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Modulation of Tumour Associated Antigen Expressed on Human Squamous Cell Carcinoma Cell Lines by Recombinant Interferon-α

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Our earlier studies have shown that monoclonal antibody (Mab) 3F8E3 generated against a head and neck cancer cell line LICR-LON-HN$_2$ showed reactivity with squamous cell carcinoma (SCC) irrespective of the tissue of origin and identified antigens on SCC cell lines AW 13516 and AW 8507. The affinity constants (K_a) for binding of Mab 3F8E3 to AW 13516 and AW 8507 cell lines were 6.2×10^8 and 4.3×10^8 mol/l and it identified 6.8×10^4 and 3.77×10^4 sites/cell, respectively, as determined by Scatchard analysis. Treatment of both the cell lines with recombinant human interferon-α (rHu-IFNα) increased the binding affinity of the Mab but did not increase the number of binding sites on the SCC cell lines. Shedding of antigen recognised by the Mab in the culture supernatant of the cell lines was reduced after rHu-IFNα treatment. The results suggest that rHu-IFNα may bring about a firm anchorage of the tumour associated antigen on the SCC cells. Cells modulated with rHu-IFNα may serve as better targets for assessing cell mediated as well as Mab mediated cytotoxicity in oral cancer patients.
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INTRODUCTION

MONOCLONAL ANTIBODIES (Mab) showing reactivity with tumour associated antigen (TAA) may prove to be useful in the diagnosis and therapy of cancer. However, one of the prerequisites for the successful use of Mab as a therapeutic agent is the optimal binding of the Mab to the tumour cell surface. Immunomodulatory agents that can alter the expression of TAA on human tumour cells may prove to be useful as adjuvants in enhancing cellular as well as Mab mediated immunotherapeutic modalities.

Interferons (IFN) are a class of biological molecules endowed with antiviral and immunomodulatory properties [1–3]. Several studies have shown that IFN-α and -β can enhance the expression of Class I and Class II histocompatibility antigens on the surface of human and murine cells [4, 5]. Reports on the effect of IFN on the expression of TAA are conflicting [2, 3, 6, 7]. The expression of melanoma associated antigens (MAA) on the surface of human melanoma cells and the shedding of carcinoembryonic antigen (CEA) from human colon tumour cell line was enhanced following treatment with IFN-α [2, 3]. However, Imai [6] and Basham [7] reported no change in the expression of TAA after IFN-α treatment. Possible explanation for these conflicting reports could be the intrinsic differences in the malignant cell types analysed for expression of TAA after IFN-α treatment.

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In the present investigations, we have made use of purified recombinant human interferon α (rHu-IFN α) and a well defined Mab raised against a head and neck cancer cell line, LICR-LON-HN$_2$ [8], to study the modulation of TAA on SCC cell lines. The affinity for binding of the Mab to TAA and shedding of antigen by the SCC cell lines, was investigated before and after treatment of tumour cells with rHu-IFNα.

MATERIALS AND METHODS

Cell lines

Cell lines AW 13516 and AW 8507 were established in our laboratory from squamous cell carcinoma (SCC) of the tongue. Their growth characteristics and ultrastructural details are described elsewhere [9]. AW 13516 and AW 8507 were grown as monolayers in complete medium containing sterile Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% pooled human blood group AB serum, antibiotics (penicillin 200 IU/ml, streptomycin 100 μg/ml, gentamycin 80 μg/ml and nystatin 10 U/ml) and 2 mmol/l glutamine. Single cell suspensions were obtained by trypsinisation using 0.25% warm trypsin in phosphate buffered saline (PBS). The cells were washed three times with balanced salt solution (BSS) before being used in the assay.

Monoclonal antibody

Mab 3F8E3 (IgG3k), was secreted by hybridomas constructed after fusing SP2/O myeloma cells with splenocytes from BALB/c mice immunised with the cell line LICR-LON-HN$_2$. The Mab showed intense reactivity with SCC cell lines AW 13516 and AW 8507 [8]. Mab 3F8E3 was purified from the ascitic fluid of BALB/c mice using protein A sepharose

affinity chromatography [10]. Protein content of the purified antibody was determined using Lowry's method.

