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Effects of tumor necrosis factor alpha on replication of varicella-zoster virus

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Summary

Replication of varicella-zoster virus (VZV) and expression of VZV nuclear antigen are inhibited in human embryonic lung fibroblast (HEL) cells pretreated with recombinant tumor necrosis factor (TNF) α for 24 h. This antiviral activity is completely blocked by the addition of monoclonal antibodies against TNF. TNF acts synergistically with interferons α and γ. When TNF is added to HEL cells after VZV adsorption, virus replication is still inhibited. When VZV-infected HEL cells are co-cultured with HEL cells which have been pretreated with TNF or grown in the presence of TNF, spread of VZV from VZV-infected HEL cells to uninfected cells is unaffected. No interferon is detected in the supernatants or cell lysates of HEL cells cultured with TNF and antibodies to α-, β- and γ-interferon have no effect on the antiviral action of TNF.

Tumor necrosis factor, TNF; Varicella-zoster virus, VZV; Interferon, IFN

Introduction

Tumor necrosis factor (TNF) was originally described as a protein which is present in the serum of BCG-primed, endotoxin-treated animals (Carswell et al., 1975). TNF-α is produced by monocytes and macrophages and is capable of mediating hemorrhagic necrosis of certain tumor cells (Old, 1985). In addition to the cytotoxic action against tumor cells, TNF has recently been found to be associated with other biologi-

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cal activities, such as antiviral effects against DNA and RNA viruses (Mestan et al., 1986; Wong and Goeddel, 1986; Arakawa et al., 1987).

Varicella-zoster virus (VZV) is one of the herpes viruses which poses a serious threat to the immunocompromised host. Interferons α and γ are known to inhibit replication of VZV, but the effect of TNF on VZV has not been investigated. The present study describes the antiviral action of recombinant TNF-α on VZV, together with its synergistic effect with interferons α and γ.

Materials and Methods

Cells and viruses

Human embryonic lung fibroblast (HEL) cells were grown in Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, U.S.A.) at 37°C. Confluent HEL cells were maintained in MEM with 2% FBS. VZV (Kawaguchi strain) was kindly provided by Prof. M. Takahashi (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). The cell-free VZV was obtained from VZV-infected HEL cells by ultrasonication in PSGC medium (phosphate-buffered saline containing 5% sucrose, 0.1% sodium glutamate and 10% fetal bovine serum) and the titer of cell-free VZV was 1×10^3 PFU/ml. The cell-free VZV was stored at −80°C until use. Cryopreserved, VZV-infected HEL cells were prepared in the following manner: monolayers of HEL cells were infected with cell-free VZV (Kawaguchi strain) and when 80% to 90% of these cells exhibited a cytopathic effect (CPE) they were removed with 0.1% EDTA-0.25% trypsin and suspended in MEM containing 10% FBS and 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO, U.S.A.). Aliquots of 10^6 cells in 1 ml volume were kept at −80°C for 24 h and then transferred to a liquid nitrogen freezer.

TNF-α and anti-TNF-α monoclonal antibody

Recombinant human TNF-α (PAD-4D Lot L637111-1) and anti-TNF-α monoclonal antibody (Lot 509) were provided by Asahi Chem. (Tokyo, Japan).

Interferons (IFNs) and anti-interferons

Human interferon α (1×10^7 U/mg; Code No. 300300, Lot No. 002, Japan Chemical Research Co., Kobe, Japan) and human interferon γ (1×10^7 U/mg; Code No. 300310, Japan Chemical Research Co.) were used. Sheep antisera to human interferons α and β (NIH Catalog Nos. G-026-502-568 and G-028-501-568) were obtained from NIH. A monoclonal antibody to IFN-γ was provided by Japan Chemical Research Co.

Monoclonal antibodies specific for varicella-zoster virus

Fluorescence-labelled monoclonal antibodies to VZV (Tonen Co., Tokyo, Japan) were used for direct immunofluorescence staining. They were a mixture of two mouse

monoclonal antibodies: one, clone 8, specific for cytoplasmic glycoprotein III; the other, clone 17, specific for nuclear antigens.

