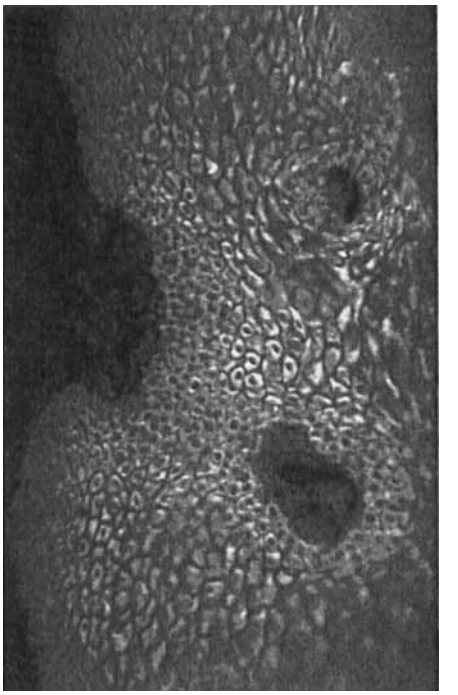

FIGURE 5 Frozen section of leukoplakia; stained with 9-AA. The epithelial cells exhibit yellow fluorescence (due to GB binding 9-AA) being most intense at the level of the basal layer of cells and extending through the full thickness of the surface layer. Note the lack of nuclear staining in these frozen sections. Compare with normal epithelium (Figure 4). Magnification × 250 (see Colour Plate at rear).

Squamous Cell Carcinoma

Frozen sections of squamous cell carcinoma exhibited strong surface yellow fluorescence on the tumour cells whilst the central pearl of keratinised cells lacked active enzyme and appeared blue-green (Figures 10 and 11). The active cells possessed marked fluorescence and consequently GB activity. This GB activity was completely suppressed by exposure to 10 μl isotonic saline containing inhibitors of GB (Figure 12). After chemotherapy with cisplatin the squamous cell carcinoma cells appeared to be markedly reduced in GB activity (Figure 13), compared to the activity of GB prior to chemotherapy (e.g. Figures 10 and 11). This apparent lack of GB activity in the tumour cells after chemotherapy (Figure 13) was due to the presence of inhibited or latent GB, since formaldehyde treatment resulted in a regain of GB activity and ability to bind 9-AA (Figure 14). It was of interest to see whether the squamous cell carcinoma cells after chemotherapy, followed by formaldehyde reactivation of latent GB were able to be recognised by the inhibitor of GB extracted from the cut cell surface of fresh frozen sections of this tissue. In such an experiment the formaldehyde fixed section was exposed to the native inhibitors of GB and the tumour cell surface GB was again inhibited (Figure 15). This experimental inhibition was reversed by formaldehyde (data similar to Figures 10, 11 and 14).

FIGURE 6 Frozen section of leukoplakia after exposure to 10 μl isotonic saline; stained with 9-AA. The epithelial cells lack the ability to bind 9-AA and do not exhibit yellow fluorescence, due to the formation of an enzyme-inhibitor complex (or latent GB) as a result of contact with the cytoplasmic inhibitor protein. The latter may be displaced by formaldehyde. Magnification × 250 (see Colour Plate at rear).

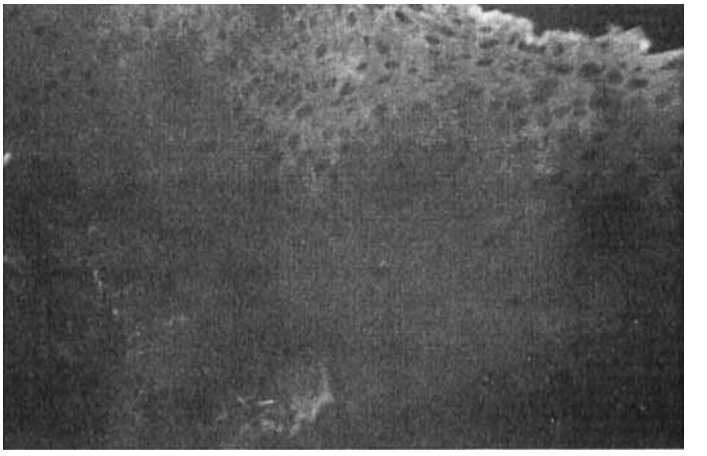

FIGURE 7 Frozen section of leukoplakia after chemotherapy with cisplatin; stained with 9-AA. The epithelial cells lack active GB and do not bind 9-AA. Magnification × 250 (see Colour Plate at rear).

