Potentiation of Interferon Induction of Class I Major Histocompatibility Complex Antigen Expression by Human Tumor Necrosis Factor in Small Cell Lung Cancer Cell Lines

Garry M. Marley,1 L. Austin Doyle,2 José V. Ordóñez, Allison Sisk, Arif Hussain, and Ray-Whay Chiu Yen

University of Maryland Cancer Center and Division of Medical Oncology, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT

The response of class I major histocompatibility complex antigen expression to in vitro administration of interferon and tumor necrosis factor α (TNF-α) was measured using class I major histocompatibility complex-deficient small cell lung cancer cell lines. Significant induction also was observed using γ interferon (IFN-γ) alone, whereas TNF-α alone yielded only modest induction. Classic small cell lung cancer cell lines NCI-H146 and NCI-H209 best demonstrated synergistic HLA and β2m-microglobulin antigen induction with IFN-γ and TNF-α with the following dose schedule: 3–6 days of TNF-α (200 units/ml) followed by 48 h of IFN-γ (100 IU/ml). Induction was quantitated using an 125I-Protein A radioimmunoassay. Synergistic induction of the HLA and β2-microglobulin surface antigens on NCI-H146 was also possible with α interferon and TNF-α but required a higher concentration of the interferon, i.e., 3–6 days of TNF-α (200 units/ml) followed by 48 h of α interferon (1000 units/ml).

Small cell lung cancer cell line NCI-H146 was further studied for expression of major histocompatibility complex messenger RNA using the optimal doses and sequence of addition of IFN-γ and TNF-α as indicated above. A significant induction with IFN-γ alone and synergistic induction with both IFN-γ and TNF-α was quantitated for both HLA-A2 and β2-microglobulin transcripts using Northern blot analysis. Incubation with relatively low subcytotoxic doses of IFN-γ and TNF-α also resulted in a marked synergistic decrease in c-myc message.

INTRODUCTION

SCLC currently comprises 20–25% of the newly diagnosed cases of bronchogenic carcinoma in the United States (1). The high metastatic potential of SCLC is of great significance to the clinician, whereas unique aspects of its cell biology distinguish SCLC from other forms of lung cancer, making it a focus of research interest. SCLC cell lines are distinguished from other lung cancer cell lines by their ability to grow in suspension culture and by production of multiple peptide hormones (1). They also possess a deletion in the short arm of chromosome 3 (2) and minimal cell surface expression of the class I MHC antigens HLA-A, -B, and -C and β2m (3).

The class I MHC antigens are expressed on the surface of most human nucleated cells. They consist of a 45-kDa protein heavy chain noncovalently associated with a 12-kDa peptide β2m, which is required for membrane transport of the heavy chain (4). SCLC shares its paucity of class I MHC expression with neuroblastoma and other neural and neuroendocrine cells (5). In addition, by immunohistochemical analysis, fresh SCLC tumor specimens reveal a lack of surface class I MHC expression (3, 6).

Since class I MHC antigens are a prerequisite for the activation of cytotoxic T-lymphocytes in the cellular immune response (7), their decreased surface expression could potentially allow tumor cells to escape rigorous cellular immune control and to metastasize early in the natural history of the disease.

Interferons of leukocyte origin (α and γ) have been shown to induce the surface expression of class I MHC antigens on both SCLC and non-small cell lung cell lines (3), as well as neuroblastoma (8), human melanoma (9), and other human tumor cells. Most class I MHC genes have promoter sequences which are responsive to interferon (10). Although α interferon has been successfully employed as a clinical agent against hairy cell leukemia (11) and chronic myelogenous leukemia (12), its in vivo role as an immunomodulatory agent is relatively unexplored. The mechanisms and cytokine interactions of the leukocyte interferon-stimulated induction of MHC antigens have also been sparsely investigated.

TNF-α is a 17-kDa protein known to be released by activated macrophages following stimulation by endotoxin (13). TNF-α has been demonstrated to be cytotoxic against some tumor cell lines in vitro (14) and in vivo (15) and in some cases has had additive or synergistic effects in combination with the leukocyte interferons (16–18). In dermal fibroblasts and endothelial cells, TNF-α has been demonstrated to act as a single agent in the induction of the surface expression of HLA antigens (19), an observation more recently noted in human tumor cells (20). TNF-α-treated fibroblasts will induce endogenous β2 interferon production (21).

Since both TNF-α and the interferons can modulate HLA expression, our objective was to study the regulation of class I MHC expression at the level of both surface antigen and transcription by combinations of interferon and tumor necrosis factor. Both the dose-dependent quantitative level of response to induction and the optimal dose schedules and sequence needed to be studied in order to ascertain optimal conditions for antigen induction.