Plaque reduction assay

HEL cells were grown in 12-well culture plates (Costar, Cambridge, MA, U.S.A.). When they became confluent, the medium was removed and MEM with 2% FBS (maintenance medium) which contained TNF at each designated concentration was added. After incubation at 37°C for 24 h, the maintenance medium was removed and HEL cells were infected with cell-free VZV (50 PFU/well). After adsorption at 37°C for 1 h, the maintenance medium was added and the cells were cultured at 37°C for 5 days. When the control HEL cells (not treated with TNF) showed an extensive cytopathic effect (CPE), they were fixed with 10% formalin and stained with 0.05% methylene blue. The number of plaques was determined with an inverted microscope. Each experiment was done in duplicate for each TNF concentration, and the mean plaque count of two wells was used to calculate the percentage of plaques using the following formula:
Percentage of plaques = plaque count (monolayers treated with TNF/plaque count (monolayers treated with medium only) × 100.

Replication of VZV in the presence of TNF

Monolayers of HEL cells were infected with cell-free VZV (50 PFU/well). After adsorption for 1 h, maintenance medium containing the indicated concentration of TNF was added and HEL cells were incubated at 37°C for 5 days. The percentage of plaques after TNF treatment was calculated as described above.

Effect of TNF on spread of VZV from VZV-infected HEL cells

Cryopreserved VZV-infected HEL cells were rapidly thawed and washed twice with maintenance medium. Monolayers of HEL cells were pretreated with TNF for 24 h. The medium was removed and the VZV-infected cells (10^2 cells per well) in maintenance medium were added and cultured. In another experiment, VZV-infected HEL cells (10^2 cells per well) in maintenance medium containing TNF were added to monolayers of HEL cells. The percentage of plaques was calculated as described above.

Percentage of VZV nuclear antigen-positive cells and membrane antigen-positive cells

Monolayers of HEL cells were pretreated with TNF for 24 h and then infected with cell-free VZV. After 4 days of incubation, the HEL cells were removed with 0.05% trypsin, resuspended in PBS and placed on slides, allowed to dry and then fixed in acetone for 5 min at room temperature. Fluorescein-labelled monoclonal antibodies specific for VZV were added to the slides which were then incubated at 37°C for 45

min in a humidified chamber. The slides were then washed three times with PBS, mounted, and examined using an inverted immunofluorescence microscope. The cells showing positive fluorescence in the nucleus or cytoplasm were counted. In another experiment, the trypsinized HEL cells were resuspended in the maintenance medium and placed in 96-well flat-bottomed microtiter plates. Following incubation at 37°C for 1 h, the plates were centrifuged at 1000 rpm for 5 min. The medium was removed and VZV antibody-positive human serum (final dilution, 1:10) was added. The plate was then incubated at 37°C for 1 h. Following incubation the wells were washed three times, FITC-labelled anti-human IgG (Cappel Laboratories, West Chester, PA, U.S.A.) was added, and the plate was again incubated for 1 h. Each well was then examined using an inverted immunofluorescence microscope and cells showing a positive membrane fluorescence were counted. A total of 200 HEL cells were evaluated and the percentage of nuclear and membrane antigen-positive cells was determined.

Addition of anti-TNF and anti-IFN antibodies

Anti-TNF monoclonal antibody, or anti-IFN α, β, γ antibodies were added to the medium containing TNF. The mixture was added to the monolayers of HEL cells which were incubated for 24 h. Then the medium was removed and HEL cells were infected with VZV and further incubated. The percentage of plaques was determined as described above.

Effect of TNF on proliferation of fibroblasts

HEL cells were seeded at a concentration of 2×10^5 cells/well in 12-well plates in MEM containing 10% FBS and the indicated concentration of TNF. The cells were then cultured at 37°C. HEL cells were removed with 0.05% trypsin and the number of viable cells was determined every day using 0.02% trypan blue.

Interferon activity in the supernatants and cell lysates

Monolayers of HEL cells were cultured with MEM containing various concentrations of TNF. After incubation for 24 h, the supernatants were collected and stored at −20°C. HEL cells which had been treated with TNF were removed with 0.05% trypsin and washed three times with PBS; 1×10^6 cells were frozen and rapidly thawed. Freeze-thawing was repeated five times and the cell lysate was stored at −20°C. Interferon α and γ was assayed by RIA (Toray-Fuji Bionics, Tokyo, Japan), and interferon β was assayed by ELISA (Toray-Fuji Bionics).

