Different and Synergistic Actions of Human Tumor Necrosis Factor and Interferon-γ in Damage of Liposome Membranes

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The effects of human recombinant tumor necrosis factor (TNF) and interferon-γ (IFN-γ) in damage of liposome membranes were examined to elucidate the molecular mechanism of their antiproliferative actions on tumor cells. The extent of membrane damage was assayed by measuring the rate of release of the fluorescent dye calcein encapsulated in the liposomes at different pH values in the presence of TNF and/or IFN-γ. At pH values below about 5, TNF bound to phospholipid liposomes composed of mixtures of phosphatidylserine and phosphatidylcholine in molar ratios of 2:1 and 1:2 and caused rapid release of calcein. In contrast, IFN-γ induced very slow leakage of dye although it bound almost completely to the membranes, suggesting that it causes much less membrane damage than TNF. Small amounts of these two antitumor factors bound to phosphatidylcholine liposomes in the pH range of 4–7, inducing relatively slow leakage of calcein. In the presence of both TNF and IFN-γ at pH 5, the maximal leakage rate was twice the sum of the rates with the two proteins individually, and the rate depended on the TNF/IFN-γ ratio, indicating synergistic effects of TNF and IFN-γ in induction of membrane damage. These different and synergistic actions on liposome membranes may account for the different antitumor properties of the two antitumor cytokines and their synergism.

Tumor necrosis factor (TNF) and interferon (IFN) are proteins with cytostatic and cytotoxic activities against tumor cells. TNF was originally found in sera of mice sensitized with bacillus Calmette-Guérin and subsequently treated with bacterial endotoxin (1). It induces hemorrhagic necrosis of some animal tumors and has cytolytic and/or cytostatic effects on tumor cells in culture (1, 2). IFNs were originally found as inducers of an antiviral state in their target cells and later as inhibitors of proliferation of transformed cells (3). They have also been shown to modulate natural host defense activities against cancer cells (4–8). Recently, TNF and IFN-γ were found to have synergistic in vitro cytotoxic effects (9–12). To explain this synergism, several groups have suggested that IFN-γ enhances the sensitivity of cells to growth inhibition by inducing TNF receptors (13–16) or changing the membrane structure (11), since high-affinity receptors for TNF are present on the surface of target cells (13, 17–19), and since IFN-γ actually induces the synthesis of TNF receptors (13–16).

However, little is known about the molecular mechanisms of the actions of TNF and IFN. It is thus essential to study these effects of TNF and IFN on biological or artificial membranes to determine the mechanisms of their antiproliferative actions.

Recently, cDNAs for human TNF (20–23) and IFN-γ (24, 25) were cloned, sequenced, and expressed in Escherichia coli, and highly purified recombinant proteins are now available for experimental and clinical studies. In this work, we studied membrane damage induced by TNF and IFN-γ using these purified recombinant proteins and a liposome system. We measured the leakage of liposome-encapsulated dye induced by TNF and IFN-γ and found that TNF had a much greater effect than IFN-γ in causing dye release from liposomes and that the effects of TNF and IFN-γ were synergistic.

EXPERIMENTAL PROCEDURES

Materials—PS and egg PC were purchased from Avanti Polar Lipids (Birmingham, AL). Calcein was obtained from Dojin Laboratory (Kumamoto, Japan) and used without further purification. Human recombinant TNF (a product of Genentech, Inc.; lot 109756K; specific activity, 1.0 × 10⁴ units/mg) and IFN-γ (lot NR-G-8517; specific activity, 5.0 × 10⁴ units/mg) were kindly supplied by Fujisawa Pharmaceutical Co. (Osaka, Japan) and Nippon Roche Co. (Tokyo, Japan), respectively. Before use, TNF and IFN-γ were dialyzed against 10 mM TES, pH 7.4, containing 100 mM NaCl and 0.1 mM EDTA, in the absence and presence of 0.02 mM dithiothreitol, respectively. Protein concentration was determined by the method of Bradford (26).