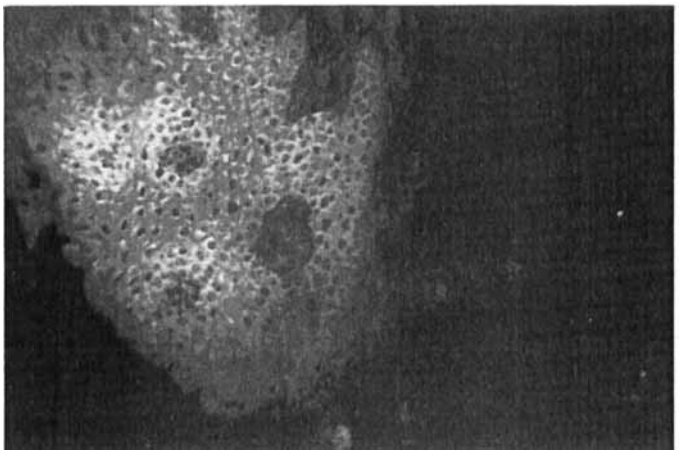

FIGURE 8 Formaldehyde treated leukoplakia after chemotherapy with cisplatin; stained with 9-AA. The full thickness of the epithelial layer binds 9-AA, fluoresces yellow and contains active GB. Compared with Figure 7, clearly the chemotherapy has not resulted in loss of GB but rather a loss of GB activity which can be regained after formaldehyde displacement of inhibitor. Chemotherapy resulted in inhibition of the cell surface GB. Magnification × 250 (see Colour Plate at rear).

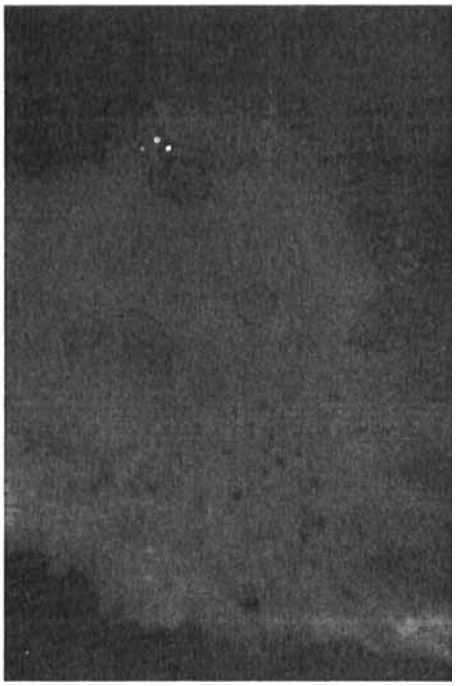

FIGURE 9 Formaldehyde treated leukoplakia after chemotherapy subsequently exposed to inhibitor (extracted in saline from fresh frozen sections); stained with 9-AA. In this slide the epithelial cells lack active GB and fail to bind 9-AA. Consequently no yellow fluorescence is exhibited by these cells. Originally these cells possessed active GB after formaldehyde treatment (see Figure 8) which has now been inhibited by the isotonic saline extract of fresh leukoplakia sections. The evidence of Figures 5, 7, 8 and 9 indicates that leukoplakia cells possess active GB which, (a) is inhibited after chemotherapy, (b) can be restored by displacement of the inhibitor and finally, (c) be artificially inhibited by adding fresh inhibitor. Magnification × 250 (see Colour Plate at rear).

