Human Cytomegalovirus Infection Decreases Expression of Thrombospondin-1 Independent of the Tumor Suppressor Protein p53

Jindrich Cinatl, Jr.,* Ruslan Katchetkov,*†
Martin Scholz,* Jaroslav Cinatl,† Jens-Uwe
Vogel,*† Pablo Hernáiz Driever,‡ and
Hans Wilhelm Doerr*

From the Zentrum der Hygiene,* Institut für Medizinische
Virologie, Zentrum für Kinderheilkunde und Jugendmedizin,†
Abteilung Pädiatrische Hämatologie und Onkologie, Klinikum
der Johann Wolfgang Goethe-Universität, Frankfurt am Main,
Germany, and Department of Neurosurgery,‡ Georgetown
University Medical Center, Washington, D.C.

Thrombospondin-1 (TSP-1) is a potent inhibitor of angiogenesis. It has been shown that promoter sequences of the TSP-1 gene can be transactivated by the wild-type tumor suppressor protein p53. As human cytomegalovirus (HCMV) infection inactivates wild-type p53 of various cell types, we investigated whether HCMV infection is associated with reduced TSP-1 production. We found, in conjunction with accumulated p53, that TSP-1 mRNA and protein expression was significantly reduced in HCMV-infected cultured human fibroblasts. To determine whether the observed TSP-1 suppression depends on p53 inactivation, the p53-defective astrocytoma cell line U373MG was infected with HCMV. In these cells TSP-1 expression was also significantly reduced by HCMV infection whereas expression of the p53 mutant variant remained unaltered. In both cell lines the decreased expression of TSP-1 mRNA occurred early after infection (4 hours), indicating that HCMV inhibits TSP-1 transcription during the immediate-early phase of infection before HCMV DNA replication. Inhibition of HCMV DNA synthesis by ganciclovir did not influence TSP-1 reduction whereas the antisense oligonucleotide ISIS 2922, complementary to HCMV immediate-early mRNA, completely prevented the HCMV-mediated TSP-1 suppression. These findings strongly suggest a novel role for HCMV in the modulation of angiogenesis due to p53-independent down-regulation of TSP-1 expression. (*Am J Pathol* 1999, 155:285–292)

Human cytomegalovirus (HCMV) has been implicated in the etiology of several human malignancies based on seroepidemiological studies and the presence of HCMV

DNA, RNA, and/or antigens in tumor tissues.¹ The definitive establishment of a direct causative role for HCMV in the course of the malignancies has been elusive as HCMV components were not detected after long-term subculture of tumor tissues.¹ Moreover, the presence of genetic information in human tumors is difficult to interpret as HCMV can cause latent infection of numerous organs in a high percentage of normal individuals.¹ On the other hand, *in vitro* infection of cells with HCMV influences the expression of different cellular genes and/or function of cellular proteins that are associated with cell growth, differentiation, and apoptosis. These changes result in disturbance of normal tissue homeostasis, which indirectly may promote tumor growth.^{2–6} In particular, *in vitro* studies have shown that HCMV gene products such as immediate-early (IE) proteins or morphological transforming region II oncoprotein bind wild-type p53 tumor suppressor protein and thereby down-regulate p53-activated transcription.^{7–11}

The development of new blood vessels (angiogenesis) is necessary to sustain the growth, invasion, and metastasis of tumors.¹² The signals controlling angiogenesis, although directed at the endothelial cells, come from tumor or stromal cells, such as fibroblasts or monocytes/macrophages. A variety of positive regulators of angiogenesis have been isolated from different tissues and have been purified and sequenced.¹³ From the many peptide growth factors known to act on the vasculature, basic fibroblast growth factor (bFGF)¹⁴ and vascular endothelial growth factor (VEGF)¹⁵ represent the most potent ones, but other factors, such as transforming growth factor- β 1 (TGF- β 1) and platelet-derived growth factor (PDGF), may also be involved in tumor angiogenesis.¹³

In contrast, several other proteins negatively control angiogenesis. One of the most potent negative regulators is the extracellular matrix protein thrombospondin-1 (TSP-1).^{16,17} Although some positive effects on angiogenesis have been reported,¹⁸ TSP-1 under defined conditions generally inhibits endothelial cell adhesion,¹⁹ mo-

Supported by the foundations Hilfe für krebssranke Kinder Frankfurt, e.V., and Frankfurter Stiftung für krebssranke Kinder.

Accepted for publication March 20, 1999.

Address reprint requests to Dr. Jindrich Cinatl, Institut für Medizinische Virologie, Zentrum der Hygiene, Klinikum der Johann Wolfgang Goethe-Universität, Sandhofstrasse 2–4, D-60528 Frankfurt am Main, Germany. E-mail: cinatl@em.uni-frankfurt.de.

tility,²⁰ and growth^{20,21} and induces apoptosis in endothelial cells.²²

TSP-1 expression and function are influenced by several growth factors, oncogenes, and tumor suppressor genes.¹⁶ For example, wild-type p53 induced TSP-1 in normal human fibroblasts.²³ Further evidence for the importance of p53 as a transactivator of the TSP-1 gene has been clearly demonstrated with cultured fibroblasts from cancer-prone Li-Fraumeni patients. When these cells, lacking one p53 allele, either lose or mutate the second p53 allele (which occurs during progression to malignancy), TSP-1 is down-regulated by ~96%.²³