Liposome Preparation—Large unilamellar vesicles (LUV) of about 1000 Å diameter containing calcein were prepared as described previously (27). Briefly, they were prepared in 60 mM calcein, pH 7.4, by a reverse-phase evaporation technique and extruded through a polycarbonate membrane (Nucleopore, Pleasanton, CA) of 0.1 μm pore size. The non-encapsulated calcein was removed from the liposome suspension by gel filtration on a Sephadex G-50 column equilibrated with 10 mM TES, pH 7.4, containing 100 mM NaCl and 0.1 mM EDTA.

Multilamellar vesicles (MLV) were prepared by vortex-mixing for 30 s at 30 °C of the lipid film in 10 mM TES, pH 7.4, containing 100 mM NaCl and 0.1 mM EDTA. The vesicles were passed through polycarbonate membranes of 0.4 μm pore size and washed four to five times by centrifugation at 13,000 × g for 4.5 min. Phospholipid concentration was determined by the method of Bartlett (28).

Leakage Assay—Release of liposome contents was measured as increase of calcein fluorescence, which was observed as the self-quenched calcein leaked out of the liposomes. Excitation and emission wavelengths of 470 and 520 nm, respectively, were used, and measurements were carried out at 25 °C in a Hitachi 650-60 fluorospectrophotometer, equipped with a constant-temperature cell holder and stirrer. The reaction was initiated by addition of antitumor protein to a suspension of liposomes (50 μg phospholipid) containing 5 mM calcein, pH 7.4, containing 100 mM NaCl and 0.1 mM EDTA.

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Calcine in a final volume of 1.0 ml of 10 mM MES (pH 6-7) or acetic acid (pH 4-5.5), each containing 100 mM NaCl and 0.1 mM EDTA. The rate of dye release was defined as the initial velocity of the reaction and expressed as the increase in calcine fluorescence per second (per microgram of protein) relative to the fluorescence increase observed on addition of a trace amount of Nonidet P-40. Although the fluorescence of calcine was quenched at pH values below 5, the leakage of calcine from liposomes could be quantitated, provided sufficiently high concentrations of calcine were encapsulated in liposomes for full self-quenching and the instrument was adjusted to obtain a high signal-noise ratio (27).

Binding Assay—The amount of membrane-bound TNF or IFN-\(\gamma\) was determined as follows: TNF or IFN-\(\gamma\) (30 \(\mu\)g/ml) was incubated at 25 °C for 30 min in buffer solutions of different pH values with or without MLV (220 \(\mu\)M phospholipid). The mixtures were then centrifuged at 13,000 \(\times\) g for 5 min, and the amount of protein in the supernatant was determined by the method of Bradford (26). The amount of membrane-bound protein at a given pH was expressed relative to the amount of protein in the supernatant at the same pH in the absence of MLV.

RESULTS

Bindings of TNF and IFN-\(\gamma\) to Liposome Membranes—Before study of the effects of TNF and IFN-\(\gamma\) on liposome membranes, it was necessary to determine whether these proteins interacted with the membranes. Therefore, we examined the amounts of TNF and IFN-\(\gamma\) bound to MLV composed of mixtures of phosphatidylserine (PS) and phosphatidylcholine (PC) at molar ratios of 2:1 and 1:2 or PC alone at various pH values. For this, the proteins were incubated with MLV at 25 °C for 30 min, the MLV were precipitated by centrifugation, and the amount of protein in the supernatant was determined. The pH dependence of the bindings of TNF to PS/PC(2:1)-, PS/PC(1:2)-, and PC-MLV is shown in Fig. 1A. Only small amounts of TNF bound to PS/PC(2:1) vesicles at above pH 6, but the amount of bound protein increased on lowering the pH below 6. Binding of TNF to PS/PC(2:1) membranes was almost complete at below pH 4.5. With PS/PC(1:2) vesicles, 0-6% of the TNF was bound at above pH 5, and the binding increased below pH 5. In contrast, very little TNF bound to PC-MLV in the pH range of 4-7.

As shown in Fig. 1B, the bindings of IFN-\(\gamma\) to PS/PC(2:1) and PS/PC(1:2) vesicles were almost complete in the pH range of 4-7 and at pH values below 5, respectively, and relatively large amounts of IFN-\(\gamma\) bound to PS/PC(1:2)-MLV at above pH 5. The binding of IFN-\(\gamma\) to PC vesicles was low, like that of TNF.