TABLE 1

Effect of pretreatment with TNF on replication of VZV

Conc. of TNF (U/ml)	No. of plaques[a]	Plaque[b] reduction (%)	No. of experiments
0	46.4 ± 7.2	100	8
0.1	41.3 ± 2.2	11.0 ± 2.0	2
1	32.0 ± 2.1	31.0 ± 0.6	3
10	20.0 ± 8.8[c]	46.9 ± 2.9	5
100	15.6 ± 4.6[c]	66.4 ± 0.9	8

[a]Mean number of plaques ± S.D. Cell-free VZV was added to HEL cells which were pretreated with TNF for 24 h. HEL cells were cultured for five days, and the number of plaques was determined.

[b]Mean percentage reduction of plaques ± S.D. Number of plaques (treated with TNF)/Number of plaques (treated with medium only) ×100.

[c]Significantly lower than the control (pretreatment with medium only). ($P<0.01$, Student's t-test).

Results

Effect of pretreatment with TNF on replication of VZV

Table 1 shows the number of plaques which appeared when cell-free VZV was cultured on HEL cells which had been treated with predetermined concentrations of TNF for 24 h. When monolayers of HEL cells were treated with 10 or 100 U/ml of TNF, the number of plaques of VZV was significantly reduced ($P<0.01$). The number of plaques was reduced in a concentration-dependent manner.

Replication of cell-free VZV in the presence of TNF

Monolayers of HEL cells were infected with cell-free VZV. After adsorption for 1 h, maintenance medium containing TNF was added and the HEL cells were cultured. When 1 and 10 U/ml of TNF were added to the maintenance medium, the percentage of plaques was not changed. When 100 U/ml of TNF was added, plaque reduction was 35% (data not shown).

Effect of TNF on spread of VZV from VZV-infected cells

The number of plaques of VZV did not change significantly when VZV-infected cells were added to monolayers of HEL cells pretreated with TNF for 24 h or when VZV-infected cells were cultured on monolayers of HEL cells in the presence of TNF (data not shown).

Addition of anti-TNF monoclonal antibody

Anti-TNF monoclonal antibody was added to the medium containing TNF. This mixture of TNF and anti-TNF was added to the monolayers of HEL cells which were then incubated for 24 h. The medium was removed and the cells were infected with cell-free VZV (50 PFU/ml) and incubated. As shown in Fig. 1, the antiviral effect of

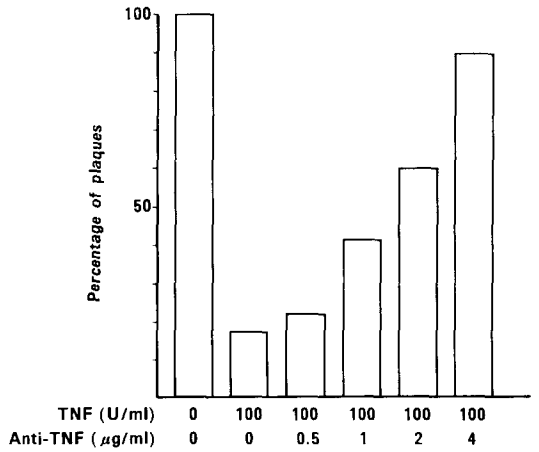

Fig. 1. Addition of anti-TNF monoclonal antibody. Anti-TNF monoclonal antibody was added to the medium containing TNF. This mixture of TNF and anti-TNF was added to the monolayers of HEL cells which were then incubated for 24 h, infected with cell-free VZV and cultured. Only the data for a representative experiment are shown.

TNF was blocked in a concentration-dependent fashion upon the addition of anti-TNF monoclonal antibody.

Effect of TNF on the expression of VZV nuclear and membrane antigens

The percentages of VZV nuclear antigen-positive and membrane antigen-positive cells were 24.1 and 14.3%, respectively, as measured 5 days following infection of HEL cells with 100 PFU of VZV. The percentage of VZV nuclear and membrane antigen-positive cells was reduced by pretreatment of the HEL cells with TNF for 24 h (Table 2).

