The 72-kilodalton IE-1 protein of human cytomegalovirus (HCMV) is a potent inducer of connective tissue growth factor (CTGF) in human dermal fibroblasts

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This research was supported by the RG Kozmetsky Foundation and by the National Institute of Health grant AR 44883.

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Clin Exp Rheumatol 2004; 22 (Suppl. 33): S31-S34.

Key words: cytomegalovirus, CTGF, collagen, fibrosis, scleroderma, atherosclerosis.

ABSTRACT

Latent human cytomegalovirus (HCMV) infection has been implicated in diseases characterized by tissue remodeling. Because of recent evidence indicating the possibility of a partial HCMV reactivation, the purpose of this study was to examine the role of the HCMV immediate early (IE) genes in the regulation of extracellular matrix (ECM) related host genes. Adenoviral vector expressing IE1 was generated to allow efficient gene delivery into human fibroblasts. IE1 stimulated the prolonged expression of connective tissue growth factor (CTGF) and TIMP1. IE1-dependent stimulation of CTGF was partially mediated by TGF-β. Moreover, whereas collagenous proteins and collagen type I mRNA were only transiently induced by IE1 in the majority of fibroblasts, in selected fibroblast strains IE1 induced persistent ECM upregulation for up to 120 hours. This study suggests that transient or limited HCMV reactivation may play a direct role in abnormal matrix remodeling in GVHD, scleroderma, atherosclerosis and other HCMV-linked diseases.

Introduction

HCMV is a ubiquitous member of the b-herpesviridae family, with 60-70% of the world's population carrying the virus. After a frequently asymptomatic primary infection, HCMV establishes lifelong persistence in the host in a latent form in a variety of tissues (1). Monocyte/macrophage progenitor cells, as well as endothelial cells, have been identified as primary sites of latent HCMV infection (2-5). Reactivation of the latent virus is often observed in immunocompromised individuals, such as transplant recipients and AIDS patients. Allogeneic stimulation has been proposed as a factor that may induce reactivation of the virus (3). A recent *in vivo* study demonstrated that allogeneic stimulation in the absence of immunosuppression leads only to the selective induction of IE genes without full reactivation of the virus (6). Furthermore, several studies have shown that TNF-α alone can substitute for allogeneic transplantation in inducing IE1 gene expression (6, 7). It has been demonstrated that activation of the cAMP-dependent pathway by different stimuli is capable of inducing IE promoter activity (7, 8). These observations raise the possibility that activation of the proinflammatory cytokines or other cAMP-activating pathways may, in latently infected host cells, lead to partial or limited reactivation of the virus. Hence, even transient expression of the IE genes in these cells may in turn cause an alteration of host cellular gene expression. Such a process may contribute to the pathogenesis of HCMV-associated disorders including atherosclerosis, GVHD and scleroderma in which HCMV has been implicated in the absence of the active viral infection (8-10).

The HCMV major immediate early locus (MIE) gives rise to several viral IE gene products. Two major proteins, 72-kDa IE1 and 86-kDa IE2, originate from this region through differential splicing of the respective mRNAs. Besides playing important roles in controlling later viral gene expression, both proteins regulate the expression of cellular genes. Of the two proteins, IE2 is considered to be the more potent general activator of viral and cellular genes. The ability of HCMV to substantially modulate cellular gene expression was recently demonstrated using DNA array technology (11). Infection of hu-

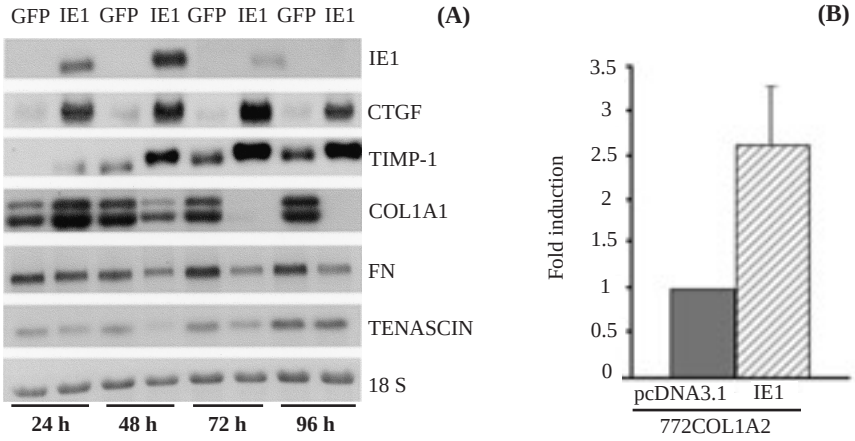

Fig. 1. (**A**) *IE1ad modulates CTGF, TIMP1, COL1A1, fibronectin, and tenascin-C mRNA expression with different kinetics.* (**A**) Confluent fibroblasts were incubated in serum-free medium for 24 hours then transduced with 100 MOI of GFPad or IE1ad. Total RNA was isolated from control GFPad (GFP) and IE1ad (IE1) transduced cells at the indicated time points and analyzed by northern blot. The blot was sequentially hybridized with IE1, CTGF, TIMP1, COL1A1, fibronectin, tenascin-C and 18S RNA probes. The experiment was repeated 3 times, representative northern blot is shown. (**B**) Human dermal fibroblasts were transiently cotransfected with 0.9 μg/ml of –772 COL1A2/CAT promoter construct and 0.1 μg of either empty vector (pcDNA3.1) or IE1 expression vectors. The bar graph depicts the means + SEM from 3 independent experiments of the COL1A2 promoter activities relative to promoter cotransfected with empty vector, which was arbitrarily set at 1.

