Distinct Pathways for Tumor Necrosis Factor Alpha and Ceramides in Human Cytomegalovirus Infection

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Received 28 August 1997/Accepted 14 November 1997

Human cytomegalovirus (HCMV) infection can be fatal to immunocompromised individuals. We have previously reported that gamma interferon and tumor necrosis factor alpha (TNF-α) synergistically inhibit HCMV replication in vitro. Ceramides have been described as second messengers induced by TNF-α. To investigate the mechanisms involved in the inhibition of HCMV by TNF-α, in the present study we have analyzed ceramide production by U373 MG astrocytoma cells and the effects of TNF-α versus ceramides on HCMV replication. Our results show that U373 MG cells did not produce ceramides upon incubation with TNF-α. Moreover, long-chain ceramides induced by treatment with exogenous bacterial sphingomyelinase inhibited HCMV replication in synergy with TNF-α. Surprisingly, short-chain permeant C6-ceramide increased viral replication. Our results show that the anti-HCMV activity of TNF-α is independent of ceramides. In addition, our results suggest that TNF-α and endogenous long-chain ceramides use separate pathways of cell signalling to inhibit HCMV replication, while permeant C6-ceramide appears to activate a third pathway leading to an opposite effect.

Human cytomegalovirus (HCMV) infections are well controlled in the immunocompetent host. Cellular immune responses (CD4+ and CD8+ T cells and NK cells) which accompany both acute and latent infections (for a review, see reference 4) are thought to be the main components of this control. HCMV infection during immunosuppression such as in cancer, transplantations, or AIDS results in severe pathology (4). We have previously shown that tumor necrosis factor alpha (TNF-α), in synergy with gamma interferon (IFN-γ), inhibits the replication of HCMV (7). In mice, TNF-α is involved in the clearance of CMV infection (25), TNF-α is a cytokine with multiple effects which is produced by many cell types, including macrophages and CD8+ and CD4+ T lymphocytes (for a review, see reference 40), and is known to possess antiviral effects (20, 47). The molecular mechanisms involved in the signalling by TNF-α depend on the type of receptor, p55 (TNF-R1) or p75 (TNF-R2) (5), to which it binds. Some cells express only one type of TNF-α receptor; however, expression of these receptors is not always mutually exclusive (5). The cytoxicity of TNF-α has not been reported to be mediated by TNF-R1 (38), whose intracellular region carries a death domain which signals for programmed cell death (39). Signalling through TNF-R1 with specific antibodies can also protect Hep-G2 cells from vesicular stomatitis virus-mediated cytopathic effects (48). Ceramide production after TNF-α treatment has been widely reported (16, 19, 31) and has also been shown to depend on signalling through the TNF-R1 receptor (45). In these experiments concerning myeloid cells, TNF-α induced the activation of a sphingomyelinase, which cleaved sphingomyelin to release ceramide and phosphocholine. The production of ceramides can lead to cell apoptosis (11, 14, 23) or cell cycle arrest (13). Induction of apoptosis by TNF-α has been mimicked by exogenous sphingomyelinase and by synthetic, short-chain, permeant ceramides, which suggests that ceramides, as second messengers, are sufficient to induce the cytotoxic effects of TNF-α (11, 23). Acidic and neutral sphingomyelinases activated in different cell compartments may be responsible for the diverse effects of TNF-α (46), with the former being involved in signalling through NF-κB (34) and the latter being involved in signalling through a ceramide-activated protein kinase and phospholipase A2 (46).

One of the characteristics of HCMV infection is the increase in the content of intracellular DNA, reported to be of viral (3, 8, 18) or cellular (12, 37) origin. Since TNF-α has been known to display antiproliferative properties and to block cells in the G1 phase (29), we initially tested its effects on the cell cycle of infected cells. Then, based on studies reporting that TNF-α induces ceramides in cells (16, 19, 31) and on a study showing the role of ceramide in cell cycle blockade (13), we originally postulated that ceramide was responsible for the antiviral effect of TNF-α. In the present study, we used astrocytoma cells (U373 MG) as a model for brain cells, which are important targets of HCMV in vivo (22). In contrast to fibroblasts, infected U373 MG cells release smaller quantities of virus particles even though all the cells were infected in our experiments. We believe that the U373 MG model is closer to HCMV infection in vivo. We show that ceramides are not produced by U373 MG cells upon incubation with even high concentrations of TNF-α. In addition, we demonstrate that exogenously added sphingomyelinase induces anti-HCMV effects whereas permeant C6-ceramide increases HCMV proliferation in U373 MG cells. This suggests that lipid second messengers can modulate HCMV infection and that TNF-α and ceramides use distinct signalling pathways in the control of HCMV infection. This is supported by our observation that the protein kinase JNK1 is activated exclusively by TNF-α in U373 MG cells and that TNF-α and exogenous sphingomyelinase act in synergy on HCMV infection.