TABLE 2

Effect of TNF on the expression of VZV nuclear antigen and membrane antigen

	Conc. of TNF (U/ml)					
	0	0.01	0.1	1	10	100
% Nuclear Ag+ cells	24.1[a]	21.9	20.2	10.8	6.5	1.9
% Membrane Ag+ cells	14.3[b]	11.3	5.4	3.4	1.2	0.5

Monolayers of HEL cells were pretreated with TNF for 24 h, and then infected with cell-free VZV.

[a]Percentage of VZV nuclear antigen-positive cells, as determined by direct immunofluorescence staining, using monoclonal antibody specific for VZV nuclear antigen and glycoprotein III.

[b]Percentage of VZV membrane antigen-positive cells, as determined by indirect immunofluorescence staining, using VZV antibody positive human sera.

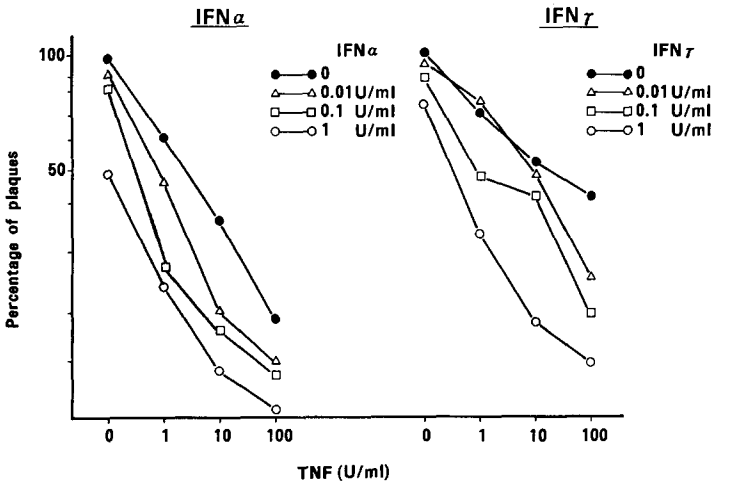

Fig. 2. Synergistic effect of TNF and IFN on VZV replication. The mixture of TNF and IFN-α (left panel) or IFN-γ (right panel) were added to the monolayers of HEL cells and incubated for 24 h. HEL cells were infected with cell-free VZV. Only the data for a representative experiment are shown. The concentration of IFN ranged from 1 to 0.01 U/ml. Δ—Δ: 0.01 U/ml; □—□: 0.1 U/ml, ○—○: 1 U/ml.

Synergistic effects of TNF and IFN on VZV replication

To determine the effect of combinations of TNF with IFNs, monolayers of HEL cells were pretreated with mixtures of TNF and IFNs α or γ. The concentration of TNF ranged from 1 to 100 U/ml and that of IFNs α and γ from 0.01 to 1 U/ml. The results of a representative experiment are shown in Fig. 2. Pretreatment of HEL cells with IFN-α or -γ for 24 h resulted in the inhibition of VZV replication.

The antiviral effect of TNF on VZV replication was enhanced by the addition of IFN-α or -γ. Combination Index (CI) was calculated by the method of Berenbaum (1977). CI between TNF and IFN-α was 0.76; CI between TNF and IFN-γ was 0.82. These data indicate that TNF and IFN-α or -γ are synergistic in their inhibitory effect on VZV replication.

Effect of antibodies to IFNs on the antiviral effect of TNF

Antibodies to IFNs α, β, and γ alone had no effect on the replication of VZV; nor had antibodies to IFNs α, β or γ, which neutralized the antiviral activity of IFN, any effect on the antiviral action of TNF (data not shown).

Lack of detectable IFN activity in the supernatants and cell lysates

Another experiment was conducted to determine whether IFN activity was present in the supernatants and in the cell lysates of TNF-treated HEL cells. Monolayers of HEL cells were cultured for 24 h in maintenance medium containing TNF (100, 10, 1 or 0 U/ml) and then the supernatants and cell lysates were collected and stored at

–20°C. No interferon-α, -β or -γ activity was detected in either the cell supernatants or cell lysates (data not shown).

