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Short communication

Differential growth inhibition and enhancement of major histocompatibility complex class I antigen expression by interferons in a small-cell lung cancer cell line and its doxorubicin-selected multidrug-resistant variant

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Summary. Expression of class I and class II major histocompatibility complex antigens on a human small-cell lung cancer cell line and its multidrug-resistant variant was examined before and after exposure to interferon α (IFNα) and IFNγ by flow cytometry. Neither IFNα nor IFNγ induced class II antigen expression on the drug-sensitive or resistant cell line. Induction of class I antigen expression along with an inhibition of proliferation was observed in both cell lines after IFNα treatment. On the other hand, IFNγ treatment resulted in growth inhibition and enhancement of class I antigen expression in the sensitive cell line but not the resistant cell line. The differential response of the two cell lines to IFNγ cannot be directly attributed to the acquisition of drug resistance but it suggests that further investigation of the possibility that drug-sensitive and resistant small-cell lung tumors may respond differently to immunotherapies that include IFNγ is warranted.

Introduction

The human major histocompatibility complex (MHC) genes, on the short arm of chromosome 6, encode two main classes of cell-surface glycoproteins. The class I antigens are determined by the HLA-A, HLA-B and HLA-C loci while the class II antigens are determined by the HLA-DR, HLA-DP and HLA-DQ loci. The HLA-A,B,C class I antigens are found on virtually every nucleated human cell and consist of a polymorphic glycoprotein in non-covalent association with a non-polymorphic protein, β_2-microglobulin. In contrast to the ubiquitous nature of class I antigens, class II antigens are found pimarily on antigen-presenting and immunocompetent cells.

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A number of tumors and tumor cell lines have been reported to express very low or non-detectable levels of surface class I molecules and β_2-microglobulin, including small-cell lung cancer (SCLC) [1, 5, 8]. In some instances, such cells can be induced to express increased class I MHC antigens by treatment with a number of agents including the interferons (IFN) [1, 3, 15, 19]. Such augmentation of MHC expression can alter the susceptibility of SCLC cells to lysis by cytotoxic T lymphocytes [23], although it does not appear to be involved in increased natural killer cell susceptibility [20]. There is general agreement that SCLC tumors and established cell lines express very low or non-detectable levels of class II antigens but reports of their inducibility by IFN appear variable [1, 5, 17].

The effective use of combination chemotherapy has increased the median survival of patients with SCLC from 6 weeks to about 1 year [22]. Nevertheless, the long-term survival in this disease is rare largely because of the development of multidrug resistance. New therapeutic strategies incorporating immunomodulating agents are being considered [2, 22]. It is possible that mutations might occur during the development of drug resistance that affect the tumor's response to such agents and hence the antigenic phenotype and malignant potential of the tumor cells [7]. To test this idea, we have examined a human SCLC cell line, NCI-H69 (H69), and its doxorubicin-selected multidrug-resistant derivative, H69AR [12], for reactivity with monoclonal antibodies (mAb) directed against class I and class II antigens before and after exposure to IFNα and IFNγ.

Materials and methods

Media, chemicals and antisera. 3-(4,5-Dimethylthiazol-2)-yl-2,3-diphenyltetrazolium bromide (MTT) and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis Mo. Recombinant human interferon α-2a (IFNα) was a gift from Schering Canada, Pointe Claire, Que., and recombinant human interferon γ (IFNγ) was purchased from Genzyme, Markham, Ont. Anti-human HLA-DR and purified NS-1 ascites were obtained from Cappel-Cooper Biomedical, Malvern, Pa. Ascites of mAb W6/32 specific to MHC class I determinants [16], and

anti-β_2-microglobulin (B2M) were obtained from Dimension Laboratories, Mississauga, Ont. Fluorescein-isothiocyanate(FITC)-conjugated F(ab')$_2$ fragment affinity-purified goat anti-(mouse IgG) (heavy chain) was purchased from Organon-Technika, Scarborough, Ont.

Cell lines. The SCLC tumor cell line NCI-H69 (H69) was provided by Drs. A. Gazdar and J. Minna (NIH, Bethesda, Md.) and its multidrug-resistant variant, H69AR, has been described previously [4, 12]. The H69AR cell line differs from many multidrug-resistant cell lines in that it does not overexpress P-glycoprotein [12]. Both cell lines were maintained in RPMI-1640 medium (Gibco, Burlington, Ont) supplemented with 5% (v/v) fetal bovine serum and L-glutamine (4 mM); H69AR cells were challenged every 2–4 weeks with 0.8 μM doxorubicin. Cells were cultured in drug-free medium for at least 72 h before each experiment.