Radioiodination of Mab 3F8E3

Mab 3F8E3 was iodinated using the Iodogen method [11]. Purified Mab (100 μg/100 μl) was added to tubes coated with 100 μl of Iodogen (1 mg/ml, Pierce, USA) in chloroform. To this, 7.4 MBq of Na ^{125}I (carrier free, specific activity 525.4 MBq/μg, Amersham) was added. The reaction was allowed to take place for 10 min at room temperature. Labelled protein was then loaded on a Sephadex G-25 column equilibrated with 1% bovine serum albumin (BSA) in PBS. The iodinated Mab was eluted with PBS containing 1% BSA. Fractions (0.5 ml) were collected and 10 μl aliquots of each fraction were counted in a gamma counter (Gammamatic Kontron, Switzerland). Trichloroacetic acid precipitable radioactivity of the aliquots was determined. Protein fractions showing peak radioactivity were pooled and used in the binding assay. Specific activity of the labelled Mab 3F8E3 was 4.95×10^4 cpm/μg.

Recombinant human-interferon-α

rHu-IFNα was a kind gift from Ernest Boehringer Mannheim (Germany). Each vial containing 3.588×10^6 U of rHu-IFN α was reconstituted in 3.6 ml of RPMI containing 10% pooled human AB blood group serum and further diluted to give a final concentration of 1×10^4 U/ml. Aliquots of these stock dilutions were stored at −80°C and used as required in the assay.

Treatment of SCC cell lines with rHu-IFNα

Monolayer cultures of AW 13516 and AW 8507 cells were maintained in IMDM in tissue culture flasks (Nunc, Denmark). Cells (1×10^5) were incubated with 1000 U of rHu-IFNα for 96 h at 37°C in 5% CO_2 atmosphere [12]. A monolayer of cells was trypsinised, washed and used in the binding assay. Before trypsinisation, supernatants of these cultures were collected, concentrated five times using the Amicon filtration assembly (Amicon, U.S.A.). These supernatants were analysed for the shed antigen.

Binding of ^{125}I 3F8E3 Mab to SCC cell lines

Briefly, 0.5×10^6 cells (AW 13516 and AW 8507) were suspended in 100 μl of the assay medium containing IMDM and 1% BSA. The cells were incubated with increasing concentrations of ^{125}I Mab 3F8E3 (50 ng to 2000 ng), as triplicate sets of each concentrations. The cells were incubated at 4°C for 90 min with intermittent shaking. The unbound radioactivity was removed by washing the cells three times with 500 μl of BSS containing 1% BSA. Radioactivity bound to the cells was measured in gamma counter. Affinity constant of the Mab (K_a) and number of sites recognised per cell (n) were determined using Scatchard analysis [13].

Determination of antigens shed by tumour cells in culture supernatants

Supernatants collected from rHu-IFNα treated and untreated SCC cell lines (AW 13516 and AW 8507) were added in varying concentrations (v/v) to saturating amount (200 ng) of ^{125}I Mab 3F8E3 in triplicate sets. The reaction mixture was incubated for 60 min at 4°C and added to 10^5 SCC cells suspended in 100 μl of IMDM containing 1% BSA. The cells were incubated for 60 min at 4°C. The unbound radioactivity was removed by washing the cells three times with BSS containing 1% BSA. After centrifugation at 1000 rpm the counts in the pellet were measured in a gamma counter. The binding of ^{125}I Mab to target cells in the presence of medium alone was considered as 100%. The results were expressed as percent binding of ^{125}I Mab pretreated with culture supernatant to SCC cells.

Flow cytometry

Flow cytometric analysis was done using an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, Florida), equipped with water cooled, 400 mW argon laser operating at a wavelength of 488 nm. Cells from SCC cell lines AW 13516 and AW 8507 before and after rHu-IFNα treatment were trypsinised, washed and suspended in 50 μl of 0.1 mol/l PBS containing 1% fetal calf serum (FCS) and 0.02% sodium azide. They were incubated with Mab 3F8E3 (50 μl of the hybridoma culture supernatant) for 30 min at 4°C. After incubation, the cells were washed with PBS containing 1% FCS and 0.02% sodium azide and incubated with fluoresceinated rabbit anti-mouse antibody (RAM-FITC Sigma, 1:20 dilution) for 30 min at 4°C. Cells were washed, centrifuged at 1000 rpm for 10 min and resuspended in 0.5 ml of the same buffer. The stained cells were analysed for surface immunofluorescence on the flow cytometer. Cells stained with a purified fluorescein labelled mouse IgG (all subclasses) and those stained with RAM-FITC alone served as negative controls in the assay. Results were expressed as histograms of relative cell number versus log fluorescence intensity.