HCMV infection of fibroblasts has been associated with increased expression of bFGF and some other cytokines and interleukins that may exert both direct and indirect angiogenic activities.²⁴ However, it has not been shown whether HCMV may also affect the expression of negative regulators of angiogenesis, for example, via HCMV-IE protein interaction with p53. In the present study, the effects of HCMV infection on TSP-1 in wild-type p53-expressing cultured human fibroblasts and in the p53-defective human astrocytoma cell line U373MG²⁵ were studied. The results show that both the laboratory-adapted strain HCMV AD169 and a patient-derived strain, Hi91, inhibited the expression of TSP-1 in infected cell lines, indicating that p53 was not involved. As HCMV-mediated suppression of TSP-1 could be prevented by treatment with antisense oligonucleotides against HCMV-IE mRNA, this suggested that HCMV-IE proteins regulate TSP-1 expression. In contrast, ganciclovir (GCV), a specific inhibitor of DNA replication and consequently late gene expression, had no effect. In conclusion, novel therapeutic strategies targeting the HCMV-IE gene expression could be important for the prevention of HCMV-associated angiogenesis.

Materials and Methods

Cell Cultures

Human foreskin fibroblast (HFF) cultures were established in our laboratory as described previously.²⁶ HFF cells between three and six subcultures were used in the experiments. The human astrocytoma cell line U373MG was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in a culture medium composed of Eagle's minimal essential medium supplemented with 20% fetal bovine serum. After reaching confluency, cells were subcultured at 6-day intervals.

Virus Preparation

The HCMV laboratory strain AD169 was obtained from American Type Culture Collection, whereas the clinical strain Hi91 was isolated from a urine sample of a patient with HIV infection. Virus stocks of both strains were prepared in HFF cells incubated in minimal essential medium supplemented with 4% fetal bovine serum. The respective titers were determined by plaque titration in HFF cells as described previously.⁴ Mock-infected inoc-

ula were prepared in an identical fashion, except that cell monolayers were not infected with HCMV.

HCMV inactivation was achieved by exposure of virus-containing cell culture medium to ultraviolet (UV) light (220 V, 12 W) for 15 minutes.²⁷ Irradiated virus samples were used as inocula in HFF or U373MG cultures. UV-irradiated samples were free of infectious virus as demonstrated by plaque titration (not shown).

Filtered virus inocula were prepared by filtering virus stocks through a Microsep microconcentrator with a cut-off at 300,000 molecular weight (Filtron Technology Corp., Northborough, MA) at 3000 × g for 12 hours at 4°C. The filtrate collected from the bottom of the filter apparatus was added to HFF or U373MG cultures. The filtrate samples were free of infectious virus as demonstrated by plaque titration.

Antiviral Drugs

GCV (Hoffman-La Roche AG, Grenzach-Wyhlen, Germany) was prepared fresh (on the day of each experiment) in distilled water. ISIS 2922, a phosphorothioate oligonucleotide that is complementary to HCMV IE RNA and the noncomplementary control oligonucleotide ISIS 26062, were kindly provided by ISIS Pharmaceuticals (Carlsbad, CA).²⁸ Both ISIS 2922 and ISIS 26062 were dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mmol/L, and aliquots were stored at -20°C until use. To enhance oligonucleotide uptake, ISIS 2922 or ISIS 26062 was complexed to cationic liposomes DOTAP (Boehringer, Mannheim, Germany) immediately before virus infectivity assay. The pretreatment of the antisense oligonucleotides with DOTAP was performed according to the instructions of the manufacturer.

Virus Infectivity Assay

Confluent cultures of HFF cells were incubated with HCMV infectious strains at a multiplicity of infection (m.o.i.) of 2 or with UV-inactivated samples or with filtered virus-free inocula. After incubation for 1 hour, required for virus adsorption, cells were washed with PBS and incubated in culture medium containing 2% fetal bovine serum. In some experiments, cells incubated with infectious virus were treated with GCV or with oligonucleotides complexed to cationic lipids, including antisense ISIS 2922 or control oligonucleotide ISIS 26062. Cells were treated with ISIS 2922 or ISIS 26062 according to established protocols.^{28,29} Briefly, cell monolayers were pretreated with the oligonucleotides overnight in IMDM containing 0.2% fetal bovine serum before infection. After pretreatment, cells were infected with HCMV at a m.o.i. of 2. After 1 hour of incubation, virus was removed and fresh medium containing antisense oligonucleotides was added. For control purposes, cells were incubated with GCV in the same way as described for ISIS 2922 and ISIS 26062. The cell numbers producing HCMV-specific antigens were examined 24 and 72 hours post infection (p.i.) by immunoperoxidase staining using monoclonal antibodies (MAbs; DuPont, Bad Homburg, Germany) di-

rected against 72-kd immediate-early antigen (IEA) and 67-kd late antigen (LA), respectively (described in detail elsewhere).^{3,4}