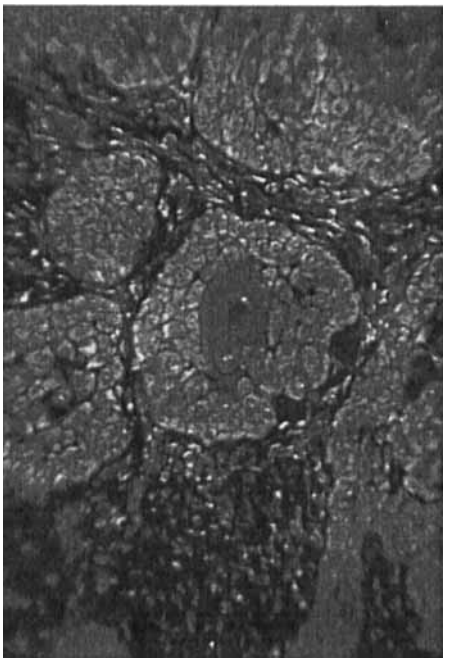

FIGURE 10 Frozen section of squamous cell carcinoma; stained with 9-AA. The central pearl of fully differentiated cells lacks active GB and appears blue-green. The surrounding squamous cell carcinoma cells exhibit yellow surface fluorescence due to the binding of 9-AA to active GB. Magnification × 250 (see Colour Plate at rear).

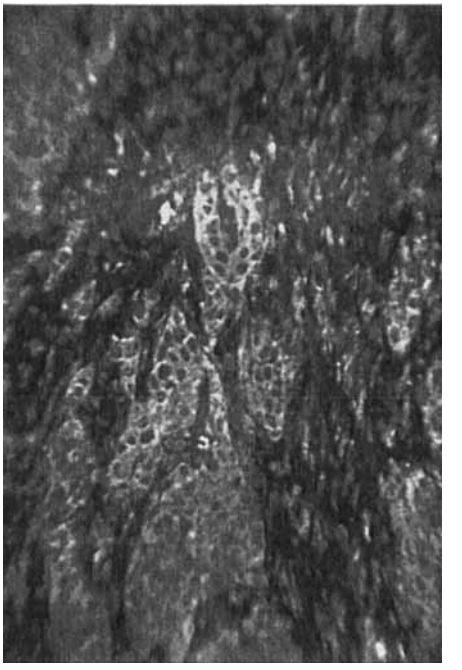

FIGURE 11 Frozen section of squamous cell carcinoma; stained with 9-AA. This section shows undifferentiated squamous cell carcinoma cells with intensely fluorescent cell surfaces due to the presence of active GB binding 9-AA. Magnification × 250 (see Colour Plate at rear).

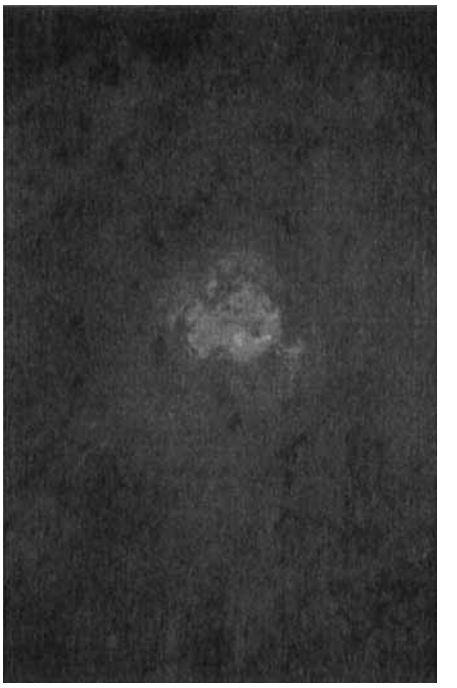

FIGURE 12 Frozen section of squamous cell carcinoma exposed to 10 μl isotonic saline; stained with 9-AA. Note the yellow fluorescence associated with the tumour cells in Figure 11 has now been prevented by exposure to the local inhibitors extracted in saline from these cells. The latent GB fails to bind 9-AA, but will do so after treatment with formaldehyde. Magnification × 250 (see Colour Plate at rear).

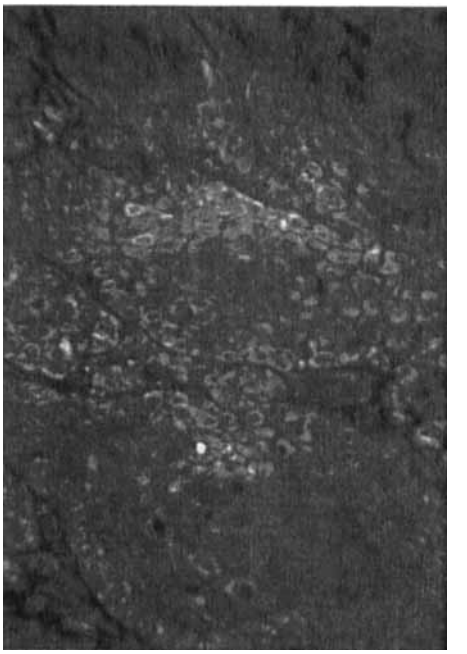

FIGURE 13 Frozen section of squamous cell carcinoma after chemotherapy; stained with 9-AA. The tumour cells fail to find 9-AA and do not exhibit fluorescence due to the presence of latent GB. This is similar to the artificial exposure of untreated squamous cell carcinoma cells to istonic saline in Figure 12. Magnification × 250 (see Colour Plate at rear).

