Synergistic Cytotoxic Effects of Recombinant Human Tumor Necrosis Factor, Interferons, and Heat-Stress

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ABSTRACT

A synergistic increase in the cytotoxic effects of recombinant human tumor necrosis factor (TNF-α), interferons (IFN-α, IFN-β, and IFN-γ) and heat-stress was demonstrated in vitro. The toxicity of these agents was assessed in the human cervical carcinoma HeLa cell line: the toxic effect was greatly increased when cells pretreated with IFNs or TNF were submitted to a 1-h heat-shock at 45°C. Moreover if the heat-shock followed simultaneous treatment with both cytokines, a synergistic effect between these treatments could be observed. The same observations were made for two other transformed cell lines: the oral epidermoid carcinoma KB cells and the hepatocarcinoma PLC/PRF/5 cells. In contrast, the survival of normal cells (normal fetal lung MRC5 cells and foreskin F7000 fibroblasts) was only slightly decreased by such treatments. These results suggest that combining a heat-shock with cytokines treatment might be one way of enhancing the sensitivity of cancer cells to the growth inhibitory effects of the individual cytokines.

INTRODUCTION

TNF-α is a cytokine, derived from activated monocytes/macrophages, which causes hemorrhagic necrosis of tumors in vivo. TNF-α has cytotoxic activity in vitro, against a wide range of human and murine transformed cells, while it can be mitogenic to fibroblasts under certain conditions (1–3). The sensitivity of cells to TNF is not directly correlated with the number of receptors at the cell surface (4) and the mechanism of action of TNF might involve complex biochemical interactions.

The cytotoxic action of TNF has been amplified by the combination of different treatments: synergistic antiproliferative effects using combinations of TNF and IFN-γ have been described in several human tumor cell lines in vitro (1, 2, 5–9) and for some tumors in vivo (10). Moreover, the cytotoxicity of TNF against tumor cells has been shown to be enhanced both in vitro and in vivo by hyperthermia (11) while the antiproliferative activities of IFNs were enhanced by incubation at elevated temperatures (12–14) as were their antitumor properties (13). These observations prompted us to assess the cytotoxic and antiproliferative effects of TNF-α and of IFNs α, β, or γ in normal and transformed human cell lines submitted to a mild, nonlethal heat-shock. We show that the cytotoxicity of TNF and IFNs is potentiated when transformed cells are submitted to a heat-shock.

MATERIALS AND METHODS

Cell Lines. The following human cell lines were used: HeLa (cervical carcinoma), KB (oral epidermoid carcinoma), and PLC/PRF/5 (hepatoma) (15) maintained in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% heat-inactivated calf serum. MRC-5 (normal fetal lung) and F7000 and FS4 (foreskin fibroblasts) cell lines were maintained in the same medium supplemented with 10% heat-inactivated fetal calf serum.

Methods. Recombinant human TNF-α was kindly provided by Boehringer Ingelheim (Vienne, Austria) with a specific activity of 6 × 10⁷ units/mg. Human leucocyte IFN-α was provided by Institut Pasteur Production (Marnes-la-Coquette, France) with a specific activity of 2 × 10⁸ NIH units/mg, human fibroblastic IFN-β was provided by Cytotect (Lausanne, Switzerland) with a specific activity of 2.9 × 10⁵ units/mg, human recombinant IFN-γ was provided by Biogen (Geneva, Switzerland) with a specific activity of 1.3 × 10⁷ units/mg.