man fibroblasts with HCMV resulted in an altered expression level of 258 cellular mRNAs within 24 hours after infection and before the onset of viral replication. Among the cellular genes that were downregulated by the HCMV infection were genes coding for several ECM proteins and cell adhesion proteins. The downregulation of genes encoding for various ECM components in response to HCMV infection were also observed in another study (12).

Since HCMV-linked disorders are frequently associated with the inflammatory response and since the partial reactivation of HCMV may occur under these conditions, the goal of this study was to examine the role of IE1 re-expression in the regulation of cellular genes. Our study demonstrates that IE1 alone is a potent inducer of CTGF and several other genes involved in ECM remodeling in dermal fibroblasts. Furthermore, our data suggest that the regulation of cellular genes in response to IE1 differs from the response to the full virus.

Materials and methods

Cell cultures

Human fibroblast cultures were established from skin biopsies obtained from healthy volunteers (upon informed consent and in compliance with the Institutional Review Board for Human Studies) and from newborn foreskins obtained from the delivery suites of local hospitals and propagated as described previously (13).

Northern blot

Total RNA was extracted and analyzed by northern blotting as previously described (14). Filters were sequentially hybridized with probes for IE1 (from Sinclair, University of Cambridge), CTGF (from Grotendorst, University of Miami), TIMP1, COL1A1, COL1-A2, Fibronectin, Tenascin-C and 18S RNA.

Plasmids and transient transfections

The –772 COL1A2 promoter CAT reporter gene construct was previouisly described (15). Transient transfections were performed using FuGene reagent (Roche Molecular Biochemicals) as previously described (14).

Adenoviral constructs

Replication incompetent adenoviral vectors were generated as described previously (16). The vectors constructed for this study express green fluorescent protein (GFPad) driven by a CMV promoter/enhancer, or GFP and IE1 (IE1ad) under the control of two separate CMV promoter/enhancers.

Procollagen gel analysis

Fibroblasts were plated in 12-well plates and processed as described previously (17). Briefly, confluent cells were incubated for 24 h in DMEM containing 0.1% BSA and ascorbic acid (50 μg/ml). Cells were transduced with adenovirus at indicated MOI for the indicated time intervals. Ten μCi/ml of L-[2,3,4,5-^{3}H] proline was added during the last 24 h of incubation. Aliquots of medium normalized for cell number were concentrated, denatured in SDS sample buffer and separated by 6% SDS-PAGE. After electrophoresis, gels were enhanced by fluorography and visualized by autoradiography.

Results

IE1 modulates expression of a subset of genes related to ECM remodeling

For the initial experiments stable transfectants expressing either IE1 or IE2 were generated in dermal fibroblasts. We observed that CTGF mRNA was elevated in IE1 stable transfectants, but not in IE2 stable transfectants (Arthritis Rheum 2001; 44 (Suppl.) abstr. #382). To gain further insight into the effects of IE1 on cellular gene expression, adenoviral transgene delivery was utilized. A recombinant adenoviral vector containing IE1 as well as a separate GFP expression cassette was generated (IE1ad). Fibroblasts transduced with IE1ad expressed high levels of IE1

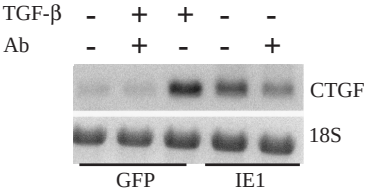

Fig. 2. I*E1 induces CTGF expression in a TGF-β dependent and TGF-β independent manner*. Confluent cells were serum-starved for 24 hours and either treated with 0.5 ng/ml of TGF-β or transduced with IE1ad (100 MOI). Where indicated, 5 μg/ml of pan-specific TGF-β neutralizing antibody (R&D System) were added 20 min before treatment. Total RNA was isolated from the cells at the indicated time points and analyzed by northern blot. The experiment was repeated two times. Representative data are shown.