MATERIALS AND METHODS

Cell Lines. The human SCLC cell lines were cultured in RPMI 1640 (Gibco Laboratories, Gaithersburg, MD) supplemented with 2% fetal bovine serum (HyClone Laboratories, Logan, UT), hydrocortisone, insulin, selenium, transferrin, and estradiol (Collaborative Research, Boston, MA), as previously described (22). Cell lines NCI-H146 and NCI-H209 are biochemically and morphologically typical of classic SCLC while the c-myc-amplified NCI-H82 possesses variant SCLC features (23). NCI-H125 is a lung adenocarcinoma cell line with high endogenous levels of class I MHC antigen production (3). All SCLC cell lines employed grew readily in suspension culture within standard Corning or Nunc tissue culture flasks. Cell viability was routinely assayed using the Trypan blue exclusion technique (24).

Cytokines. IFN-α was supplied by Hoffman-LaRoche (Nutley, NJ)...
and diluted in RPMI 1640 from a stock solution of 10^6 IU/ml.

IFN-γ was donated by Genentech (San Francisco, CA) and diluted in RPMI 1640 from a stock solution of 10^7 IU/ml.

TNF-α was also donated by Genentech and diluted in complete tissue culture medium from a stock solution of 3 x 10^6 units/ml (0.489 mg/ml protein).

Antisera. Primary monoclonal antibodies consisted of anti-human HLA-A, -B, -C (Cappel, Malvern, PA) and anti-human β₂m (No. HB28; American Tissue Culture Collection, Rockville, MD). Secondary antisera for the indirect immunofluorescence assays consisted of FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Avondale, PA). Secondary antisera for the indirect immunofluorescence assays consisted of FITC-conjugated goat anti-mouse IgG (Becton-Dickinson, Mountain View, CA).

Radioimmunoassay. The RIA was performed in 96-well polystyrene Falcon 3912 MicroTest III plates (Becton Dickinson), as previously described (25). All samples were counted in a Beckman 6000 gamma counter. Negative controls consisted of untreated cells incubated with purified mouse IgG (Cappel) and cells incubated without any primary antisera. Positive controls used peripheral blood lymphocytes from a healthy donor. Medium was replenished on day 4 of the experiment. Total duration of the experiment was 6 days. All cell cultures were harvested 2 days following the addition of interferon. Total duration of the experiment was 6 days.

Indirect Immunofluorescence Assay. Following PBS washings, log phase cultures of SCLC were incubated under saturation conditions with a 1:500 dilution for anti-HLA, -B, -C and anti-β₂m antibodies. The total reaction volume was 0.2 ml in 3-ml polystyrene Falcon tubes. After incubation with shaking for 1 h at room temperature, the cells were pelleted at 1500 x g, washed with PBS, and incubated for 30 min with a 1:25 dilution of FITC-conjugated goat anti-mouse IgG. The cells were pelleted again, washed in PBS, vigorously resuspended in 0.2 ml PBS with a Pasteur pipette, and screened through a 43-μm wire mesh in order to eliminate cell clumping. The cells were then immediately assayed for mean fluorescence in a FACS IV Flow Cytometer (Becton-Dickinson). Cells (5 x 10^4) were analyzed using a live gate placed on forward light scatter. Three hundred mW of laser power and 488 nm excitation light were used. Fluorescence emission was collected through a 530/30 filter. The percentage of positive cells and mean fluorescence (26) were determined using the analysis capabilities of the FACS IV.

Prior to the incubations, each cell sample was divided into three parts: (a) for a negative control (i.e., without primary antibody); (b) for relevant antibody addition; and (c) for addition of irrelevant antibody [purified mouse IgG (Cappel)].

RNA Isolation and Northern Blotting. All total RNA preparations were made from 5 x 10^6 cells by the method originally described by Chirgwin et al. (27). All final RNA precipitations were stored at -20°C in 70% ethanol.

Twenty μg of total RNA were added to each lane of an ethidium bromide-pretreated formaldehyde/1% agarose gel prepared as described by Lefrach et al. (28). RNA molecular weight markers were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Filters were hybridized using 50% formamide with a radiolabeled probe according to the procedure described by Thomas (29) and were washed at 65°C. Following hybridization, dried nitrocellulose filters were autoradiographed on Kodak XAR-5 film with a Dupont Cronex intensifying screen at -70°C. The intensity of the signal on the autoradiogram was quantitated by a Quick Scan densitometer (Helena Laboratories; Beaumont, TX) and expressed as a value relative to the control. Histograms represent the mean of triplicate samples where each value was within 10% of the mean value.