Cytotoxic Effects. Cell monolayers were detached by treatment with 0.25% trypsin 0.025% versene solution and resuspended in complete medium at a density of 10⁴ cells/ml. Aliquots (100 μl) were dispensed into 96-well microtiter plates (Falcon) and incubated 24 h in 5% CO₂ at 37°C. Medium was discarded and replaced with 0.1 ml of fresh medium containing various concentrations of rTNF, IFN-α, IFN-β, or IFN-γ for 24 h at 37°C. Then cells were submitted or not to a 1-h heat-shock at 44°C or 45°C by floating the microplates on a water bath. The cells were then replaced at 37°C and the monolayers were stained 48 h later with napthol blue (0.05% in 0.1 M sodium acetate, 9% acetic acid). Bound dye was eluted with NaOH 0.05 n and the absorbance (A) was measured at 630 nm using a microtiter plate spectrophotometer. The cell survival was calculated as follows:

% cell survival = 100 × A drug treated/A control at 37°C

Five replicate experiments were conducted for each cell line.

Colony Formation. Cells were grown in 25-cm² culture flasks (Falcon) at 37°C in complete medium. Twenty-four h later, the medium was replaced with medium containing or not rTNF and IFN-α or rIFN-β. After 24 h incubation at 37°C, cells were submitted or not to a 1-h heat-shock at 44°C then trypsinized, counted, and 500 cells were plated in 35-mm tissue culture plates (Falcon) in complete medium. Colony formation was assessed at 6 days thereafter by fixing the cells with methanol and staining with Giemsa 10%. Colonies larger than approximately 50 cells were counted under a microscope. Control plates of HeLa cells incubated at 37°C had cloning efficiencies of 49%.

Determinations of additive or synergistic were obtained by the method of Spector et al. (16) where the CI = ln (number of colonies after TNF treatment) + ln (number of colonies after IFN treatment) – ln (number of colonies with no treatment) – ln (number of colonies after combined TNF and IFN treatment). The effects are considered as additive if: +2 SE ≥ CI ≥ –2 SE; synergistic if: CI > +2 SE; antagonistic if: CI < –2 SE.

RESULTS

Increased Cytotoxicity of a Combined Treatment with rTNF, IFNs, and Hyperthermia in Human HeLa Cells. When HeLa cells treated with either TNF, IFN-α, or IFN-β were kept at 37°C, only a very low cytotoxicity could be observed as more than 90% cells survived after 3 days of treatment (Fig. 1, A and B). The toxicity of these substances was slightly increased when the cells were treated simultaneously with TNF and either IFN-α or IFN-β; after treatment with 20 ng/ml of TNF and 500 IU/ml of IFN-α or IFN-β, 80% of the cells still survived. Submitting HeLa cells to a 1-h heat-shock at 45°C led to a 20–30% decrease in cell survival as compared to the cells kept at 37°C. When cells were treated with either TNF or IFN (α or β) for 24 h and then submitted to the same heat-shock, the cytotoxicity aug-
at relatively high doses, a synergistic cytotoxic effect was observed: for example 20 ng/ml TNF and 500 units/ml IFN-γ killed 67% of the cells. When IFN-γ treated cells were submitted to a 1-h 45°C heat-shock, the survival rate was decreased by about 20%. With the combined TNF-IFN-γ treatment, accompanied by a heat-shock, the survival of HeLa cells was strongly decreased, even when relatively low doses of TNF and IFN-γ, such as 8 ng/ml and 20 units/ml, respectively, were used. These results are illustrated in Fig. 2 by the photographs of the microplates showing that the cytotoxic actions of TNF and IFN-γ observed at 37°C (Fig. 2A) were considerably enhanced when treated HeLa cells were submitted to a heat-shock (Fig. 2B).

Therefore it seemed useful to confirm these observations by a study of colony formation after combined heat, IFN and TNF treatment. To determine whether HeLa cells were able to grow after these different treatments, colony formation following a 24-h cytokine exposure and a 1-h heat-shock at 44°C was assessed. As shown in Fig. 3, when the cells were kept at 37°C, TNF (10 ng/ml) only slightly diminished the number of colonies; IFN-α and IFN-γ (500 units/ml) proved to be more effective, as 67 and 58%, respectively, of cells formed colonies after these treatments. The rates were reduced to 41 and 35%, respectively, when TNF and IFN-α or IFN-γ were used in combination. While in cells submitted to the heat-stress alone, 95% of the cells were able to form colonies, this capacity was reduced to 49% with a prior TNF treatment and even to 35 and 33%, respectively, when IFN-α or IFN-γ were used. When cells were pretreated with TNF and IFN-α before the heat-shock, only 9% of them were colony formers, and this rate fell as low

