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Human Recombinant Alpha- and Gamma-Interferons Enhance the Cytotoxic Properties of Tumor Necrosis Factor on Human Melanoma

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Summary: Three short-term human melanoma cell lines were tested for sensitivity to human recombinant α-tumor necrosis factor (TNF) in a semisolid agar colony formation assay. Cells from three pigmented and one amelanotic strain displayed low sensitivity to TNF. The ID_{50} for the inhibition of melanoma colony formation ranged from 2,500 to 20,000 U/ml. We then tested the ability of human recombinant alpha-interferon (IFN-α) and gamma-interferon (IFN-γ) to interact with TNF to inhibit melanoma colony formation. Analysis of the TNF–IFN mixtures using the median effect method demonstrated that both IFNs interacted synergistically with TNF to inhibit melanoma colony formation. On a unit basis, IFN-γ was more active with TNF than IFN-α. The addition of the second interferon to the mixture enhanced the ability of TNF to promote the cytolysis of human melanoma cells. The enhanced killing effect seen with the combination of IFN-α, IFN-γ, and TNF suggests an interesting strategy for the treatment of human melanoma. **Key Words:** Interferon—Melanoma—Tumor necrosis factor.

Tumor necrosis factor (TNF) is a substance produced by macrophages in response to such stimuli as bacillus Calmette-Guerin and endotoxin (1,2). TNF can cause tumor cytolysis in vitro and in vivo (3–5), although the mechanism of action is not clearly understood. TNF has shown activity against various tumor cells including human tumor-burdened nude mice (6). Gamma-interferon (IFN-γ) and TNF in combination produce variable effects on tumor cells, ranging from synergistic antiproliferative (7,8) to cytotoxic responses (9,10).

We examined the ability of human recombinant alpha-interferon (IFN-α) and IFN-γ to interact with human recombinant TNF. The combination data were

modeled using the median effect method (11). We found that either IFN sensitizes human melanoma cells to the cytotoxic properties of TNF.

MATERIALS AND METHODS

Human recombinant clone A alpha and gamma IFN were generous gifts from Hoffmann LaRoche Inc., Nutley, NJ, U.S.A. Human recombinant α-TNF was obtained from Genentech Inc., So. San Francisco, CA, U.S.A.

Human melanoma cell strains C8146C, C8161, and C83-2CY have been characterized (12,13). The cells were maintained in monolayer in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine (0.8 μg/ml), and gentamicin (10 μg/ml). All experiments that assessed colony formation with short-term human melanoma cell strains were done on cells subcultured less than seven times from the isolation from the original patient cells.

Soft Agar Assay

Human melanoma cells were plated in soft agar as previously described (12) and counted on day 14 by the FASII colony counter (14). Cell concentrations chosen for plating were those in the midrange cell dose, which produce a linear relationship between the number of cells plated and number of colonies formed (15).

Viability

Melanoma cells were plated in soft agar and were tested for viability on day 5 using a modification (13,16) of the 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) vital stain method (17). The dye was added on day 5, and the viable cells and growth units were scored on day 7.

Median Effect Analysis of Dose–Effect Curves

The colony-forming assay data were analyzed by the median effect method (11): fraction affected/fraction unaffected = (dose/Dm)^m, where Dm is the ID_{50} dose. The initial doses for the TNF–IFN combinations were chosen from the single-agent dose–response curves. If needed, these doses were changed in later experiments according to the outcome. Each concentration was done in triplicate, and each individual determination was used as a datum point. All dose–effect curves had linear regression coefficients greater than 0.9. An average combination index was determined. An average combination index of 1 denotes additivity, <1 synergism, and >1 antagonism.