RESULTS

A primary goal of this study was to ascertain the dose-dependent nature of class I MHC antigen induction by interferon and to determine whether this effect is in any way potentiated by TNF-α. In a classic SCLC cell line (NCI-H146), we noted a dose-dependent induction by RIA for both HLA and β₂m surface antigens, using either IFN-α (Fig. 1) or IFN-γ (Fig. 2). MHC antigens of SCLC cell NCI-H209, another classic SCLC cell line, were similarly induced by IFN-γ (Fig. 3). The threshold for response, however, was much lower for γ interferon and was detectable at less than 100 IU/ml. Accordingly, subsequent experiments were all conducted with the same lot of recombinant IFN-γ (Genentech). A flow cytometric analysis (Table 1) of the HLA surface antigen expression in NCI-H146 was used to confirm the RIA observations. Nonetheless, the optimal enhancement of HLA surface expression appeared to be achieved at 100 IU/ml IFN-γ within 48 h of administration. The same dose of IFN-γ over 24 h resulted in less than maximal class I MHC induction, which was observed at both 48 and 72 h after interferon addition. No significant or consistent improvement in response was noted among the cell lines above that concentration (data not shown). The results of the RIA and flow cytometry experiments warranted selection of 100 IU/ml IFN-γ as a standard optimal dose for subsequent transcriptional analysis experiments.

When the SCLC cell lines were pretreated for a period of 6 days (prior to IFN-γ addition) with recombinant TNF-α (Genentech), synergistic induction of HLA-A and β₂m surface...
TNF-α resulted in no measurable increase over the 200 units/ml level (data not shown). Neither reversing the order of cytokine addition nor simultaneous addition at the same concentration before synergy could be achieved. A dose of 200 units/ml TNF-α alone resulted in induction levels as high as those previously obtained with IFN-γ (100 IU/ml; 48 h); non-small cell lung cancer cell line (NCI-H125) with high endogenous HLA surface expression. A FITC-conjugated goat anti-mouse IgG was used as a secondary antibody. Mean fluorescence was determined by the analysis capabilities of the FACS IV flow cytometer (Becton-Dickinson). Mean fluorescence values represent the mean of triplicate samples where each sample’s value was within 10% of the SE.

antisera was observed over most of the dose range indicated by radioimmunoassay (Figs. 1–3) and by FACS IV analysis (Table 1). Later, this pretreatment period was reduced to 3 days without significant diminution of induction. A minimal dose of 200 units/ml recombinant TNF-α appeared to be necessary before synergy could be achieved. A dose of 200 units/ml TNF-α alone over the same time period achieved only a modest level of class I MHC induction (Figs. 1–3). A dose of 500 units/ml TNF-α resulted in no measurable increase over the 200 units/ml level (data not shown). Neither reversing the order of cytokine addition nor simultaneous addition at the same concentration resulted in induction levels as high as those previously indicated (data not shown).

Northern blots were prepared using total RNA purified from the classic SCLC cell line NCI-H146 and probed with β2m and HLA-A2 (Fig. 4) DNA fragments. DNA probes revealed that a 3-day pretreatment with TNF-α (200 units/ml) also resulted in a synergistic increase in steady state levels of β2m and HLA-A message within 48 h after IFN-γ addition, indicating control of the induction phenomenon at the mRNA level. IFN-γ (100 IU/ml) treatment alone over the 48-h time course also yielded significant induction of both HLA and β2m message, whereas TNF-α alone (200 units/ml; 5 days total) resulted in slight induction. Densitometer tracings of the autoradiograms quantitated this observation; with the β2m DNA probe, IFN-γ and TNF-α individually gave relative hybridization intensities of 7.8 and 1.6, respectively, over those of the control; the combined regimen gave a value of 26.1. With the HLA probe, IFN-γ and TNF-α individually gave hybridization intensities of 7.8 and 1.6, respectively, over those of the control; the combined regimen had a value of 9.6.

In NCI-H82, a variant SCLC cell line, kinetics of the IFN-γ and TNF-α induction of HLA-A message in TNF-α-pretreated cells was followed over a 24-h time course. The HLA-A message inducit
tion was evident within 2 h and reached a plateau within 8 h of IFN-γ (100 IU/ml) addition. This high level of message persisted for at least 24 h (data not shown).

Fig. 4 also indicates the results of probing these blots with an actin probe, confirming that equal amounts of total RNA were added to each lane.