Flow Cytometry

To investigate the expression of TSP-1 or p53, 5×10^5 cells were fixed for 10 minutes in 4% buffered formaldehyde. After washing the cells twice in buffer containing 0.5% Tween-20, cells were incubated for 30 minutes with mouse MAbs against TSP-1 (clone 11.4; Dianova, Hamburg, Germany), a MAb specifically detecting mutant p53 (clone PAb 240; Calbiochem, Bad Soden, Germany), or a MAb specifically detecting wild-type p53 (clone PAb 1620; Calbiochem). After washing cell pellets two times in buffer, the fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (Becton Dickinson, Heidelberg, Germany) was added for 30 minutes. Fluorescence intensities were measured by flow cytometry (FACScan, Becton Dickinson). Data collected from 1×10^4 cells were analyzed using Cell Quest software. All experiments were repeated at least three times.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from mock-infected or infected cells using TRIZOL according to the manufacturer's instructions (Gibco-BRL Life Technologies, Gaithersburg, MD). RNA was reverse transcribed using random hexamer priming. One microgram of total RNA was denatured at 70°C for 10 minutes and chilled on ice. The denatured RNA was then co-incubated with 2.5 $\mu\text{mol/L}$ random hexamer oligonucleotides, 1 $\mu\text{mol/L}$ each dNTP, 5 mmol/L MgCl_2 , 1 μl of RNase inhibitor (Boehringer Mannheim), and 1 μl of MuLV reverse transcriptase (Gibco-BRL) in 1X polymerase chain reaction (PCR) buffer II (Perkin-Elmer Corp.) for 1 hour at 37°C. The reverse transcription was inactivated for 5 minutes at 95°C before amplification. TSP-1 primers used were 5'-CGT CCT CTT CCT GAT GCA TG-3' (position 99 to 118) and 5'-GGC AGG ACA CCT TTT TGC AGA-3' (position 1115 to 1135),³⁰ the sequence of GAPDH primers used as control were 5'-TGG GGA AGG TGA AGG TCG GA-3' (position 61 to 81) and 5'-GAA GGG GTC ATT GAT GGC AA-3' (position 151 to 171).³¹ PCR amplification of the cDNA was carried out by adding 0.5 μg of *Taq* DNA polymerase (Boehringer Mannheim). PCR amplification was done using 26 cycles in a DNA thermocycler as follows: denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C, and extension for 1.5 minutes at 72°C in a Perkin Elmer thermocycler. PCR products were resolved alongside DNA marker on an 8% polyacrylamide gel, stained with silver nitrate, and photographed. The photographs were further analyzed by scanning densitometry using E.A.S.Y. RH system (HeroLab, Wiesloch, Germany), and the ratio of TSP-1/GAPDH band intensity was calculated. To ascertain that TSP-1 transcripts were specifically amplified, sequence analysis of PCR prod-

ucts was performed. Amplified sequences fully matched TSP-1 nucleotide sequence (results not shown).

Results

Effects of HCMV Infection on TSP-1 and p53 Expression

To study the effects of HCMV infection on TSP-1 and p53 expression, both HFF and U373MG cells were infected with HCMV AD169 and Hi91 strains at a m.o.i. of 2. Both human fibroblasts and U373MG represent fully permissive cell types for HCMV replication in cell culture,³² which was confirmed by immunoperoxidase staining against HCMV-specific IEA and LA as controls for each experiment. In both HFF- and U373MG-infected cultures more than 99% of cells stained positively for HCMV IEA and more than 98% and 92% of cells stained positively for LA, respectively (data not shown).

The levels of cell-associated TSP-1 were evaluated by flow cytometry at different times after virus inoculation and compared with those of mock-infected HFF and U373MG cells. Although at 4 hours p.i. no HCMV-mediated modulation of TSP-1 could be detected within the infected HFF cell populations, significant differences were observed 24 and 72 hours p.i. Infection of HFF with AD169 strain decreased TSP-1-specific fluorescence intensity to 51% after 24 hours and to 21% after 72 hours p.i. of the mock-infected control. Similar results were obtained when HFF cells were infected with the HCMV-Hi91 strain (Figure 1A).

In fibroblasts and other normal cell types HCMV infection results in accumulation of the wild-type p53 in association with loss of its function in transactivation of different cellular genes.^{7-9,11} As this tumor suppressor protein represents a potent transactivator of the TSP-1 gene we determined by flow cytometry whether decrease in cell-associated TSP-1 protein was paralleled by increasing (accumulated) concentrations of wild-type p53 in HCMV-infected HFF cells. According to the measurement of TSP-1, no HCMV-related modulation of p53 levels could be observed as early as 4 hours p.i. Cells infected with strain AD169 exhibited 170% and 223% expression after 24 and 72 hours, respectively, of the mock-infected control. Similar results were obtained after infection with strain Hi91 (163% and 216%, respectively; Figure 2A).

To determine whether the above HCMV-mediated effects on TSP-1 were also observed in cells with mutant p53, the U373MG astrocytoma cells were infected with HCMV AD169 or Hi91. This cell line contains a mutation in codon 273 (CGT→CAT) of the p53 gene that results in the production of inactive p53 protein.²⁵ Despite the lack of functional p53, the U373MG infected with HCMV-AD169 exhibited decreased TSP-1 expression to 50% and 10% (Hi91: 52% and 8.6%) of the mock-infected cells 24 and 72 hours p.i., respectively (Figure 1B). In contrast, the levels of mutant p53 remained unmodified on HCMV infection in U373MG cells (Figure 2B).