RESULTS

In the present experiments, we have studied the effect of rHu-IFNα on the modulation of TAA expressed on the SCC cell lines AW 13516 and AW 8507. It was observed that rHu-IFNα added at a concentration of 1000 U/ml did not affect the cell viability of cultures AW 13516 and AW 8507 even after 96 h of treatment. Viability of untreated and rHu-IFNα treated cell cultures was greater than 90% as judged by trypan blue dye exclusion. This concentration of IFN-α was apparently not cytostatic to cells as both in IFN-α untreated and IFN-α treated cultures, at the end of 96 h, the density of cells achieved was about 2×10^6/culture flask of 25 cm^2.

The effect of rHu-IFNα on the affinity and number of sites per cell of Mab 3F8E3 expressed on SCC cell lines was determined in a binding assay using Scatchard analysis. As seen in Figs 1 and 2, which show data of one representative experiment of the set of three performed, cell lines AW 13516 and AW 8507 expressed 6.8×10^4 and 3.77×10^4 sites/cell for the binding of Mab 3F8E3, respectively. The inter experimental coefficient of variation was 1.59%. The affinity with which Mab 3F8E3 bound the cells of AW 13516 and AW 8507 were 6.2×10^8 mol/l and 4.3×10^8 mol/l, respectively. After rHu-IFNα treatment, cell lines AW 13516 and AW 8507 expressed 6.3×10^4 and 3.85×10^4 sites/cell for Mab 3F8E3, respectively, indicating that rHu-IFNα treatment did not alter the number of TAA expressed on the SCC cell lines. It was however interesting to note that after rHu-IFNα treatment the affinity of Mab 3F8E3 binding to the SCC cell lines increased significantly. The increased affinity constants for Mab 3F8E3 on AW 13516 and AW 8507 cell lines were 1.03×10^9 mol/l and 1.55×10^9 mol/l, respectively (Figs 1 and 2).

The effect of rHu-IFNα treatment on the expression of TAA on SCC cell lines AW 13516 and AW 8507 was further

examined using flow cytometry. As seen in Fig. 3, before rHu-IFNα treatment, about 97.04% and 52.3% of cells of AW 13516 and AW 8507 cell lines, respectively, stained positively for Mab 3F8E3. Following rHu-IFNα treatment of SCC cell lines for 96 h, the percentage of AW 13516 and AW 8507 cells showing binding with Mab 3F8E3 remained unchanged (Fig. 3). There was no significant change in the fluorescence intensity of the stained cells before and after IFN treatment. Thus, these data indicate that rHu-IFNα treatment did not increase the surface expression of TAA on SCC cell lines, neither did it induce *de novo* expression of the TAA.

The stability of TAA expressed on the SCC cell lines was assessed by estimating the amount of antigen shed in the culture supernatants of untreated and rHu-IFNα treated SCC cell lines. As seen from Fig. 4, both untreated and IFNα

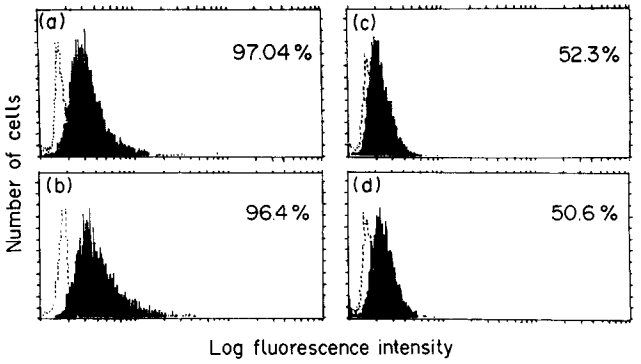

Fig. 3. Flow cytometric analysis of Mab 3F8E3 binding to the surface of SCC cell lines AW 13516 (a, b) and AW 8507 (c, d) before (a and c) and after (b and d) treatment with r-Hu-IFNα. Dotted line histograms represent isotype controls.