RESULTS

The sensitivity of short-term human melanoma cell lines to recombinant TNF was tested in the soft agar assay. Dose–response curves were generated for each melanoma cell line. The colony-forming units in these melanoma cell lines displayed low sensitivity to TNF (Table 1). For the three pigmented human melanomas (C8146C, C82-7A, and C83-2CY), the ID_{50} ranged from 2,500 to 20,000

TABLE 1. *Sensitivity of short-term human melanoma colony-forming cells to treatment by recombinant α-tumor necrosis factor*

Human melanoma	ID_{50} (U/ml)
C8146C	2,500
C8161	7,500
C82-7A	20,000
C83-2CY	10,000

U/ml. A TNF dose of 7,500 U/ml was needed with the amelanotic human melanoma C8161 to reduce colony formation by 50%.

The recombinant IFNs were tested with TNF to determine if the combinations would promote a synergistic inhibition on human melanoma colony formation. Dose–response curves for each IFN, TNF, and IFN combination were developed. IFN-α and TNF in combination synergistically inhibited melanoma colony formation (Table 2). The average combination index for the two human melanoma cell lines C8161 and C83-2CY was 0.6 and 0.7, respectively. The IFN-γ–TNF mixture generated a stronger inhibition on human melanoma colony formation than did the IFN-α–TNF combination. The IFN-γ–TNF mixture displayed an average combination index that was <0.07 (Table 2). This combination index value is indicative of very strong synergism. Based on a unit basis, the TNF response in combination with IFN-γ can be up to 15 times stronger in inhibiting human melanoma colony formation than the one obtained with IFN-α.

As both IFNs combined with TNF produced a synergistic inhibition of melanoma colony formation, we explored whether or not a further synergism could be achieved by combining both IFN-α and IFN-γ with TNF. Figure 1 displays the dose–response curves for IFN-α and IFN-γ, which ranged between 100 and 1,000 U/ml. Higher concentrations of TNF were needed to obtain colony formation inhibition. Combining IFN-α, IFN-γ, and TNF in a 1:1:1 ratio resulted in a 1–2 log shift in the dose–response curves. For example, 33 U each of TNF, IFN-α, and IFN-γ in combination caused an 80% reduction in melanoma colony formation. This was strong synergistic interaction, as 33 U of either agent alone had no effect on melanoma colony formation. Similar results were obtained with the C8161 human melanoma line.

TABLE 2. *Analysis of the IFN–TNF combination interactions in the melanoma colony-forming assay*

Melanoma	Average combination index	Correlation coefficient	Result
C8161			
IFN-α + TNF	0.60	0.939	Synergism
IFN-γ + TNF	0.04	0.924	Very strong synergism
C8146C			
IFN-γ + TNF	0.06	0.985	Very strong synergism
C83-2CY			
IFN-α + TNF	0.73	0.996	Synergism
IFN-γ + TNF	0.02	0.916	Very strong synergism