Release of Liposome-encapsulated Dye Induced by TNF and IFN-\(\gamma\)—The above results showed that TNF and IFN-\(\gamma\) bound to liposome membranes containing PS, although the binding of TNF depended on the pH. In many cases the binding of a protein with bilayer membranes alters the bilayer integrity and permeability of the membranes (29, 30). To ascertain whether TNF and/or IFN-\(\gamma\) alters the permeability of membranes, we measured the release of the fluorescent dye calcine encapsulated in PS/PC(2:1)-, PS/PC(1:2)-, and PC-LUV in the presence of TNF or IFN-\(\gamma\) at different pH values. PS/PC liposomes alone were not leaky at pH 4-7, as observed by Straubinger et al. (31), but PC liposomes were slightly leaky at below pH 5: the leakage rates were 0.08 and 0.03%/s at pH 4 and 4.5, respectively. Fig. 2 shows the pH dependence of the rates of calcine release induced by TNF and IFN-\(\gamma\). With PS/PC-LUV, TNF did not induce leakage above pH 6, but induced release of dye below pH 6, and the leakage rate increased steeply on decrease in pH below 5. The threshold pH for dye release from PS/PC(2:1) vesicles was slightly higher than that for dye release from PS/PC(1:2) vesicles. This difference is attributable to the difference in the pH dependence of bindings of protein to the two kinds of liposomes. Thus, these results together with those in Fig. 1A indicate that TNF can induce dye release on binding to PS/PC liposomes. In contrast, there was no leakage from PC-

![Fig. 1. pH dependence of the bindings of TNF and IFN-\(\gamma\) to multilamellar vesicles. TNF (A) or IFN-\(\gamma\) (B) at 30 \(\mu\)g/ml was incubated with PS/PC(2:1) (O), PS/PC(1:2) (C), or PC (A) MLV (220 \(\mu\)M phospholipid). The amounts of protein bound were determined as described under "Experimental Procedures."](image-url)

![Fig. 2. pH dependence of the rates of calcine release from liposomes induced by TNF and IFN-\(\gamma\). TNF at 1.0 \(\mu\)g/ml (closed symbols) or IFN-\(\gamma\) at 2.0 \(\mu\)g/ml (open symbols) was added to suspensions of large unilamellar vesicles (50 \(\mu\)M phospholipid) containing calcine and composed of PS/PC(2:1) (circles), PS/PC(1:2) (squares), or PC (triangles) at the indicated pH values. Increase of calcine fluorescence was monitored at 25 °C with excitation and emission wavelengths of 470 and 520 nm, respectively. The increase in fluorescence of calcine on addition of a trace amount of Nonidet P-40 was taken as 100%.](image-url)
LUV above pH 5, but below pH 5 a low rate of change in permeability was detected on addition of TNF, due to a slight increase in binding of TNF to the membranes.

Although IFN-γ bound almost completely to PS/PC membranes, IFN-γ-induced release of dye from PS/PC liposomes was much slower than TNF-induced dye release: at pH 4 and pH 4.5 the rates were 1/2 to 1/4 and 1/3 to 1/4 of those induced by TNF, respectively. Thus the rate of leakage induced by IFN-γ is much lower than that by TNF even when IFN-γ is completely membrane-bound. However, release of the liposome contents was seen at all pH values examined, although TNF caused no dye release at neutral pH. The rate of dye release by IFN-γ from PC-LUV was low, because only a small amount of IFN-γ bound to PC vesicles. In the pH range examined, however, the rates of IFN-γ-mediated release of calcine from PC-LUV were higher than those from PS/PC(2:1)- or PS/PC(1:2)-LUV.

Fig. 3 shows the dependence of the leakage rates on the concentrations of TNF and IFN-γ at pH 4.5. With all three kinds of liposomes, the rates were proportional to the amount of TNF or IFN-γ present, indicating that the observed release from liposomes was induced by TNF or IFN-γ only. Similar linear relations were obtained at different pH values (see Fig. 5). It is noteworthy that PS stimulated release by TNF and inhibited that by IFN-γ.