MATERIALS AND METHODS

Reagents. Fetal calf serum (FCS), normal goat serum (NGS), penicillin-streptomycin, sodium pyruvate, trypsin-EDTA, EDTA, 10 × phosphate-buffered saline (PBS) without Ca2+ and Mg2+, RPMI 1640 Glutamax, BME, and Glutamax

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were from Gibco-BRL. Recombinant human TNF-α was from R and D Systems. Recombinant human IFN-γ was from Boehringer Mannheim. Sphingomyelinase (Staphylococcus aureus), ceramide (type III), dihydroethylenetriaminepentaacetic acid (DETAPAC), ATP, amidase, diithiothreitol, octyl-p-d-glucopyranoside, cardio-
lipin, and propidium iodide were from Sigma. N-Hexanoylphosphosine (C6-ceramide) was from BIOMOL Research Laboratories. Recombinant c-Jun was from Santa Cruz Biotechnology. Se/Plaque agarose was from FMC. Protein A-Sepharose was from Pharmacia Biotech. n-1,2-Diacylglycerol kinase (E. coli) was from Calbiochem. [γ-32P]ATP (4,500 Ci/mmol) was from ICN Radiochemicals. [9,10-(9)]N-propidium iodide were from Sigma.

Mycoplama were tested were mouse IgG1, or 50 μl of secondary antibody. Immunofluorescence was measured on an EPICS Elite from Coulter. [8,9-(2)]methyl-[3H]palmitic acid (51 Ci/mmol) was from Amersham Life Science. The organic phase was dried under N2 and then solubilized by sonication in 7.5% NaCl, 100 mM imidazole-HCl [pH 6.6] plus 10 μl of 20 mM diithiothreitol in H2O, 20 μl of 10 mM [γ-32P]ATP (9 μCi/assay, diluted in 100 mM imidazole and 1 mM EDTA, pH 8.0) and 100 μg/ml of CCH2 (anti-early protein) was purchased from DAKO. Phycoerythrin-conjugate goat fragment F(ab')2 to mouse IgG was from Immunotech. Agarose conjugate anti-JNK1 was purchased from Santa Cruz Biotechnology.

RESULTS

Tzn-α and Ceramides in HCMV Infection

Infection by HCMV in vitro characteristically increases the cytokine, ceramide, or HCMV was treated with medium. Lipids were extracted by the method described above, dried under N2, and then spotted on silica gel TLC plates. Phospholipids were separated by migration up the first half of the plates in chloroform-meth-
alol-acetic-acid-formic-acid-water (65:30:10:4,2, by volume). The plates were then dried and developed in chloroform-methanol-acetic acid (95:5:5, vol/vol) to the top of the plate, up to the solvent front, Va. Culture supernatant E13 (mouse IgG1 anti-IE1 and IE2 proteins) was labelled metabolically for 4 h with 0.01 Ci of [3H]thymidine in RPMI-5% FCS. To eliminate excess radioelements, the cells were incubated for 1 to 2 h in RPMI-5% FCS and then treated with different concentrations of TNF-α for the given times in RPMI-5% FCS. Lipids were extracted by the method described above, dried under N2, and then recovered and counted on a β-counter (Packard 1900TRI-CARB) in 6 ml of Picofluor (Packard). The quantity of ceramide in each assay was determined by using the standard curve. The second method allows parallel measurement of other lipids as well as ceramides. U373 MG cells (seeded at 0.5 × 106 cells per assay) were labelled metabolically for 4 h with 0.01 Ci of [3H]thymidine in RPMI-5% FCS. To eliminate excess radioelements, the cells were incubated for 1 to 2 h in RPMI-5% FCS and then treated with different concentrations of TNF-α for the given times in RPMI-5% FCS. Lipids were extracted by the method described above, dried under N2, and then counted on a Berthold scanner and then scraped, and the radioactivity was measured on a β-counter in 6 ml Picofluor. The results are expressed as a percentage of total radioactivity per lane.