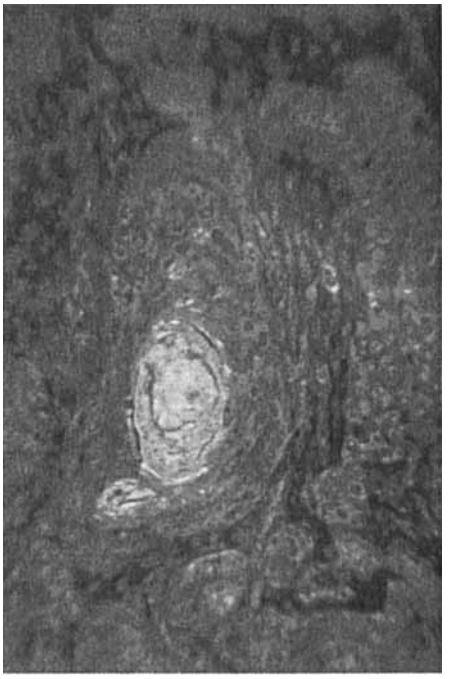

FIGURE 14 Formaldehyde treated squamous cell carcinoma after chemotherapy; stained with 9-AA. All the epithelial cells bind 9-AA due to the presence of active GB on their surfaces; even those in the keratinised pearls. Compare with Figure 13 in which similar cells possess latent GB prior to displacement of inhibitor with formaldehyde. Magnification × 250 (see Colour Plate at rear).

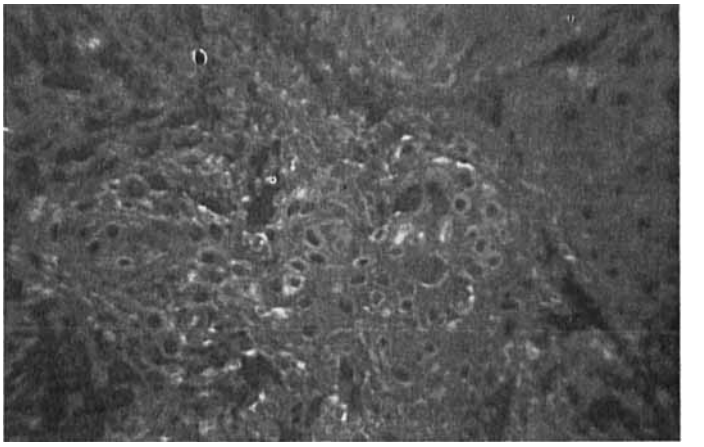

FIGURE 15 Formaldehyde treated squamous cell carcinoma after chemotherapy, subsequently exposed to inhibitor (extracted in saline from fresh frozen sections); stained with 9-AA. The GB on the squamous cell carcinoma cells has been inhibited by the fresh inhibitor and fails to bind 9-AA. The fact that the fresh inhibitor can recombine with the GB, thus mimicking the appearance of the frozen section of the cisplatin treated tumour (Figure 13) indicates that one effect of chemotherapy is the formation of latent GB on the cell's surfaces. Magnification × 250 (see Colour Plate at rear).

DISCUSSION

It has long been known that the activity of plasminogen activator increased when normal cells were transformed or modified to become tumour cells.[10] This increase in plasminogen activator is not confined to tumour cells but is also associated with migratory and invasive cells, part of the enzyme being located at the external surface of the cell attached to a receptor protein.[11] In this present study we have used a fluorescent probe to locate cells possessing a protease referred to as GB, which may well be plasminogen activator, since it is inhibited by PAI-1 with consequent loss in ability to bind 9-AA and loss of cell surface ability to fluoresce.[7] It has previously been demonstrated[1] that cytoplasmic proteins act as inhibitors of cell surface GB and can regulate the activity of cell surface GB.