Effect of TNF on proliferation of HEL cells

HEL cells (2×10^5 cells/well) in growth medium containing TNF (0, 1, 10 or 100 U/ml) were placed in 12-well culture plates and incubated. Proliferation of fibroblasts was not affected by the addition of TNF to the growth medium. After 4 days of incubation, HEL cells cultured in the presence of each concentration of TNF achieved confluence and the number of viable cells was not affected by the addition of TNF.

Discussion

In this paper we have described the effect of TNF, alone or combined with IFN, on VZV replication. Recombinant TNF-α must have a specific inhibitory effect on the replication of cell-free VZV, since its antiviral effect is blocked by the addition of anti-TNF monoclonal antibody. TNF may affect fibroblast cell growth (Sugarman et al., 1985; Vilček et al., 1986). In our study, however, growth and viability of HEL cells cultured in the presence of TNF were not altered. Thus, TNF was not toxic to the cells under our experimental conditions.

There are several stages in viral infection starting with viral attachment to host cells and culminating in the release of viral particles. In reviewing these stages, there are several possible mechanisms to explain the inhibition of VZV replication by TNF including: (1) blocking viral attachment to the cell surface receptors; (2) blocking viral entry into the cells; (3) inhibition of viral protein synthesis; (4) inhibition of cell-to-cell spread of virus, and (5) killing of virus-infected cells by TNF. According to the results of our study, the spread of VZV infection from VZV-infected cells to uninfected cells was not affected by either pretreatment of HEL cells with TNF or cultivation of VZV-infected HEL cells in the presence of TNF. TNF was not cytocidal to VZV-infected HEL cells.

Pretreatment with TNF for at least 12 h was necessary for effective inhibition of VZV replication (data not shown). However, if added after adsorption of VZV to HEL cells, TNF was still inhibitory to the replication of VZV. The present study did not address binding of the virus to viral receptor or viral entry into cells. Feduchi et al. (1989) reported that uptake of radioactive HSV-1 virions was not blocked by the presence of TNF and IFN-γ.

The exact mechanism of the inhibitory effect of TNF on VZV infection is still unclear. Mestan et al. (1986), Kohase et al. (1986, 1987) and others (Ito and O'Malley, 1987) have shown an almost complete reversal of the antiviral activity of TNF in the presence of an antiserum to human IFN-β. Hughes et al. (1988) reported that immunoreactive IFN-β was detected in the supernatants of TNF-treated HEp-2 cells. Kohase et al. (1986) showed that TNF induces IFN-β_2 mRNA and concluded that IFN-β_2 (interleukin-6, 26 kDa protein) was the mediator of the antiviral activity of TNF. Others, however, (Van Damme et al., 1987; Reis et al., 1988) have shown that

interleukin-6, induced by TNF, lacks antiviral activity and cannot account for the antiviral activity of TNF.

Wong and Goeddel (1986) concluded that the antiviral action of TNF was not due to the induction of IFN. Campos et al. (1988) stated that bovine TNF does not induce 2′-5′A synthetase. In our experiments, no IFN-α or -γ, as assayed by RIA, or IFN-β as assayed by ELISA, was detected in the supernatants or in the cell lysates of HEL cells treated with TNF. Antibodies to IFN-α, -β and -γ which neutralized interferon activity did not block the antiviral activity of TNF.

Recently Jacobsen et al. (1989) demonstrated by polymerase chain reaction (PCR) an increased transcription of IFN-β_1 gene in TNF-treated cells. Reis et al. (1989) provided direct evidence that IFN-β mRNA is increased in FS-4 cells after treatment with TNF. Mestan et al. (1988) found enhancement of the antiviral activity of TNF when combined with low concentrations of IFN-β_1. We cannot exclude the possibility that in our experiments low concentrations of IFN-β_1, which cannot be detected by conventional methods and are not neutralized by the anti-IFN-β antibody used, are induced by TNF and act synergistically with TNF. Other mechanisms might be involved, and clearly more work is needed to determine the mechanism of antiviral activity of TNF.

TNF has an inhibitory effect on replication of VZV and potentiates the antiviral activities of interferons α and γ. When peripheral blood mononuclear cells were cultured with VZV, interferon α and TNF were detected in supernatants within 24 h (unpublished data). TNF may play a role in the host defence against VZV. Eventually, TNF and IFN may prove useful in combination with antiviral drugs in the treatment of severe VZV infection in immunocompromised patients.

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