Chemosensitivity testing. Dose/response curves were determined for IFNα and IFNγ by a microtitre plate chemosensitivity assay, which utilizes a tetrazolium salt, MTT [13]. MTT is converted to a colored formazan product by reducing enzymes present only in viable cells and its use with the H69 and H69AR cell lines has been previously described [4].

Flow cytometry. Cells (1×10^6/tube) were pelleted by centrifugation and the supernatant was removed by aspiration. All subsequent procedures were carried out at 4° C. Samples of 50 μl appropriately diluted mAb in 1% bovine serum albumin in phosphate-buffered saline (cold wash) were added and incubated for 30 min. Cells were then washed twice with cold wash and 50 μl FITC conjugate, diluted 1:500 in cold wash with 5% normal goat serum, were added and incubated for 60 min in the dark. After two further washes, the cells were resuspended in 500 μl cold wash and analyzed immediately on a Becton Dickinson FACS IV flow cytometer, using an argon-ion laser running at 200 mW at 488 nm with a 530/20 band-pass filter in front on the photomultiplier. Data were collected from 20000 cells, which had been gated by forward scatter. Histograms were displayed on a 256-channel log scale, calibrated at 60 channels/decade [18]. The mean log (fluorescence intensity) channel was calculated from the log-scale histograms. Relative fluorescence intensity was calculated by the difference, given in channel numbers, between the mean log(fluorescence intensity) of the test sample and of the background NS-1 ascites control sample. The proportion of antigen-bearing cells (% positive) was calculated by analyzing samples using a lower gate set at the channel where 95% of the negative control cells (incubated with NS-1 ascites) were excluded.

Cells for the modulation studies were seeded at a density of 1×10^5 cells/ml and IFNα or IFNγ was added 24 h later. After a further 3 days incubation, cells were harvested and analyzed by flow cytometry as above. Each experiment was repeated at least three times. Statistical analysis was performed using an unpaired *t*-test to compare the H69 and H69AR cell lines and a paired *t*-test to compare treated cells with untreated cells.

Results and discussion

The ultimate success of immunotherapy in SCLC will depend on the ability either to render cancer cells more recognizable to the immune system, or to enhance the immune response towards a more effective recognition of the tumor cells, or both. The present study deals with the former possibility. Since class I and class II MHC molecules in combination with a foreign antigen stimulate the immune system, enhanced expression of MHC antigens in a malignancy such as SCLC could increase the tumor's ability to generate an effective immune response. However, most patients with SCLC will be drug-resistant by the time immunotherapy is attempted, and therefore it is important to know whether or not drug-resistant and -sensitive tumors respond in a similar manner to immunomodulating agents such as the interferons.

Table 1. Basal expression of major histocompatibility complex (MHC) class I antigens on H69 and H69AR cells[a]

Cell line	mAb			
	W6/32		B2M	
	RFI[b]	% Positive[c]	RFI[b]	% Positive[c]
H69	18±5	23±10	17±3	26±9
H69AR	18±7	27±11	17±5	30±11

[a] Values shown are the mean ± SD of results obtained in 11–12 independent experiments

[b] Relative fluorescence intensity (RFI) represents the difference (given in channel numbers) between mean log(fluorescence intensity) of the test sample and the background NS-1 ascites control sample. A 60-channel difference represents a 10-fold change in fluorescence intensity

[c] The number of events that occurred above the channel number at which 95% of the negative control (NS-1) events had been counted

In the present study, we found that the expression of class I molecules on both the drug-sensitive H69 cells and the drug-resistant H69AR cells, although low, was detectable by flow cytometry (Table 1) and was similar on the two cell lines. Class II molecules, as recognized by mAb HLA-DR, were not detectable on either cell line (not shown); this is consistent with the observations of others [1, 5, 17]. These results indicate that the acquisition of drug resistance is not accompanied by changes in the basal expression of either class I or class II MHC antigens.

When measured by the MTT assay, IFNα and IFNγ did not appear growth-inhibitory to either cell line (results not shown). With IFNα, even at a dose of 10000 UI/ml, little inhibition was observed. H69AR cells were slightly more sensitive to IFNγ than H69 cells but only at a concentration greater than 6000 UI/ml. These results were surprising since these concentrations of IFN have been reported to be growth-inhibitory in other studies with SCLC cells [1, 19, 23]. Therefore, to verify the results obtained with the MTT assay, an alternative method of measuring growth inhibition and cytotoxicity was employed. When cells were counted directly with trypan blue exclusion as an indicator of viability, both IFNα and IFNγ inhibited the proliferation of H69 cells by approximately 50% after culturing for 3 days at concentrations of 10000 UI/ml and 2000 UI/ml, respectively (Fig. 1). Thus the growth-inhibitory effects of IFNα and IFNγ observed by counting cells directly were not detected in the MTT assay. Jabber et al. [9] have similarly found that the MTT assay underestimates the growth-inhibitory effects of both interferons on lung tumor cell lines. Of particular interest in the present study is that the H69AR cell line appears cross-resistant to IFNγ since this agent inhibited the proliferation of H69 cells but not H69AR cells (Fig. 1). The basis of this difference in sensitivity to the growth-inhibitory effects of IFNγ is unknown.