Northern blots using total RNA preparations of the SCLC cell NCI-H146 and probed with a c-myc DNA probe revealed that TNF-α treatment alone produced a modest decline in c-myc message, unlike IFN-γ alone, which yielded no measurable decline in expression (Fig. 5). Moreover, 48 h of pretreatment with TNF-α followed by 48 h of IFN-γ exposure resulted in a synergistic decline in steady state c-myc RNA levels.

Cell viability studies were performed during the induction experiments to determine cytostatic or cytotoxic effects of the cytokines employed which might interfere with the proper quantitation of RNA message or cell surface antigen expression. Trypan blue exclusion staining at all cytokine concentrations employed indicated no significant cytotoxic effects on the SCLC cells. Neither cell viability nor the standard SCLC growth curves were significantly effected by the cytokine dose ranges utilized in these experiments. Higher concentrations of 1,000 units/ml IFN-γ with or without 200–500 units/ml TNF-α did appear to have a moderate cytostatic effect on the growth of classic H146 SCLC cells (Fig. 6).

DISCUSSION

We have shown that SCLC cell lines with low endogenous levels of class I MHC antigen expression can be induced to synergistically increase this expression with combinations of TNF-α and IFN-γ. There is a growing body of evidence to suggest that class I MHC expression is required for the cyto-

toxic T-lymphocyte response (7). Indeed, human neuroblastoma cell lines, possessing low levels of class I MHC surface antigen expression (5, 8), are susceptible to lysis by natural killer cells but not by cytotoxic T-lymphocytes (34). SCLC cells treated with interferon are more susceptible to lysis by cytotoxic T-lymphocytes than untreated SCLC cells (35). Since SCLC has a greater propensity for early metastasis than other lung cancers, its paucity of class I MHC antigen expression could play a significant role in this process. The surface expression of class I MHC antigens has been successfully induced with the leukocyte interferons or TNF-α alone in vitro (3), and we have now synergistically induced HLA and β2m expression in SCLC with interferon and TNF-α in combination.

Biopsies obtained from patients after high dose IFN-α treat-

ment have shown that, in SCLC as well as in mid-gut carcinoid tumors, β2m expression can be enhanced in vivo and that concentrations of up to 10^5 IU/ml IFN-α are achievable in serum (36). If clinically achievable doses of TNF-α and interferon could be introduced into patients, it is possible that these cytokines could, through MHC induction, reduce or delay tumor recurrence following reduction of tumor burden by conventional therapy. A recent clinical trial employing maintenance interferon after cytoreductive therapy of SCLC shows a trend towards increased disease-free survival among the interferon-treated patients (6), although IFN-α is ineffective as a single agent against bulky SCLC tumors (36). Our findings are significant in that they show that MHC-deficient cell lines can be synergistically induced to express MHC antigens by IFN-γ and TNF-α at relatively modest doses, which may potentially be administered with less toxicity than higher doses of either agent alone.

Interferon has been clinically employed as an antiviral and antitumor agent (11, 12). It has significant cytotoxic activity in vitro against a variety of cell lines (14, 15). TNF-α has been noted to enhance additively or supraadditively the cytotoxicity of interferon against some of these cell lines (16–18). This suggests a therapeutic potential for this type of regimen in minimal disease states.

Amplification and overexpression of the c-myc or N-myc gene with a concomitant decrease in generation time is one of the chief characteristics which distinguishes variant from classic SCLC subgroups (23). Variant SCLC cell lines, which are c-myc amplified, possess a lower endogenous HLA level than do classic SCLC cell lines, which do not have amplified c-myc.4

Fig. 5. Northern blot analysis of c-myc and constitutive actin expression in the NCI-H146 classic SCLC cell line. Cells (10^6) in 200-ml tissue culture flasks were treated with cytokines according to the following schedule: lane A, control (no treatment); lane B, IFN-γ (100 IU/ml) 2 days; lane C, recombinant TNF-α (200 units/ml) 6 days; lane D, recombinant TNF-α (200 units/ml) 6 days plus IFN-γ (100 IU/ml) added on day 4. Media, including the proper cytokine concentrations, were replenished during day 3 of the dose schedule. All cell cultures were harvested 48 h after IFN-γ treatment. Twenty µg of each sample of total RNA isolated was electrophoresed on a formaldehyde/agarose denaturing gel for 12 h at 25 mA. Electrophoresed gels were blotted onto nitrocellulose paper and probed successively for 16 h with the following random-primed plasmids: a 1.5-kilobase fragment of the second exon of c-myc DNA inserted into pBR322; a 2.1-kilobase fragment of actin DNA inserted into the BamH1 site of pBR322. Total trichloroacetic acid-precipitable counts equaled 1.6 X 10^6 and 2.0 X 10^6 cpm, respectively. Washed blots were autoradiographed onto Kodak XAR-5 X-ray film for 16 h using two Dupont Cronex intensifying screens. kb, kilobase.