As can be seen in Fig. 2, the rates of IFN-γ-mediated release of calcine from all kinds of liposomes tended to increase on lowering the pH below 5, although there was no large increase in its binding (Fig. 1B). This change may be due to alteration of the integrity of the liposome bilayer membranes at low pH, which could facilitate the permeability change. Indeed, spontaneous leakage of dye from PS/PC liposomes was observed below pH 4 and from PC liposomes below pH 5, and carboxyfluorescein, another fluorescein derivative, tends to leak out of liposomes rapidly below pH 6 (31–33).

Combined Effects of TNF and IFN-γ on Dye Release from Liposomes—TNF and IFN-γ are known to have synergistic rather than additive cytotoxic effects (9–12). To ascertain whether their effects on liposome membranes are also synergistic, we examined their combined effects on release of calcine from PS/PC(2:1)-LUV at pH 5, where the rate of leakage induced by IFN-γ alone was comparable with that induced by TNF alone. A mixture of TNF and IFN-γ was added to liposomes with entrapped calcine, and the rate of dye release was compared with the sum of the rates induced by the two proteins individually. As shown in Fig. 4, the percentage of leakage induced by the mixture was always greater than the sum of those induced by the two antitumor proteins separately, and the rate of release was double the sum of the two rates, indicating synergism of their actions in release of dye from liposomes.

The leakage rates with mixtures of various amounts of TNF and IFN-γ were measured (Fig. 5). When the IFN-γ concentration was fixed at 1.3 μg/ml and the TNF concentration was varied, the rate of calcine release was 2.3 times higher...
than the sum of the individual rates at a TNF concentration of 0.33 μg/ml and at higher TNF concentrations almost parallel to the increase in the rate observed with increase in TNF concentration (Fig. 5A). At an IFN-γ concentration of 2.6 μg/ml, the rate was enhanced 1.9 times at TNF concentrations of up to 0.67 μg/ml and at higher TNF concentrations also increased in parallel to the increase in the sum of the rates (Fig. 5A). Likewise, when the TNF concentration was fixed at 0.67 μg/ml and the IFN-γ concentration was varied, 2-fold increase of the rate was observed at an IFN-γ concentration of 2.6 μg/ml and then the rate increased in parallel with the sum of the rates with the two singly (Fig. 5B). These results indicate that the synergistic effect is largest at a weight ratio of IFN-γ to TNF of 4.

**DISCUSSION**

In the present work we studied membrane damage induced by TNF and IFN-γ using purified human recombinant proteins and liposome systems. Membrane damage was assessed by measuring the release of dye encapsulated in the liposomes. We prepared three kinds of liposomes composed of PS, an anionic phospholipid, and PC, a neutral phospholipid. These liposome systems were effective for elucidating the molecular mechanisms of action of TNF and IFN-γ since both phospholipids are main components of biological membranes.

Results showed that TNF induced quick release of dye from liposomes containing PS when it bound to them, whereas IFN-γ caused very slow release of dye even when IFN-γ was completely bound to the liposomes. This difference seems to be due to differences in the mode of binding of the two antitumor proteins and their extents of membrane damage. Papahadjopoulos et al. (30) classified the interactions of proteins with membranes into three categories, with different effects on membrane permeability: type 1 interactions represent simple binding of proteins to the surface of lipid bilayers without their penetration into the hydrocarbon region, and have minimum effects on membrane permeability; type 2 interactions represent surface binding of proteins followed by their partial penetration, resulting in a large increase in the permeability of the membranes; type 3 interactions result in embedding of the proteins into the bilayer, and also induce a large increase in membrane permeability. Since TNF and IFN-γ are soluble in aqueous medium, the present results suggest that they show type 2 and 1 interactions, respectively: TNF could bind to the membrane surface and penetrate into the hydrocarbon region, whereas IFN-γ simply bound to the membrane surface.