The ability of interferons to enhance the expression of class I heavy-chain molecules and β_2-microglobulin has been reported for a number of human tumor cell lines including SCLC. Doyle et al. [5] showed that IFNα and IFNγ enhanced the expression of HLA-A,B,C and β_2-microglobulin on SCLC cell lines as well as on several non-

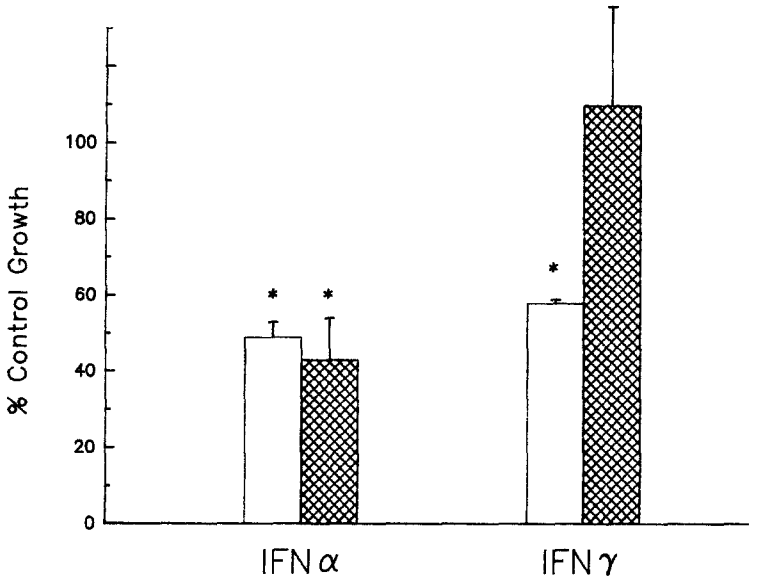

Fig. 1. Inhibition of cell proliferation by interferon α (IFNα) and IFNγ. H69 cells (*open bars*) and H69AR cells (*cross-hatched bars*) were seeded at 1×10^5/ml and cultured in the absence or presence of IFNα (10 000 UI/ml) or IFNγ (2000 UI/ml). After 3 days, cell counts were determined using a hemocytometer with trypan blue exclusion as a measure of viability. Results were expressed as a percentage of untreated cells and the *bars* represent the mean (± SD) of values obtained in three independent experiments. The *asterisk* denotes a significant difference from untreated cells, $P < 0.05$

Table 2. Effect of IFNα on MHC antigen expression on H69 and H69AR cells[a]

mAb	Cell line	RFI[b]		% Positive[c]	
		Control	Treated	Control	Treated
W6/32	H69	17±4	31±7[d]	20±8	48±12[d]
	H69AR	16±6	26±7[d]	22±7	39±8[d]
B2M	H69	17±2	29±5[d]	26±8	50±11[d]
	H69AR	18±3	21±7[e]	32±7	38±8[e]
HLA-DR	H69	0±1	0±2[e]	5±1	5±1[e]
	H69AR	0±1	−1±2[e]	5±1	4±2[e]

[a] Indirect immunofluorescence staining of tumor cells treated with 10000 UI/ml IFNα for 3 days. Values shown are the mean ± SD of results obtained in 3–6 independent experiments
[b] Relative fluorescence intensity (RFI) represents the difference (given in channel numbers) between mean log (fluorescence intensity) of the test sample and the background NS-1 ascites control sample. A 60-channel difference represents a 10-fold change in fluorescence intensity
[c] The number of events that occurred above the channel number at which 95% of the negative control (NS-1) events had been counted
[d] $P < 0.01$, paired *t*-test, compared with untreated controls
[e] Not significantly different from untreated controls ($P > 0.1$)