Previous studies on oral smears[12] demonstrated that squamous cell carcinoma cells possessed active GB whilst normal cells did not. The data presented above indicate that normal oral epithelial layers lack cells with active GB (Figures 1 and 4) apart from cells in the basal epithelial layer. On the other hand, epithelial cells in leukoplakia and squamous cell carcinoma clearly possess active GB (Figures 2, 3, 10 and 11) which is capable of being inhibited by extracts obtained from these frozen sections (Figures 6 and 12). It was therefore of considerable interest to observe that in frozen sections of leukoplakia and squamous cell carcinoma after cisplatin chemotherapy (Figures 7 and 13) the GB associated with these cells was generally inactive and the cells had little or no ability to bind 9-AA. This inactivity was reversed by formaldehyde treatment[1] (Figures 8 and 14), known to displace inhibitor from GB, in a similar manner to that described above for the frozen sections of leukoplakia and squamous cell carcinoma with no chemotherapy.

The data presented above clearly show a marked difference in the ability of tumour cells to bind 9-AA, before and after chemotherapy. Clearly the experimental inhibition of GB on frozen sections of leukoplakia and squamous cell carcinoma (Figures 6 and 12) mimicked the effect of chemotherapy. We have simulated this change by artificially exposing the formaldehyde treated leukoplakia and tumour sections (obtained after chemotherapy) to fresh cytoplasmic inhibitors (Figures 9 and 15 respectively) with the consequent loss of GB activity and ability to bind 9-AA.

It seems highly likely that the observed difference in ability of the cells to bind 9-AA is due to the regulation of cell surface GB activity by cytoplasmic inhibitors of GB which have been released as one effect of cisplatin chemotherapy. If this hypothesis is correct then the observed tumour regression resulting from cisplatin chemotherapy may in part be due to regulation of the tumour cell surface protease (GB) by specific protein inhibitors leading to a period of reduced tumour cell aggressiveness. We noted that the effect of cisplatin on the well differentiated cells (Figure 5) of leukoplakia was uniform (Figure 7); all the cells exhibited reduced GB activity and reduced GB ability to bind 9-AA. On the other hand, a slightly different situation was observed in the squamous cell carcinoma of the oral cavity. In all cases the highly differentiated epithelial cells of the tumour forming the typical pearl configuration (Figure 10) showed marked inhibition of GB (Figure 13) after chemotherapy. The undifferentiated squamous cell carcinoma cells (Figure 11) often showed marked inhibition of GB, similar to the differentiated tumour cells, whilst other groups of undifferentiated squamous cell carcinoma cells showed no such inhibition of GB. It is not clear why these undifferentiated squamous cell carcinoma cells behaved in this manner after chemotherapy in different patients, but it is known that chemotherapy can lead to the induction of drug resistant tumour cells which have a high metastic potential.

We conclude that one effect of cisplatin chemotherapy of leukoplakia and squamous cell carcinoma patients leads to a decrease in cell surface GB activity and this decrease is caused by the release of a cytoplasmic protein which is capable of inhibiting cell surface GB.

This study is of interest with regard to the regulation of cell function through the inhibition of tumour cell surface proteases. It demonstrates that the functionally important location of GB is the *external* surface, since the internal cell surface is in direct contact with the cytoplasmic proteins which contain the inhibitior of GB in excess of the amount needed to inhibit all the GB associated with the cells. The cells possess enough inhibitor to inhibit their own GB and that GB on the target section used in the inhibitor transfer experiments. These comments support the claims made in earlier studies.[1,9] It also demonstrates that cell surface GB can be modified as a result of chemotherapy using cisplatin, an agent known to react with cellular DNA. The effect of cisplatin on the cell surface GB must be indirect, achieved by the export of a soluble cytoplasmic inhibitor protein resulting in the formation of cell surface-bound latent GB. Thus one of the actions of cisplatin would seem to be the decrease in cell surface GB activity mediated by an inhibitor protein, with the result that the tumour cells may become less aggressive and functionally less active. The probable role of inhibitors in regulation of cell surface proteases in tumour therapy has obvious significance in view of the known increase in activity of plasminogen activator (similar to GB in some respects[7]) which takes place in transformed cells and invasive cells.[10,11]

Acknowledgements

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