In the pH range examined, the rates of IFN-γ-induced release of dye from PC-LUV were higher than those from PS/PC-LUV, although the protein bound almost completely to PS/PC-LUV but only slightly to PC-MLV. This difference may be attributable to a difference in the modes of binding of IFN-γ to PS/PC and PC membranes: the cytokine could bind to charged PS/PC membranes by the type 1 electrostatic interaction discussed above, but bound to neutral PC membranes exclusively by the type 2 hydrophobic interaction, or it could be attributable to a difference in the effects of IFN-γ on the phospholipid packing in the two types of membranes since PC liposomes themselves were merely leaky as PS/PC liposomes. Alternatively, 1-3 might serve as a potent modulator of dye release from PC liposomes.

Human TNF and IFN-γ are reported to have different antiproliferative effects on some cell lines of human cancers (9, 10). We also observed that the human recombinant TNF and IFN-γ used in the present study have different antiproliferative effects on several lines of human tumor cells. This difference might be attributable to different effects of these cytokines on the cell membranes.

Several groups have reported that TNF binds to receptors on the surface of target cells and then is internalized by receptor-mediated endocytosis and degraded (13, 17-19). This internalization of TNF is thought to be required for expression of its cytotoxicity (34, 35). IFN-γ is also internalized by receptor-mediated endocytosis and degraded, but its internalization does not seem to be a prerequisite for its biological activity (36). The pH profiles of the bindings of TNF to PS/PC liposomes were different from those of IFN-γ (Fig. 1). This was mainly due to the difference in the PI values of the two proteins: human recombinant TNF has a PI value of 6.0 (21) or 6.6, whereas the PI values of human glycosidase-treated and recombinant IFN-γ are 9.3 (37) and 10.4, respectively. Since the pH values inside endocytic vesicles, endosomes, and lysosomes are known to be relatively low (38), it seems significant in relation to the expression of the antitumor activity of TNF after its internalization into the cells that it bound to anionic membranes and induce membrane damage at low pH. Thus, the different effects of the two antitumor proteins on membranes might also account for the difference in their processes of expression of cytotoxic activity. Tsujimoto et al. (19) reported that binding to specific high-affinity receptors, internalization, and subsequent intracellular degradation of TNF occurred in cell lines that were highly sensitive to the cytotoxic action of TNF and also in those that were completely resistant to TNF. TNF could perturb or disrupt the membrane structure of endocytic vesicles, endosomes, or lysosomes in an acidic environment, and in this way might convey or trigger a potent signal to express cytotoxic activity only in TNF-sensitive cell lines.

Surprisingly, we found that the effects of TNF and IFN-γ on leakage of dye from liposomes were synergetic. The enhanced dye release induced by TNF plus IFN-γ is unlikely to be due to some process other than synergetic effects, because the leakage rate was proportional to the amount of TNF or IFN-γ added (Figs. 3 and 5). Moreover, the synergetic effect was greatest at a weight ratio of IFN-γ to TNF of 4. Since the molecular weights of human recombinant TNF and IFN-γ in buffer solutions have been reported to be 35,000 (22) or 45,000 (21) and 30,000-40,000 (39) or 73,000 (40), respectively, these results suggest that mutual interaction of at least two molecules of IFN-γ with one molecule of TNF results in maximum synergism in the actions of the two on liposome membranes. This finding is interesting in relation to the observation that there is an optimum in the synergetic interactions of TNF and IFN-γ on the antiproliferative response to human carcinoma cell lines (10). The mechanism of this synergism in vivo has been explained by the action of IFN-γ in enhancing the sensitivity of cells to growth inhibition by inducing TNF receptors (13-16) or by altering the membrane structure (11). The present results support the latter explanation for the synergetic actions of TNF and IFN-γ.

The present work was carried out in a model membrane system, but the intrinsic properties of TNF and IFN-γ observed, such as their isoelectric points, affinities for phospholipid membranes, and primary and high-ordered structures are important in expression of their distinct antitumor properties. Further studies on the interactions of these cytokines with biological or artificial membranes would be helpful in elucidating the molecular mechanisms of their cytotoxic and antiproliferative effects.
cytostatic actions on tumor cells.

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Synergism between TNF and IFN-γ in Membrane Damage

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