Additional experiments were performed to determine whether HCMV-specific viral gene expression was re-

Figure 1. Time course of TSP-1 protein expression in HFF (A) and U373MG (B) cells. TSP-1 was measured by flow cytometry in mock-infected or AD169- or Hi91-infected cells 4, 24, and 72 hours after infection p.i. The results are expressed as relative mean fluorescence intensity units. Data are depicted as means $\pm$ SD from one representative experiment performed in triplicate. Statistically significant differences were found between virus-infected and mock-infected cultures ($P < 0.05$) after 24 and 72 hours as determined by Student's *t*-test.

sponsible for changes in TSP-1 and/or p53 expression. In four independent experiments, it was found that neither ultrafiltrated supernatants from virus-infected cells nor UV-inactivated virus affected TSP-1 or p53 protein expression in HFF or U373MG cells as determined by flow cytometry (data not shown).

Effects of HCMV on TSP-1 mRNA Expression

To study whether HCMV-mediated reduction of TSP-1 protein levels were due to impaired mRNA synthesis, TSP-1 mRNA levels in HFF and U373MG cells infected with strain AD169 at different times after virus inoculation were measured by reverse transcription PCR. Reverse transcription was carried out using specific primers for TSP-1 and GAPDH. As shown, HCMV infection resulted in the reduction of TSP-1 mRNA levels similarly in both cell types already at 4 hours p.i. (Figure 3A). Densitometric analyses of TSP-1-specific bands revealed that HCMV infection but not treatment with UV-inactivated virus or

Figure 2. Time course of p53 protein expression in HFF (A) and U373MG (B) cells. p53 was measured by flow cytometry in mock-infected or AD169- or Hi91-infected cells 4, 24, and 72 hours p.i. The results are expressed as relative mean fluorescence intensity units. Data are depicted as means $\pm$ SD from one representative experiment performed in triplicate. Statistically significant differences were found between virus-infected and mock-infected HFF cultures ($P < 0.05$) after 24 and 72 hours as determined by Student's *t*-test.

virus-free filtrates (not shown) resulted in inhibition of the respective mRNA to 60% of the mock-infected cultures. TSP-1 mRNA was found to be further reduced to 25% and 5% of the mock-infected cultures at 24 and 72 hours p.i., respectively (Figure 3B).

Effects of GCV and ISIS 2922 on TSP-1 and p53 Expression

To study the mechanism involved in the HCMV-mediated effects on TSP-1 and p53 expression, HCMV-infected cells were treated with two anti-HCMV agents. GCV, a drug clinically used for treatment of HCMV disease, targets HCMV DNA synthesis and does not prevent immediate-early or early virus gene expression.^{33,34} In contrast, the antisense oligonucleotide ISIS 2922 specifically blocks the function of HCMV IE mRNA and thus prevents the *de novo* synthesis of viral IE proteins before viral DNA

Figure 3. Time course of TSP-1 mRNA expression in mock-infected (–) and AD169-infected (+) HFF cells. Total RNA was isolated 4, 24, or 72 hours p.i. Reverse transcription PCR was done using specific primers for TSP-1 and GAPDH, and products were visualized by staining with silver nitrate on polyacrylamide gel (A). The bands were analyzed by densitometry. Relative density values of TSP-1 mRNA are depicted as the mean of three experiments $\pm$ SD (B).

replication occurs.²⁸ The antiviral effects of both agents were determined in infected cultured HFF and U373MG cells by quantification of numbers of cells carrying HCMV-specific IEA and LA by means of immunoperoxidase staining technique. The number of HCMV IEA/LA-positive cells detected after treatment with either drug was compared with those detected in the untreated controls (Figure 4).

GCV treatment of HCMV-infected cells at concentrations ranging from 2.5 to 40 μ mol/L suppressed LA expression in HFF and U373MG cells in a dose-dependent

Figure 4. Effects of antiviral drugs GCV and ISIS 2922 on HCMV IEA (●) and LA (▼) expression in HFF cultures (A and C) and U373MG cultures (B and D) infected with AD169. The numbers of HCMV IEA- and LA-positive cells were determined by means of immunoperoxidase staining and counted 24 hours (IEA) and 72 hours (LA) p.i. The results are expressed as percentage of IEA/LA-positive cells of infected controls without drug treatment. In control cultures, more than 98% and 92% of cells stained positive for IEA or LA, respectively. Data are shown representative of three independent experiments.

manner with complete inhibition at 40 μ mol/L whereas no inhibitory effect on the expression of IEA was observed (Figure 4, A and B). When cells were treated with ISIS 2922 both IEA and LA expression was inhibited in a dose-dependent manner (range, 0.06 to 1.0 μ mol/L). The nontoxic concentration of 1 μ mol/L inhibited IEA and LA in both cell lines by at least 99% (Figure 4, C and D). Treatment with the irrelevant antisense oligonucleotide ISIS 26062 had no detectable inhibitory effects (not shown).