Protein kinase assays. U373 MG cells were seeded at 3 × 105 cells per 75-cm2 flask. After 24 h, they were treated with the agonist for 15 min and then washed with cold PBS. The cells were scraped and disrupted on ice in 1 ml of lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.8 mM MgCl2, 5 mM EGTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 15 μl of leupeptin per ml, 1 mM pepstatin, 1 mM sodium orthovanadate). Debris was cleared by centrif-
gulation (800 × g for 10 min), and the supernatant was preclear with protein A-Sepharose beads at 4°C overnight on a rocker. These beads were removed by centrifugation (15,000 × g for 30 s), and the supernatant was then cleared for 15 min with protein A-Sepharose at 4°C. After centrifugation (13,000 rpm for 30 s), the protein kinase JNK1 was immunoprecipitated with 20 μl of the appropriate agarose conjugate per assay for 1 h at 4°C on a rocker. After centrifugation, the supernatant was kept at −20°C to quantitate the proteins in each assay by the micro-BCA technique as specified by the manufacturer. The pellets were washed twice with 500 μl of lysis buffer and then twice with 500 μl of kinase buffer (50 mM HEPES [pH 7.5], 30 mM NaCl, 100 mM KCl, 5 mM MgCl2, 10% glycerol, 200 μM sodium orthovanadate, 1% Nonidet P-40). Immunoprecipitated protein kinases were incubated with 150 mM magnesium acetate-75 μM ATP–5 μCi [γ-32P]ATP–0.5 μg of c-Jun (9 μCi/assay) for 25 min in 0.5 ml of kinase buffer per assay for 20 min at 30°C. The reaction was stopped by adding 5 μl of 8% Laemmli sample buffer and analyzed by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophore-
sis. The bands were then quantified by densitometry on a GelDoc 1000 scanner (Bio-Rad).

RESULTS

Tnz-α inhibits the cell cycle progression of HCMV-infected cells

We have previously shown (7) the decrease in viral protein expression and infectious virus production after pretreat-
ment of infected U373 MG cells with TNF-α. In the present study, we confirm this viral inhibition by TNF-α by using flow cytometry analysis of the cell cycle of HCMV-infected U373 MG cells. Inffection by HCMV in vitro characteristically increases the percentage of cells which appear in the S phase of the cell cycle (12, 37). The cell cycle of uninfected U373 MG cells rapidly reached a status quo (Fig. 1a) with 87% of cells in G0/G1 phase, 4.5% in S, and 8.5% in G2/M. However, 6 days after infection with HCMV at a MOI of 2, this pattern was modified and only 5% of cells in the S and G2/M phases, whereas 90% of the total cells (Fig. 1c). Pretreatment of U373 MG cells with 100 U of TNF-α per ml (Fig. 1b) had no effect on day 6 of the cell cycle of uninfected cells; however, in infected cells (Fig. 1d), it tended to cause the original profile of uninfected cells to be retained, with only 17% of cells in S and
13% in G2/M. Apoptosis was not observed by propidium iodide staining when cells were cultured in the presence of TNF-α.

Therefore, TNF-α exerts a direct anti-HCMV effect on infected U373 MG cells, which does not rely on cytotoxicity or apoptosis.

C6-ceramide has the opposite effect to TNF-α on HCMV. It has been reported (16, 19, 31) that TNF-α can induce the production of intracellular ceramides as second messengers. We investigated whether the viral inhibition observed with TNF-α could be reproduced with the shortchain, cell-permeable C6-ceramide. U373 MG cells were treated with different concentrations of C6-ceramide before and during HCMV infection. Figure 2A shows that C6-ceramide had no effect on the cell cycle of mock-infected cells 6 days p.i. On the same day, however, in HCMV-infected cells (Fig. 2B), C6-ceramide induced a dose-dependent increase in the percentage of cells in S phase (29% and 39% in the absence and presence of 10 μM C6-ceramide, respectively) and G2/M phase (28% and 38% in the absence and presence of 10 μM C6-ceramide, respectively). Therefore, in infected cells, C6-ceramide specifically enhances the increase in the intracellular DNA content already induced by HCMV infection.

We next studied the expression of the viral envelope protein gB in infected U373 MG cells treated or not treated with C6-ceramide. Flow cytometry analysis (Fig. 3A) showed that the intracellular expression of gB increased in a dose-dependent manner from 3 μM C6-ceramide upward. Similar results for gB expression were obtained with FSF1 cells treated with C6-ceramide (data not shown). These increases in viral protein expression could not, however, be reproduced when dihydro
C6-ceramide was used (data not shown), thus confirming the specificity of the permeant C6-ceramide. Furthermore, a plaque assay (Fig. 3B) showed that infected U373 MG cells treated with 10 μM C6-ceramide produced six times more infectious virions than did untreated control cells. Therefore, the short-chain lipid C6-ceramide increases HCMV production in our cell models.