implemented with increasing concentrations of these drugs. When both substances were present simultaneously in the culture medium at the above concentrations, the toxicity reached 90% in heat-shocked cells. Thus, treatment with TNF and IFNs α or β appeared to have no significant effect on the survival of HeLa cells at the normal growth temperature. However after a relatively mild heat-shock, the cytotoxicity of these substances was strikingly potentiated, mostly when they were used in combination.

In contrast with IFN-α and IFN-β, IFN-γ treatment decreased the survival rate of HeLa cells at 37°C (Fig. 1C): when cells were treated with 500 IU/ml, only 70% survived after a 3-day treatment at 37°C. When both TNF and IFN-γ were used...
as 2% after pretreatment with TNF and IFN-γ. Moreover, the size of the colonies was significantly smaller for the cells submitted to a combination of the three treatments than for the cells untreated or treated by only one cytokine or a heat-stress alone.

Synergy of Action between TNF, IFNγ, and Heat-Stress. To determine if these augmented antiproliferative responses were the result of synergistic interactions between TNF, IFNs, and heat-stress, we compared the observed survival rates of HeLa cells with the additive values expected if there was no interaction between the treatments (Table 1). The percentage of cell survival after the different treatments, estimated from colony forming efficiency, was calculated from data shown in Fig. 3.

A low synergy of action between TNF and IFNs was observed when the cells were kept at 37°C: thus a predictive additive model of TNF and IFN-α would give 63% survival, while the observed value was 41%. The same degree of synergy was observed between TNF and IFN-γ. When a heat-shock was applied to HeLa cells treated with either TNF, IFN-α, or IFN-γ, the observed survival rates were 20–40% lower than the predicted additive values. Moreover when cells treated simultaneously with both substances were subsequently submitted to a heat-stress, the survival rates dropped drastically: only 2% of the cells were able to form colonies after a combination of TNF, IFN-γ, and heat treatments, while the expected additive survival value would be 52%. In this case, a 26-fold potentiation was observed. Thus, when applied in combination, TNF, IFNs (mostly IFN-γ), and heat-stress were potent cytotoxic agents for HeLa cells and, as shown above, their action was synergistic.

Cytotoxicity of the Combined Heat, IFN, and TNF Treatments for Other Transformed and Normal Cells. Two other transformed cell lines were submitted to the same treatments, i.e., TNF and/or IFN-γ followed or not by a heat-shock at 45°C (Fig. 4). In the PLC/PRF/5 hepatocarcinoma cell line, IFN-γ and TNF were only slightly cytotoxic at 37°C, even when used together. On the contrary, a 1-h heat-shock at 45°C reduced the survival rate to 65% in untreated cells and this percentage decreased to 35% when the cells were pretreated with IFN-γ or TNF. Moreover, when exposed to both IFN-γ and TNF, over 80% of the cells died following a heat-stress. In oral epidermoid carcinoma KB cells, IFN-γ was cytotoxic by itself, as only 65% of the cells survived when treated with 500 IU/ml of this substance, while 90% of cells survived after treatment with TNF. When both substances were used in combination, the survival rate reached 50%. These values decreased to 15% when in addition, a thermal stress was applied to the cells.