Next, the effects of GCV and ISIS 2922 on TSP-1 and p53 expression in HCMV (AD169)-infected HFF and U373MG cells were compared. Expression of these proteins was assessed by means of flow cytometry at 3 days p.i. The relative mean fluorescence intensity values obtained by histogram analyses of populations stained with MAb 11.4 (recognizing TSP-1), MAb Pab 240 (recognizing mutant p53), and MAb Pab 1620 (recognizing wild-type p53) are depicted in Tables 1 and 2, respectively. The results showed that HCMV-mediated down-regulation of TSP-1 (as indicated by the reduced MAb 11.4-related fluorescence intensity) was prevented by ISIS 2922 but not by GCV in HFF (Table 1). Moreover, GCV

Table 1. Effects of GCV and ISIS 2922 on TSP-1 in Mock-Infected and AD169-Infected HFF and U373MG Cells

Treatment	Mean fluorescence units			
	HFF		U373MG	
	Mock-infected	AD169-infected	Mock-infected	AD169-infected
Without	61.3 $\pm$ 8.1	13.2 $\pm$ 2.1	46.3 $\pm$ 5.7	8.7 $\pm$ 1.4
GCV (40 μ mol/L)	62.1 $\pm$ 7.3	14.1 $\pm$ 2.3	45.9 $\pm$ 5.2	10.4 $\pm$ 2.8
ISIS 2922 (1 μ mol/L)	59.8 $\pm$ 7.8	63.2 $\pm$ 6.8	48.7 $\pm$ 6.3	46.1 $\pm$ 3.8

Values are mean $\pm$ SD from one representative FACSscan analysis performed in triplicate. Mean fluorescence units for irrelevant antibodies ranged from 2.1 to 4.8.

Table 2. Effects of GCV and ISIS 2922 on p53 in Mock-Infected and AD169-Infected HFF and U373MG Cells

Treatment	Mean fluorescence units			
	HFF		U373MG	
	Mock-infected	AD169-infected	Mock-infected	AD169-infected
Without	12.7 ± 2.5	36.2 ± 4.2	41.7 ± 5.3	42.3 ± 5.2
GCV (40 µmol/L)	13.9 ± 2.1	32.6 ± 3.8	38.9 ± 4.3	41.8 ± 4.6
ISIS 2922 (1 µmol/L)	16.7 ± 2.1	14.9 ± 1.9	43.2 ± 5.8	38.7 ± 4.6

Values are mean ± SD from one representative FACScan analysis performed in triplicate. Mean fluorescence units for irrelevant antibodies ranged from 2.4 to 3.9.

treatment had no influence on the HCMV-mediated enhancement of MAb Pab 1620-specific fluorescence intensity in infected HFF. In contrast, the values obtained for ISIS 2922-treated infected HFF did not differ from mock-infected control values, suggesting that ISIS 2922 completely prevented wild-type p53 up-regulation. Neither GCV nor ISIS 2922 modified the expression of mutant p53 in U373MG cells (Table 2).

To confirm that ISIS 2922 prevented the HCMV-induced suppression of TSP-1 on the transcription level, reverse transcription PCR for TSP-1 mRNA was carried out with cell extracts from 1) untreated, 2) GCV-treated, and 3) ISIS 2922-treated HFF and U373MG cells. Figure 5A shows blots demonstrating that HCMV-induced suppression of TSP-1 mRNA was completely prevented by treatment with ISIS 2922 but not with GCV. For semiquantitation purposes the density of the signals was analyzed by scanning densitometry. The ratios of TSP-1/GAPDH are given as mean relative TSP mRNA expression values from three experiments in Figure 5B.

Discussion

In the present study HCMV infection was shown to decrease TSP-1, a negative regulator of angiogenesis. Down-regulation of TSP-1 was independent of the tumor suppressor protein p53. These findings may suggest that HCMV promotes angiogenesis in a more complex manner than assumed. It was previously shown that HCMV infection of different cell types was associated with increased expression of bFGF and other positive regulators of angiogenesis.^{24,35}

Based on its effects on tumor and endothelial cell behavior, TSP-1 has attracted attention as a possible negative regulator of tumor growth and metastasis. In addition to its direct effects on tumor cells, TSP-1 reduces tumor growth by inhibition of angiogenesis possibly through an indirect mechanism induced by infected fibroblasts.^{23,36} Initial studies have confirmed that increased TSP-1 expression suppressed growth and metastasis of some tumors *in vivo* and inhibited angiogenesis.^{37–39} In the present study HCMV infection of both fibroblasts and astrocytoma cells resulted in down-regulation of TSP-1. Therefore, it is conceivable that the infection of stromal and/or tumor cells, frequently observed in patients with different malignancies,^{1,4} stimulates angiogenesis due to an imbalance between positive and negative regulators. Although the exact role of

HCMV in tumorigenesis is unclear, angiogenic properties resulting from HCMV infection could be responsible for tumor progression in these patients.