The effect of TNF-α is ceramide independent. Since U373 MG expressed the p55 but not the p75 TNF-α receptor (data not shown), we looked for a potential production of ceramides in U373 MG cells treated with TNF-α. Figure 4 represents the kinetics of ceramide release and sphingomyelin degradation. Treatment with 0.1 U of sphingomyelinase per ml as a positive control gave a peak of ceramide production at 8.23% total radioactivity 1 h after treatment (Fig. 4B) and a corresponding degradation of 82% of the cellular sphingomyelin (Fig. 4A). Treatment with 2,000 U of TNF-α per ml induced neither ceramide release nor sphingomyelin degradation in U373 MG. Moreover, no ceramide was detected in the medium of cells incubated with TNF-α (data not shown). This data was also verified by using the diacylglycerol kinase method (28) of quantifying ceramide production. Therefore, the anti-HCMV effect of TNF-α on U373 MG cells is ceramide independent, although these cells express the p55 TNF-α receptor.

To our surprise, 1 h of sphingomyelinase treatment before infection had the opposite effect to C6-ceramide on infected cells. As shown in Fig. 5A, pretreatment with 0.1 U of sphingomyelinase per ml inhibited the expression of gB protein. Preincubation of U373 MG cells with 100 U of TNF-α per ml also inhibited gB expression but to a lesser extent. Furthermore, gB expression was practically abolished by preincubation with a combination of 0.1 U of sphingomyelinase per ml and 100 U of TNF-α per ml, suggesting synergy between these two reagents. This synergy was confirmed by measuring the amount of infectious virus produced by cells treated with a combination of different concentrations of TNF-α and sphingomyelinase (Fig. 5B). This was carried out by flow cytometry analysis of intracellular IE1 and IE2 protein expression in low-passage FSF1 cells infected with a lysate of treated U373 MG cells which had been infected for 6 days. The positive control of FSF1 cells infected with untreated, infected-cell lysate gave a mean fluorescence of 20 for IE1 plus IE2 expression on day 4 p.i. Figure 5B shows that all the combinations of TNF-α and sphingomyelinase displayed synergistic activity on HCMV production. For example, separate treatments with 500 U of TNF-α per ml and 0.1 U of sphingomyelinase per ml induced a moderate decrease of virus production (mean fluorescence intensities of 9.1 and 18.5, respectively), whereas their combination was synergistic and strongly decreased virus production (fluorescence intensity of 3.3).

This agrees with the fact that we observed no ceramide production induced by TNF-α and suggests that TNF-α uses
another pathway that is complementary to the sphingomyelin pathway in HCMV inhibition.

Differences in signalling via the MAPK pathways. The current literature suggests a role for the mitogen-activated protein kinase (MAPK) pathways in signalling via ceramides, and several authors have shown that in HL-60 cells (30, 44) or rat glomerular mesangial cells (6) the protein kinase JNK1 is activated by TNF-α and ceramides. We therefore decided to see whether there was a difference in MAPK signalling between TNF-α and ceramides in U373 MG cells. We measured the activity of JNK1 after a 15 min treatment with TNF-α was capable of activating JNK1 in U373 MG cells (Fig. 6). Higher doses (40 μM) of C6-ceramide also had no effect on JNK activity (data not shown). We conclude that in U373 MG cells, TNF-α and long-chain ceramides effectively use different signalling pathways.

DISCUSSION

We have previously reported that TNF-α alone and in synergy with IFN-γ mediates the protection of cells against HCMV infection in vitro (7). In the present study, we investigated the potential role of ceramides in the anti-HCMV effects of TNF-α in U373 MG cells. Our results show that these cells did not produce ceramides upon incubation with TNF-α. Moreover, contrary to the opinion that exogenous sphingomyelinase and permeant ceramides may be used as reagents with comparable biological consequences in cells, we found that they had opposite effects on HCMV infection: exogenous sphingomyelinase inhibited HCMV infection, whereas C6-ceramide amplified it. We also show that TNF-α and long-chain ceramides use separate pathways of cell signalling to inhibit HCMV proliferation while short-chain ceramides appear to activate a third pathway, leading to an opposite effect. This is the first report of ceramide-independent antiviral activity of TNF-α.