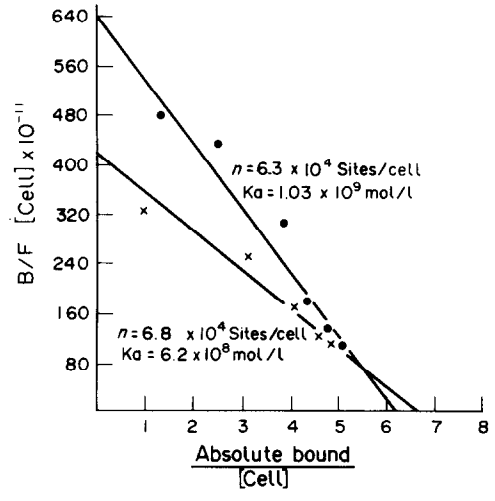

Fig. 1. Scatchard plot of binding of Mab 3F8E3 to the SCC cell line AW 13516. Untreated (×) or cells treated with 1000 U/ml of rHu-IFNα (●) for 96 h.

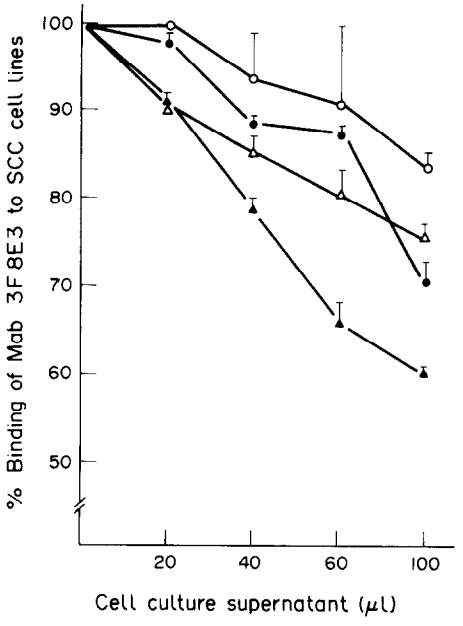

Fig. 4. Effect of rHu-IFNα on the shedding of TAA expressed on the SCC cell lines AW 13516 (○, ●) and AW 8507 (△, ▲). The antigen shed in the culture supernatant was quantitated using an inhibition binding assay. Culture supernatant from untreated (●, ▲) or rHu-IFNα treated (○, △) cells were incubated with ^{125}I Mab 3F8E3. To this reaction mixture, 10^5 cells were added. The results were expressed as mean percentage (±S.E.) of the Mab 3F8E3 bound to SCC cells in presence of supernatants.

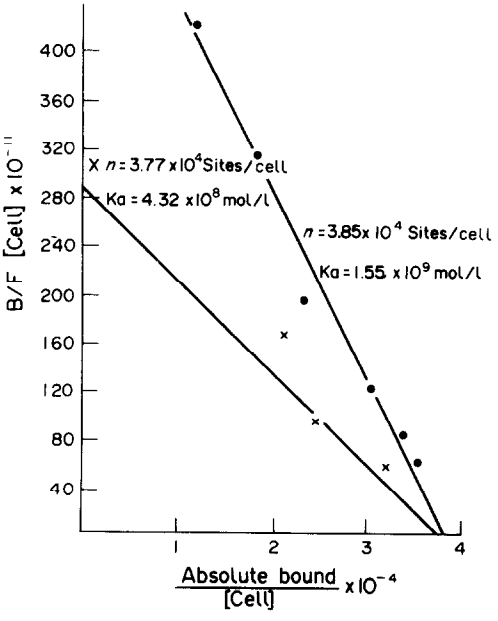

Fig. 2. Scatchard plot of binding of Mab 3F8E3 to the SCC cell line AW 8507. Untreated (×) or cells treated with 1000 U/ml rHu-IFNα (●) for 96 h.

treated SCC cells did show shedding of TAA in culture supernatant. Even in the presence of 20% supernatant there was inhibition of binding from 100% to about 90% with AW 8507 cells. With increasing amounts of culture supernatants used for incubation with the labelled antibodies, it was seen that the per cent binding was much less with supernatants of untreated cultures compared with IFN-α treated cultures, indicating that the amount of antigen shed was proportionately less after treating the SCC cells with rIFN-α. This phenomenon was observed for both cell lines.