The down-modulation of TSP-1 mRNA and protein expression could be due to inactivation of tumor suppressor proteins in HCMV-infected fibroblasts. Previous studies demonstrated that accumulation of tumor suppressor proteins, including wild-type p53 and Rb in HCMV-infected cells, was associated with loss of their function.^{7–9,11} These effects of HCMV result mainly from the binding of tumor suppressor proteins by HCMV IE proteins reminiscent of the

Figure 5. TSP-1 mRNA expression in mock-infected (–) and AD169-infected HFF (+) cells without treatment, after treatment with GCV or after treatment with ISIS 2922. Reverse transcription PCR was done using specific primers for TSP-1 and GAPDH (standard control). Products were visualized by staining with silver nitrate on polyacrylamide gel. The bands were analyzed by densitometry (A). Relative density values of TSP-1 mRNA are depicted as the mean of three experiments ± SD (B).

effects of oncoproteins of small DNA tumor viruses.^{7-9,11} It was initially suggested that inactivation of p53 in HCMV-infected cells represents a major mechanism in down-modulation of TSP-1 expression as this tumor suppressor gene is involved in the positive regulation of TSP-1.^{23,40} In patients with Li-Fraumeni syndrome inactivation of p53 due to lack or mutation of p53 alleles was associated with reduced expression of TSP-1.²³ Indeed, we observed that decreased TSP-1 amounts in HCMV-infected fibroblasts were paralleled by increased levels of wild-type p53 in the cytoplasm of the cells. However, in U373MG cells that express a mutated variant of p53²⁵ TSP-1 was similarly reduced on HCMV infection whereas the level of p53 remained unaltered. In a glioblastoma cell line (U87MG) carrying wild-type p53²⁵ the level of TSP-1 was similar to the level in U373MG cells (not shown). Unfortunately, we were not able to determine the effects of HCMV infection on the expression of TSP-1 in U87MG because these cells have a low permissivity for HCMV (<1%). Moreover, it has been reported that induction of wild-type p53 in glioblastoma cells did not alter TSP-1 levels.⁴¹ These findings support our results indicating that the HCMV-mediated modulation of the TSP-1 expression may occur via intracellular pathways independent of p53. Rb, a different tumor suppressor protein, was also shown to be involved in the positive regulation of TSP-1 gene expression.^{23,40} As HCMV has been shown to functionally inhibit Rb,^{8,9} it is conceivable that this mechanism could contribute to reduced expression of TSP-1.

In addition to tumor suppressor proteins, cellular transcription factors may also influence TSP-1 gene expression.¹⁶ In the human and mouse, TSP-1 promoter potential binding sites for different transcription factors are present.⁴²⁻⁴⁵ It has been shown that HCMV activates cellular metabolism in a manner similar to growth factors and hormones,⁴⁶⁻⁴⁸ resulting in activation of different proto-oncogenes and transcription factors.⁴⁶⁻⁴⁷ For example, both mitogenic stimuli and HCMV infection induce expression of proto-oncogenes such as *c-jun*, *c-fos*, and *c-myc*.⁴⁹ In particular, the Jun proto-oncogene product was shown to repress thrombospondin expression in rat embryo fibroblasts.⁵⁰ It is known that cellular proto-oncogenes can be induced by transactivating HCMV IE proteins but also by inactivated HCMV particles within minutes after infection independent of HCMV gene expression.⁵¹ Our results demonstrate that HCMV-induced proto-oncogene activation before HCMV gene expression does not play a major role in TSP-1 regulation. And TSP-1 expression was not modified either by inactivated HCMV or by HCMV-induced nonspecific soluble factors present in cell culture supernatants. Thus, it is likely that effects on TSP-1 gene expression stem from transcription activity of HCMV IE proteins. For example, HCMV IE gene expression controls activation transcription factor-1/c-AMP responsive element binding protein (ATF-1/CREB)⁵² that in turn negatively regulates TSP-1 expression.⁴⁵ This negative regulation may occur either directly by binding of ATF-1 to a negative regulatory site in the TSP-1 promoter or indirectly by negative regulation of Rb via an ATF binding site in the promoter of Rb.^{45,53} Our results demonstrate that HCMV-mediated TSP-1 down-regulation is due to HCMV IE gene expression,

which can be prevented by specific inhibition of HCMV IE mRNA with the antisense oligonucleotide ISIS 2922 but not by inhibition of HCMV DNA replication with GCV.

Recently, we and others suggested that HCMV IE gene expression plays an important role in HCMV-associated pathogenicity.^{27,33,34} Evidence has been presented in this report that GCV or related drugs are not sufficient in this regard,^{33,34} although drugs suppressing the HCMV IE phase may be of more relevance.³⁴ In this report it was shown that ISIS 2922 specifically blocks HCMV IE mRNA, which resulted in complete inhibition of HCMV-mediated down-regulation of TSP-1. In summary, the inhibition of HCMV IE gene products provides an important approach for the treatment of HCMV-associated angiogenesis involved in various pathogenic conditions such as tumor growth or inflammation.

Acknowledgments

We are grateful to Gabi Bauer for microphotographs and Sandra Dujardin and Gesa Meincke for technical assistance.