The cytotoxic (38), cytostatic, and antiviral (48) effects of TNF-α have been linked to signalling via the p55 TNF-α receptor, which we have found to be the only receptor present on U373 MG cells (data not shown). Wiegmann et al. (46) have shown that intracellular ceramide release is mediated via the p55 receptor either by a neutral, membrane-associated sphingomyelinase or by an acidic, endosomal sphingomyelinase. The antiviral activity of TNF-α has been attributed to two mechanisms: protection of cells from cytotoxic effect in vesicular stomatitis virus infection (48) or lysis of infected cells (49). In the present study, TNF-α provoked a decrease in the percentage of cells in the S and G2/M phases during infection, although it had no effect on the cycle of noninfected cells and was incapable, even at high concentrations, of inducing apoptosis in these cells. The absence of apoptosis was not, however, due to a defect in the cell line, since high concentrations of C6-ceramide (80 μM) were capable of inducing cell death by apoptosis (data not shown). Therefore, the antiviral activity of TNF-α in our model is due neither to cell lysis nor to apoptosis, and we show that TNF-α is capable of mediating a cytostatic effect without ceramide production or cytotoxicity despite the p55 receptor being used in U373 MG cells.

We used exogenous sphingomyelinase treatment of U373 MG cells as a positive control for long-chain ceramide release. This enzymatic treatment induced ceramide production and also displayed anti-HCMV activity, which implies that long-chain ceramides are responsible for this effect. A combination of exogenous sphingomyelinase and TNF-α synergistically enhanced the anti-HCMV action of the two reagents, suggesting that two complementary pathways are being used.

C6-ceramide was incapable of reproducing the anti-HCMV effect of TNF-α or exogenous sphingomyelinase. This was surprising, since short-chain ceramides have been considered the equivalent of endogenous ceramide production and have been extensively used in this way (13, 31), although different effects of bacterial sphingomyelinase and exogenous ceramides in some studies have led to the observation of distinct pools of sphingomyelin (50). C6-ceramide amplified the percentage of cells in the S and G2/M phases, a hallmark of HCMV infection, which has been attributed either to an increase in cellular DNA replication (12, 37) or to a mass production of viral DNA (3, 8, 18). C6-ceramide also increased the expression of the envelope viral protein gB, as well as the amount of infectious virus produced by U373 MG cells. These results have also been validated with FFSF1 cells (data not shown), which showed the same increase in gB expression following C6-ceramide treatment, proving that this is not specific to U373 MG cells. This model of HCMV infection has thus revealed a signalling pathway which is different for long- and short-chain ceramides. Exogenous sphingomyelinase treatment cleaves sphingomyelin of the outer leaflet of the plasma membrane; the long-chain ceramides generated are hydrophobic and will progress only to the cytosol. C6-ceramide, however, can cross the plasma membrane as well as the internal membranes, and Futerman et al. (9) have shown that it accumulates in the Golgi apparatus, where the majority of sphingomyelin synthesis occurs. These differences may explain the discrepancy observed with long-chain and short-chain ceramides on the infection of U373 MG cells. Another possibility is that exogenous ceramides altered the cell secretory pathway, as described previously (33), and prevented the release of virions into the medium, thereby increasing the amounts of intracellular structural proteins and of intracellular infectious virions. Alternatively, Venable et al. (41) have shown that C6-ceramide is metabolized in WI-38 human diploid fibroblasts to give two minor, unidentified metabolites, which could possibly explain the differences observed between C6-ceramide treatment and sphingomyelinase-induced ceramide release. Mitogenic activity of C6-ceramide has already been reported in fibroblasts (10, 24), and ceramide has also been shown to be involved in cellular senescence (41).
In conclusion, and as schematically depicted in Fig. 7, HCMV infection has revealed three different signalling pathways for TNF-α and ceramides: a ceramide-independent inhibition of HCMV replication by TNF-α, a long-chain-ceramide-dependent inhibition of HCMV replication, and a permenant C6-ceramide-induced increase of HCMV infection.

ACKNOWLEDGMENTS

This study was supported by grants from INSERM, “Association Recherche et Transfusion” and “Conseil Régional Midi-Pyrénées.” Justine Allan Yorke was supported by an M.S.R. fellowship.

We thank Hélène Brun and Georges Cassar for help with flow cytometry, Danièle Clément for technical assistance, and Hugues Chap for support and critical reading of the manuscript.

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