DISCUSSION

We have shown that SCLC cell lines with low endogenous levels of class I MHC antigen expression can be induced to synergistically increase this expression with combinations of TNF-α and IFN-γ. There is a growing body of evidence to suggest that class I MHC expression is required for the cyto-

toxic T-lymphocyte response (7). Indeed, human neuro-

blastoma cell lines, possessing low levels of class I MHC surface antigen expression (5, 8), are susceptible to lysis by natural killer cells but not by cytotoxic T-lymphocytes (34). SCLC cells treated with interferon are more susceptible to lysis by cytotoxic T-lymphocytes than untreated SCLC cells (35). Since SCLC has a greater propensity for early metastasis than other lung cancers, its paucity of class I MHC antigen expression could play a significant role in this process. The surface expression of class I MHC antigens has been successfully induced with the leukocyte interferons or TNF-α alone in vitro (3), and we have now synergistically induced HLA and β2m expression in SCLC with interferon and TNF-α in combination.

Biopsies obtained from patients after high dose IFN-α treat-

ment have shown that, in SCLC as well as in mid-gut carcinoid tumors, β2m expression can be enhanced in vivo and that concentrations of up to 10^5 IU/ml IFN-α are achievable in serum (36). If clinically achievable doses of TNF-α and interferon could be introduced into patients, it is possible that these cytokines could, through MHC induction, reduce or delay tumor recurrence following reduction of tumor burden by conventional therapy. A recent clinical trial employing maintenance interferon after cytoreductive therapy of SCLC shows a trend towards increased disease-free survival among the interferon-treated patients (6), although IFN-α is ineffective as a single agent against bulky SCLC tumors (36). Our findings are significant in that they show that MHC-deficient cell lines can be synergistically induced to express MHC antigens by IFN-γ and TNF-α at relatively modest doses, which may potentially be administered with less toxicity than higher doses of either agent alone.

Interferon has been clinically employed as an antiviral and antitumor agent (11, 12). It has significant cytotoxic activity in vitro against a variety of cell lines (14, 15). TNF-α has been noted to enhance additively or supraadditively the cytotoxicity of interferon against some of these cell lines (16–18). This suggests a therapeutic potential for this type of regimen in minimal disease states.

Amplification and overexpression of the c-myc or N-myc gene with a concomitant decrease in generation time is one of the chief characteristics which distinguishes variant from classic SCLC subgroups (23). Variant SCLC cell lines, which are c-myc amplified, possess a lower endogenous HLA level than do classic SCLC cell lines, which do not have amplified c-myc.4

Fig. 5. Northern blot analysis of c-myc and constitutive actin expression in the NCI-H146 classic SCLC cell line. Cells (10^6) in 200-ml tissue culture flasks were treated with cytokines according to the following schedule: lane A, control (no treatment); lane B, IFN-γ (100 IU/ml) 2 days; lane C, recombinant TNF-α (200 units/ml) 6 days; lane D, recombinant TNF-α (200 units/ml) 6 days plus IFN-γ (100 IU/ml) added on day 4. Media, including the proper cytokine concentrations, were replenished during day 3 of the dose schedule. All cell cultures were harvested 48 h after IFN-γ treatment. Twenty µg of each sample of total RNA isolated was electrophoresed on a formaldehyde/agarase denaturing gel for 12 h at 25 mA. Electrophoresed gels were blotted onto nitrocellulose paper and probed successively for 16 h with the following random-primed plasmids: a 1.5-kilobase fragment of the second exon of c-myc DNA inserted into pBR322; a 2.1-kilobase fragment of actin DNA inserted into the BamH1 site of pBR322. Total trichloroacetic acid-precipitable counts equaled 1.6 X 10^6 and 2.0 X 10^6 cpm, respectively. Washed blots were autoradiographed onto Kodak XAR-5 X-ray film for 16 h using two Dupont Cronex intensifying screens. kb, kilobase.
REFERENCES

Potentiation of Interferon Induction of Class I Major Histocompatibility Complex Antigen Expression by Human Tumor Necrosis Factor in Small Cell Lung Cancer Cell Lines

Garry M. Marley, L. Austin Doyle, José V. Ordónñez, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/22/6232

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.