References

- Rosenthal LJ, Choudhury S: Potential oncogenicity of human cytomegalovirus: molecular aspects of human cytomegalovirus diseases. *Frontiers of Virology*, vol 2. Edited by Becker Y, Darai G, Huang E-S. Berlin, Springer-Verlag, 1993, pp 412-436
- Zhu H, Shen Y, Shenk T: Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J Virol* 1995, 69:7960-7970
- Cinatl J Jr, Cinatl J, Vogel J-U, Rabenau H, Kornhuber B, Doerr HW: Modulatory effect of human cytomegalovirus infection on malignant properties of cancer cells. *Intervirology* 1996, 39:259-269
- Cinatl J Jr, Vogel J-U, Cinatl J, Weber B, Rabenau H, Novak M, Kornhuber B, Doerr HW: Long-term productive human cytomegalovirus infection of a human neuroblastoma cell line. *Int J Cancer* 1996, 65:90-96
- Cinatl J Jr, Cinatl J, Vogel J-U, Kotschetkov R, Hernáiz Driever P, Kabickova H, Kornhuber B, Schwabe D, Doerr HW: Persistent human cytomegalovirus infection induces drug resistance and alteration of programmed cell death in human neuroblastoma cells. *Cancer Res* 1998, 58:367-372
- Shen Y, Zhu H, Shenk T: Human cytomegalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation with the adenovirus E1A proteins. *Proc Natl Acad Sci USA* 1997, 94:3341-3345
- Speir E, Modali R, Huang E-S, Leon MB, Shawl F, Finkel T, Epstein SE: Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* 1994, 265:391-394
- Hagemeyer C, Caswell R, Hayhurst G, Sinclair J, Kouzarides T: Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. *EMBO J* 1994, 13:2897-2903
- Jault FM, Jault J-M, Ruchti F, Fortunato EA, Clark C, Corbei J, Richman DD, Spector DH: Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. *J Virol* 1995, 69:6697-6701
- Muralidhar S, Doniger J, Mendelson E, Araujo JC, Kashanchi F, Azumi N, Brady JN, Rosenthal LS: Human cytomegalovirus mtrII oncoprotein binds to p53 and down-regulates p53-activated transcription. *J Virol* 1996, 70:8691-8700
- Fortunato EA, Spector DH: p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus. *J Virol* 1998, 72:2033-2039
- Hanahn D, Folikman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, 86:353-364