Table 3. Effect of IFNγ on MHC antigen expression on H69 and H69AR cells[a]

mAb	Cell line	RFI[b]		% Positive[c]	
		Control	Treated	Control	Treated
W6/32	H69	18±5	32±9[d]	26±11	52±15[d]
	H69AR	20±8	19±9[e]	32±13	37±13[e]
B2M	H69	17±4	21±8[e]	27±11	38±13[e]
	H69AR	15±6	21±10[e]	28±14	38±15[e]
HLA-DR	H69	0±2	−3±4[e]	4±2	4±2[e]
	H69AR	−5±8	−1±2[e]	4±1	3±1[e]

[a] Indirect immunofluorescence staining of tumor cells treated with 2000 UI/ml IFNγ for 3 days. Values shown are the mean ± SD of results obtained in 3–6 independent experiments
[b] Relative fluorescence intensity (RFI) represents the difference (given in channel numbers) between mean log(fluorescence intensity) of the test sample and the background NS-1 ascites control sample. A 60-channel difference represents a 10-fold change in fluorescence intensity
[c] The number of events that occurred above the channel number at which 95% of the negative control (NS-1) events had been counted
[d] $P < 0.01$, paired *t*-test, compared with untreated controls
[e] Not significantly different from untreated controls ($P > 0.1$)

SCLC cell lines. Augmentation of class I antigens by interferons was also observed in the present study although the response of the H69 and H69AR cell lines to these agents differed. IFNα caused an increase in the class I molecules recognized by mAb W6/32 in both cell lines (Table 2). However, while IFNα also enhanced β_2-microglobulin expression in H69 cells, it did not do so in H69AR cells. Reports of differential induction of class I heavy-chain molecules and β_2-microglobulin are rare [10]. It is possible that a β_2-microglobulin-like molecule is induced by IFNα but is not recognized by the mAb B2M. MHC class II expression remained undetectable in both SCLC cell lines after IFNα treatment (Table 2). Thus the development of drug resistance does not alter the inability of IFNα to induce class II expression in the H69 SCLC cell line.

In response to treatment with IFNγ, H69 cells displayed a significant increase in HLA class I heavy-chain expression as detected by mAb W6/32 (Table 3). The relative fluorescence intensity of IFNγ-treated H69 cells was 32 channels, compared to 18 for untreated cells. The difference of 14 channels represents a 1.7-fold increase in fluorescence intensity. In contrast, expression of this antigen on the drug-resistant H69AR cells was unaffected by IFNγ treatment (Table 3). Furthermore, β_2-microglobulin expression was not significantly increased in either cell line by IFNγ treatment (Table 3). The absence of enhanced expression of β_2-microglobulin on H69AR cells by IFNγ is not unexpected since class I heavy-chain molecules were also not induced on this cell line. However, heavy-chain class I expression was induced on H69 cells and the absence of a concomitant increase in β_2-microglobulin expression is of interest.

The difference in IFNγ-induced increases in MHC class I antigen expression in the H69 versus the H69AR

cells may be due to several factors including differences in the intrinsic properties of each cell line at the receptor or post-receptor level. Ucer et al. [21] reported that a panel of colon adenocarcinoma cell lines could bind IFNγ with comparable affinities even though significant differences in the number of available plasma membrane receptors were observed. It has become clear that specific binding of IFNγ to its receptor is a prerequisite for activity, but is not sufficient to confer sensitivity to IFNγ action, and post-receptor events are required. Thus, alterations of genetic response elements may be involved, which could result in a differential regulation of antigen expression in H69 and H69AR cells [6, 11].

The generally low or absent expression of class II antigens on tumor cells including SCLC has been a consistent observation in a number of studies [5, 14, 17]. IFNγ has been reported to induce expression of class II antigens in cell lines derived from a variety of carcinomas including lung, colon, cervix, and mammary gland [19] (and others). With respect to SCLC, there have been reports that IFNγ treatment induces class II expression [17, 19] while in other studies this inducibility in SCLC cell lines has not been observed [5]. Our results are consistent with the findings of the latter studies (Table 3). It is difficult to reconcile these apparently contradictory observations since several of the same cell lines were used in the different studies. In any case, our results demonstrate that the expression of class II antigens is no more inducible by IFNγ in H69AR cells than in H69 cells.

In conclusion, we have shown that the drug-sensitive H69 SCLC cell line and its multidrug-resistant variant H69AR respond similarly to IFNα treatment, i.e., inhibition of proliferation is observed along with induction of class I antigen expression. In contrast, the H69AR cell line was resistant to IFNγ-mediated growth inhibition and class I induction compared to the H69 cell line. While it is clearly premature to attribute the differential response of the H69 and H69AR cell lines to the acquisition of drug resistance, our findings demonstrate the need to consider the possibility that differences in IFNγ responsiveness may exist. The extent to which this phenomenon may occur in resistant SCLC or other tumors awaits further study.

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