DISCUSSION

IFNs are naturally occurring biological substances with potent immunomodulatory properties. They are capable of

modulating antigen expression on a variety of human tumour cell lines grown *in vitro* [14–17] and human tumour xenografts grown *in vivo* [18]. When administered to patients with bladder cancer, renal cell carcinoma, malignant melanoma, carcinoid tumours and those with SCC of head and neck and lung, IFNs were found to be effective in bringing about regression of the tumours [19–22].

In the present paper, we have studied the effect of rHu-IFNα on modulation of the TAA identified by Mab 3F8E3 on SCC cell lines AW 13516 and AW 8507. Binding studies with ^{125}I Mab 3F8E3 showed that rHu-IFNα treatment of the SCC cell lines did not alter the cell surface expression of TAA. Similar results have been reported by Basham *et al.* [7] and Imai *et al.* [6] who found no change in the expression of MAA on melanoma cells treated with rHu-IFNα.

On the other hand, Greiner *et al.* [14, 16] have reported increased levels of CEA and TAA TAG-72 on the surface of breast and colorectal carcinoma cell lines treated with IFNγ. Similarly, Marth *et al.* [23] showed increased expression of CA 125 on ovarian carcinoma cell lines treated with IFNγ. Cell surface expression of 115 Kd and 100 Kd MAA were also increased on melanoma cells grown in the presence of IFNα, β or γ [24, 25].

Guadagni *et al.* [26] suggested that there may exist an inverse correlation between the constitutive levels of tumour antigen expression and the degree to which the rHu-IFNs can augment its expression. In their study, they observed that only those ovarian adenocarcinomas which initially expressed low levels of antigen TAG-72, showed increased expression of the antigen after rHu-IFNα and rHu-IFNγ treatment. For tumour cells which expressed high levels of either TAG-72 or CEA antigen, rHu-IFNα or rHu-IFNγ treatment resulted in no further increase in their levels.

In another study Grenier *et al.* [15] demonstrated that cloned populations of breast carcinoma cell line MCF-7 showed heterogeneity in the expression of TAA. Tran *et al.* [27] have also confirmed the inability of rIFNα to augment the binding of Mab to breast carcinoma cell line which constitutively expressed high levels of tumour antigens.

Additional studies using flow cytometry further confirmed our observations. Flow cytometric analysis of AW 8507 is of particular interest, since only about 50% of these cells expressed TAA initially. IFNα treatment did not induce the negative (or weakly positive) cells to express the TAA. Similar studies conducted by Greiner *et al.* [16] showed that IFNα treatment modulated the TAA expression on tumour cells both in terms of accumulation of more antigen per cells as well as increase in the percentage of cells expressing TAA.

In most of the reports dealing with modulation of TAA by IFNα or IFNγ, simple binding of radiolabelled Mab to surface antigens has been studied. The affinity of binding has not been studied so far. We have analysed the expression of TAA in a more quantitative way by analysing the sites per cell and the affinity of binding. Our results have shown that although IFNα treatment did not increase the total binding of ^{125}I labelled Mab to SCC cells, indicative of increased expression of TAA, we did find almost a 2–3-fold increase in the affinity. The increased affinity could be a result of better stability of the TAA on SCC cell membranes. This possibility is corroborated by the observation of decreased shedding of TAA in culture supernatants by SCC cells grown in presence of IFNα. Other reports demonstrating the increased shedding of TAA after after IFN treatment [12, 14, 17, 28], unfortunately, do not provide information on affinity status of Mab binding to TAA.

The ability of IFN-α to stabilise the expression of TAA, as shown by us, might have an important potential clinical application in radiolocalisation and immunotherapy of tumours with Mabs. Rowlinson *et al.* [29] have demonstrated that the administration of rHu-IFNα to nude mice bearing human breast tumours significantly improved the radioimaging of these tumours by the Mab. Furthermore, such modulation of TAA by rHu-IFNα may also potentiate the immune recognition of tumour cells by cellular cytotoxic cells.

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