The same experiments were performed on two normal cell lines, MRC5 and F7000 (Fig. 5). In (MRC5) lung fibroblasts, IFN-γ was cytotoxic (only 70% of the cells survived when treated with 500 units/ml). In contrast with transformed cells, TNF stimulated the growth of these cells at concentrations as low as 0.3 ng/ml. Indeed TNF has already been shown to be mitogenic for fibroblasts (1, 2). When cells were treated simultaneously with both substances, the survival rate was the same as for IFN-γ alone. A heat-stress applied to the cells after the cytokine treatment slightly increased the cytotoxicity of IFN-γ, but even when the cells were treated with 500 units/ml of IFN-γ and 20 ng/ml of TNF, 45% of them survived. Interestingly, IFN-γ and TNF were almost without effect in the F7000 foreskin fibroblasts, submitted or not to a heat-stress. When the three treatments were used in combination, in the presence of high doses of IFN-γ and TNF more than 70% of the cells survived. The same results were obtained with other foreskin fibroblasts: the FS4 cell line. Thus the combination of cytokines and heat-stress treatments had only low cytotoxicity for the normal cell lines we have studied.

DISCUSSION

In this paper, some evidence of the synergistic cytotoxic activities between TNF-α, IFNs, and heat-stress is presented. We show that higher efficacies are attainable on three trans-
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formed cell lines (HeLa, KB, and PLC/PRF/5) by using low concentrations of cytokines in combination with a heat-shock, as compared to a high dosage of a single cytokine. In contrast to transformed cells, the three normal cell lines (MRC5, F7000, and FS4) tested were poorly affected by such treatments.

The synergy between TNF and IFN-γ has been previously observed for antiproliferative effects in tumor cells (2), differentiation inducing effects in human myeloid cell lines (17) and Ia-antigen expression inducing effects in murine WEHI-3 cell line (18). It has been shown that IFN-γ pretreatment increased the number of TNF receptors in some tumor cell lines (19–21). Such an increase of TNF receptor expression may be one of the factors explaining the synergistic effects between these cytokines, although other mechanisms are likely to play a role since synergistic effects without increased expression of the TNF receptors could be obtained (21). The breakdown of cellular DNA into small fragments by TNF treatment could be involved in its toxicity (22, 23) the more so as it has been shown that the DNA cleavage induced by TNF is enhanced by IFN-γ treatment (22).

The killing effect of hyperthermia has been investigated on many cells in culture (24). Data indicate that malignant cells may be more sensitive to increased temperature than their normal counterparts (25). In our hands, the transformed cell lines appear to be less sensitive to a heat-shock (such as 45°C for 1 h) compared to other authors (26). This discrepancy could be due to the fact that we applied the heat-shock to the cells in stationary phase while most of the investigators administer a thermal stress to exponentially growing cells (24).

The cytotoxic and antitumor effects of TNF-α in conjunction with hyperthermia has been investigated by several groups (4, 11): they show that the cytotoxic effect of TNF-α is markedly stronger after incubation of transformed cells at 40°C for several hours, while the combined use of hyperthermia and TNF inhibits tumor growth in mice. We show that a short heat-shock (1 h at 44 or 45°C) applied to TNF-treated cells exerts a cytotoxic effect in the three transformed cell lines tested, comparable to a prolonged incubation at 40°C in the presence of TNF.

Similarly, a 45°C heat-shock significantly decreases the survival of IFN-treated HeLa, KB and PLC/PRF/5 cells, while the growth of normal MRC5 and F7000 cells is only slightly affected. Other authors have already shown that the antiproliferative effects of IFNs are augmented at elevated temperatures (12–14, 27–29) but the mechanism of this enhancement is not elucidated. We have previously reported (30–31) that IFN potentiates the heat-shock response: the repression of general translation patterns is extended and the synthesis of heat-shock proteins is prolonged in IFN-treated cells submitted to a heat-shock. In contrast, TNF treatment does not modify the synthesis of heat-shock proteins, but does slightly depress the translation of normal proteins. This effect is amplified when TNF and IFNs are used in combination before the heat-shock (data not shown). Indeed, the cytotoxic activity of TNF has been shown to be potentiated by protein synthesis inhibitors (32).

Thus the overall repression of the translation could be involved in the synergistic toxicity of TNF, IFNs, and heat-stress and combining cytokines with hyperthermia may be one way of enhancing the sensitivity of cancer cells to the growth inhibitory effects of the individual treatments.

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