FIG. 1. Dose-dependent inhibition of C83-2CY human melanoma colony formation by IFN-α (■), IFN-γ (□), TNF (○), and IFN-α, IFN-γ, and TNF in combination at a 1:1:1 ratio (●).
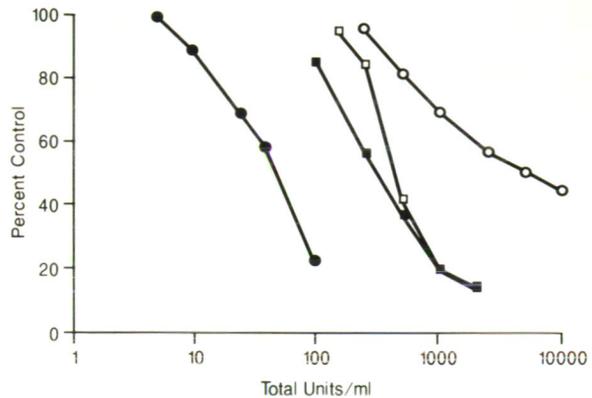
Does this syngerism between the IFNs and TNF manifest itself in the cytolysis of melanoma cells? C83-2CY cells were plated in soft agar, and groups of plates were exposed to 500 U of either IFN alone, TNF alone, IFN-α + IFN-γ, and combinations of IFN–TNF. On day 5, INT stain was added to the plates, and on day 7, the red-colored viable single cell and multi-cell units were counted. Day 5 was chosen because from monolayer studies, a 48–72-h exposure was needed for cytolysis. As single agents, TNF and IFN-α did not appreciably reduce melanoma viability (Fig. 2). IFN-γ reduced the number of red units by 25%. The IFN-α + IFN-γ combination resulted in a 29% reduction in viable melanoma units, which was not significantly different from the IFN-γ treatment (not shown). A significant enhanced reduction in viability was observed with the IFN–TNF combinations. The IFN-α–TNF combination reduced the viability of the culture by 40%. The IFN-γ–TNF combination produced the largest reduction in melanoma viability (55%). These reductions in viability were greater than would be predicted for an additive interaction. The [IFN-α, IFN-γ, TNF] combination resulted in a further synergistic reduction in melanoma viability. Ninety percent of the plated melanoma cells were killed by the simultaneous addition of IFN-α, IFN-γ, and TNF.
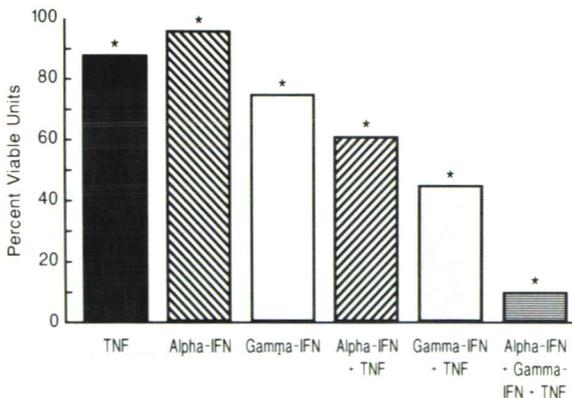

FIG. 2. Effect of TNF, IFNs, and TNF–IFN combinations on the viability of C83-2CY human melanoma cells plated in soft agar. A quantity of 500 U/ml of each agent was added to the various groups. The standard error is represented by (*).

DISCUSSION

Tumor necrosis factor is a cytotoxic protein that has promise as an antitumor agent. The question has been raised by recent data of whether TNF works in vivo by direct cytolysis of tumor cells, immune modulation, or by direct action on tumor vasculature (18,19). In this study, pure human melanoma cells were tested and found to be insensitive to low doses of TNF (1–1,000 U/ml). Low doses of TNF (1–100 U/ml) cause cytolysis with sensitive cell lines BT-20 and MCF-7 (breast cancer) and ME (cervical cancer) (20). Either IFN-α or IFN-γ interacted synergistically with TNF to inhibit colony formation. The IFN-γ–TNF combination generated the strongest synergistic interaction, which resulted in the cytolysis of the human melanoma cells. The IFN-γ–TNF combination is active on human breast, colon, and cervix carcinomas, but not leukemias, lymphomas, or normal cells (10). IFN-α and IFN-γ act through unique receptors (21–23), and in combination with TNF, they may display different tumor-specific activity profiles. The IFN-α–TNF combination has not been extensively explored. Our observation that IFN-α promotes the cytolytic properties of TNF on human melanoma cells indicates that this combination should be tested on other tumor types.

In combination, α/β and γ-IFN generate synergistic antiproliferative effects (24–27). We tested whether or not this synergism between the interferons extended to sensitizing human melanoma cells to the cytolytic effects of TNF. In our study, a pronounced 1–2 log dose shift was observed on melanoma colony formation when IFN-α, IFN-γ, and TNF were used in combination. The addition of the second interferon to the mixture enhanced the ability of TNF to promote the cytolysis of human melanoma cells. The enhanced killing effect seen with the IFN-α–IFN-γ–TNF mixture suggests an interesting strategy for the treatment of human melanoma.

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