13. Beck L Jr, D'Amore P: Vascular development: cellular and molecular regulation. *FASEB J* 1997, 11:365-373
14. Ferning GD, Gallagher JT: Fibroblast growth factors and their receptors: an information network controlling tissue growth, morphogenesis and repair. *Prog Growth Factor Res* 1994, 3:353-377
15. Klagsbrun M, D'Amore PA: Vascular growth factor and its receptors. *Cytokines Growth Factor Rev* 1996, 7:259-270
16. Roberts D: Regulation of tumor growth and metastasis by thrombospondin 1. *FASEB J* 1996, 10:1183-1191
17. Tuszynski GP, Nicosia RF: The role of thrombospondin-1 in tumor progression and angiogenesis. *BioEssays* 1996, 18:71-76
18. Lawler J, Weinstein R, Hynes RO: Cell attachment to thrombospondin: the role of Arg-Gly-Asp, calcium, and integrin reports. *J Cell Biol* 1988, 107:2351-2361
19. Lahav J: Thrombospondin inhibits adhesion of endothelial cells. *Exp Cell Res* 1988, 177:199-204
20. Tarabozetti G, Roberts D, Liotta LA, Giavazzi R: Platelet thrombospondin modulates endothelial cell adhesion, motility and growth: a potential angiogenesis regulatory factor. *J Cell Biol* 1990, 111:765-772
21. Bagavandoss P, Wilks JV: Specific inhibition of endothelial cell proliferation by thrombospondin. *Biochem Biophys Res Commun* 1990, 170:867-872
22. Neng-Hua G, Krutzsch HC, Inman JK, Roberts DD: Thrombospondin 1 and type 1 peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. *Cancer Res* 1997, 57:1735-1742
23. Dameron KM, Volpert OV, Tainsky MA, Bouck N: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994, 265:1582-1584
24. St. Jeor S, Admiraal J, Bruening E, Riolo J: Induction of cytokines by human cytomegalovirus. *Multidisciplinary Approach to Understanding Cytomegalovirus Disease*. Edited by Michelson S, Plotkin SA. Amsterdam, Elsevier Science, 1993, pp 123-126
25. Van Meir EG, Kikuchi T, Tada M, Li H, Diserens A-C, Wojcik BE, Su Huang H-J, Friedmann T, de Tribolet N, Cavanee WK: Analysis of the p53 gene and its expression in human glioblastoma cells. *Cancer Res* 1994, 54:649-652
26. Cinatl J Jr, Cinatl J, Mainke M, Weissflog A, Steigmann G, Rabenau H, Doerr HW, Kornhuber B: Aphidicolin selectively kills neuroblastoma cells in vitro. *Cancer Lett* 1992, 67:199-206
27. Murayama T, Ohara Y, Obuchi M, Khabar KSA, Higashi H, Mukaida N, Matsushima K: Human cytomegalovirus induces interleukin-8 production by a human monocytic cell line, THP-1, through acting concurrently on AP-1- and NF-kappaB-binding sites of the interleukin-8 gene. *J Virol* 1997, 71:5692-5695
28. Azad R, Driver VB, Tanaka K, Crooke RM, Anderson KP: Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region. *Antimicrob Agents Chemother* 1993, 37:1945-1954
29. Anderson KP, Fox MC, Driver VB, Martin MJ, Azad RF: Inhibition of human cytomegalovirus immediate-early gene expression by an antisense oligonucleotide complementary to immediate-early RNA. *Antimicrob Agents Chemother* 1996, 40:2004-2011
30. Bertin N, Clezardin P, Kubiak R, Frappart L: Thrombospondin-1 and -2 messenger in normal, benign, and neoplastic human breast tissue: correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. *Cancer Res* 1997, 57:396-399
31. Bordow SB, Haber M, Madafoglio J, Cheung B, Marshall GM, Norris MD: Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. *Cancer Res* 1994, 54:5036-5040
32. Kari B, Rakeke R, Gehr R: Processing of human cytomegalovirus envelope glycoproteins in and egress of cytomegalovirus from human astrocytoma cells. *J Gen Virol* 1992, 73:253-269
33. Grundy JE: Current antiviral therapy fails to prevent the pro-inflammatory effects of cytomegalovirus infection, whilst rendering infected cells relatively resistant to immune attack. *Monogr Virol* 1998, 21: 67-89
34. Scholz M, Vogel J-U, Blaheta R, Cinatl J Jr: Cytomegalovirus, oxidative stress, and inflammation as interdependent pathomechanisms: need for novel therapeutic strategies? *Monogr Virol* 1998, 21:90-105
35. Alcamí J, Barzu T, Michelson S: Induction of endothelial growth factors by human cytomegalovirus infection of fibroblasts. *J Gen Virol* 1991, 72:2765-2770
36. Nicosia RF, Tuszynski GP: Matrix-bound thrombospondin promotes angiogenesis in vitro. *J Cell Biol* 1994, 124:183-193
37. Good DJ, Polverini PJ, Rastinejad F, Le BM, Lemons RS, Frazier WA, Bouck PJ: A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci USA* 1990, 87:6624-6628
38. Yabkowitz R, Mansfield PJL, Dixit VM, Suchard SJ: Motility of human carcinoma cells in response to thrombospondin: relationship to metastatic potential and thrombospondin structural domains. *Cancer Res* 1993, 53:378-387
39. Weinstat-Saslow DL, Zabrenetsky VS, Van Houtte K, Frazier WA, Roberts DD, Steeg PS: Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential and angiogenesis. *Cancer Res* 1994, 54:6504-6511
40. Salnikow K, Cosentino S, Klein C, Costa M: Loss of thrombospondin transcriptional activity in nickel-transformed cells. *Mol Cell Biol* 1994, 14:851-858
41. Van Meir EG, Polverini PJ, Chazin VR, Huan H-JS, de Tribolet N, Cavanee WK: Release of an inhibitor of angiogenesis upon induction of wild type p53 expression in glioblastoma cells. *Nature Genet* 1994, 8:171-176
42. Lawler J, Duquette M, Ferro P, Copeland NG, Gilbert DJ, Jenkins NA: Characterization of the murine thrombospondin gene. *Genomics* 1991, 11:587-600
43. Shingu T, Borstein P: Overlapping Egr-1 and Sp1 sites function in the regulation of transcription of the mouse thrombospondin I gene. *J Biol Chem* 1994, 269:32551-32557
44. Laherty CD, Gierman TM, Dixit VM: Characterization of the promoter region of the human thrombospondin gene. *J Biol Chem* 1989, 264: 11222-11227
45. Salnikow K, Wang S, Costa M: Induction of activating transcription factor 1 by nickel and its role as a negative regulator of thrombospondin I gene expression. *Cancer Res* 1997, 57:5060-5066
46. Mocarski S Jr: Cytomegaloviruses and their replication. *Fields Virology*. Edited by Fields BN, Knipe DM, Howley PM, Chanosk RM, Melnick JM, Monath TP, Roizman B, Straus SE. Philadelphia, Lippincott-Raven, 1996, pp 2447-2492
47. Boldogh I, Fons MP, Albrecht T: Increased levels of sequence specific DNA-binding proteins in human cytomegalovirus-infected cells. *Biochem Biophys Res Commun* 1993, 197:1505-1510
48. Yurochko AD, Hwang E-S, Rasmussen L, Keay S, Pereira L, Huang, E-S: The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of SP1 and NF-kappaB during infection. *J Virol* 1997, 71:5051-5059
49. Boldogh I, Abubakar SW, Albrecht T: Activation of proto-oncogenes: an immediate early event in human cytomegalovirus. *Science* 1990, 247:561-564
50. Mettouchi A, Cabon F, Montreau N, Vernier P, Mercier G, Blangy D, Tricoire H, Vigier P, Binetruy B: SPARC and thrombospondin genes are repressed by the c-jun oncogene in rat embryo fibroblasts. *EMBO J* 1994, 13:5668-5678
51. Albrecht T, Boldogh I, Fons MP, Valyi-Nagy T: Activation of proto-oncogenes and cell activation signals in the initiation and progression of human cytomegalovirus infection. *Frontiers of Virology*, vol 2. Edited by Becker Y, Darai G, Huang E-S. Berlin, Springer-Verlag, 1993, pp 384-411
52. Rodems SM, Spector DH: Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. *J Virol* 1998, 72:9173-9180
53. Sakai T, Ohtani N, McGee TL, Robbins PD, Dryja TP: Oncogenic germ-line mutations in SP1 and ATF sites in the human retinoblastoma gene. *Nature* 1991, 353:83-86