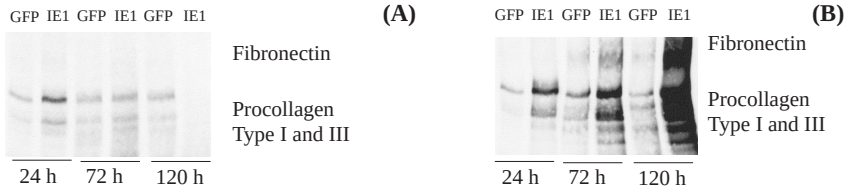

Fig. 3. *IE1 induces prolonged stimulation of ECM proteins in selected cell lines.* Confluent cells were incubated in serum-free medium supplemented with ascorbic acid (50 μg/ml) for 24 hours then transduced with 100 MOI of GFPad or IE1ad. After 3 hours, [^{3}H]-proline was added and the cells were incubated for an additional 24 hours. An aliquot of conditoned medium normalized for cell number was analyzed via SDS-PAGE and fluorography. For the 72 h and 120 h time points, [^{3}H]-proline was added for the final 24-hour incubation. The aliquots of conditoned medium normalized for cell number were analyzed via SDS-PAGE and fluorography.

mRNA after 48 hours; however, expression of IE1 declined after 72 hours, and remained very low for up to 96 hours (Fig. 1A). Expression of CTGF in cells transduced with IE1ad was next examined. A rapid and persistent upregulation of CTGF mRNA for up to 96 hours was observed (Fig. 1A). Likewise, TIMP1expression in response to IE1 increased steadily up to 96 hours post transduction. A modest increase of TIMP1 mRNA in cells transduced with the control adenovirus was also noted (Fig. 1A).

Expression of ECM proteins, including collagen type 1 (COL1A1, COL1A2), fibronectin (FN) and tenascin-C, was also examined. In contrast to CTGF and TIMP1, induction of COL1A1 mRNA levels were transient (at 24 h), followed by decreased levels at 48 h and a further decrease below detectable levels at later time points (72 and 96 hours). COL1A2 showed a similar pattern of expression (data not shown). Fibronectin and tenascin-C mRNA levels were also decreased, albeit to a lesser extent than collagen type I (Fig. 1A). The mRNA levels of metalloproteinase-1 (MMP-1) were not affected under these experimental conditions (data not shown).

To further assess the effects of IE1 on collagen gene expression we utilized the COL1A2 promoter. In agreement with the mRNA data, IE1 induced COL1A2 promoter activity (Fig. 1B)

IE1 induces CTGF via TGF-β dependent and independent pathways

Since TGF-β is known to induce CTGF (18), and induction of TGF-β by HCMV IE2 was previously demonstrated (19), we tested whether CTGF induction by IE1ad is TGF-β dependent. Neutralizing TGF-β antibody was used to inhibit TGF-β signaling. As shown in Figure 2, addition of the antibody almost completely abolished TGF-β stimulation of CTGF, while IE1dependent CTGF stimulation was only partially inhibited. These results suggest that IE1 induces CTGF with a potency similar to that of TGF-β and that IE1 induction of CTGF is partially mediated by TGF-β.

Long-term effects of IE1 on ECM synthesis are cell line-dependent

We next examined the short– (24 h) and long-term (72-120 h) effects of IE1 on the synthesis of collagenous proteins using the ^{3}H-proline incorporation assay followed by SDS-PAGE. As shown in Figure 3, IE1ad consistently induced modest secretion of collagenous proteins 24 hours post-infection. This induction correlated with the induction of collagen type I mRNA and induction of the COL1A2 promoter (Fig. 1A and B) suggesting that IE1 is a positive regulator of collagen type I gene transcription. IE-1-dependent synthesis of collagenous protein declined at the later time points (72-120 h) in a majority of cell lines tested (five independent lines) (Fig. 3A). Interestingly, however, two individual cell lines showed a different response. In these cells ECM production gradually increased with time and was more pronounced after 72 h, reaching the highest levels 120 h post-infection with IEad (Fig. 3B)

Discussion

The present study demonstrates for the first time that the HCMV IE1 gene is a potent inducer of CTGF. CTGF is a pleiotropic growth factor, which has been implicated in several fibrotic diseases (20, 21). Our observations provide a direct link between HCMV infection and elevated CTGF expression, which is observed in many disorders associated with HCMV reactivation, including atherosclerosis, GVHD and scleroderma (22, 23).

Our finding that IE1 is an inducer of CTGF independently of IE2 is of special interest because, unlike IE2, IE1 has been considered a weak transcription activator of cellular genes, with only a few target genes known. In addition, TIMP1 mRNA was elevated, while MMP-1 levels remained unchanged. We also observed transient upregulation of collagen type I promoter, mRNA and protein. The effects of IE1 on host gene expression described herein differ from the previously reported effects of the viral infection. In studies by Zhou *et al*. (11) collagen type I and III were dramatically downregulated within 24 post infection, while either transient or persistent stimulation of collagen genes, was observed in our study. In addition, other IE1-inducible genes, including CTGF and TIMP1, were not reported among the genes modulated by the HCMV infection. That may be due to the longer time periods (72 h) required for the maximal expression of these two genes.

Since expression of IE1 did not affect cell viability, our system permits examination of the long term effects of IE1 on host gene regulation, a situation that may occur *in vivo* during partial HCMV reactivation. This may lead to potentially important observations related to the long-term host-dependent response to IE1 with respect to ECM production. While a majority of the cell lines responded to IE1 with modest and transient increases in ECM synthesis, in selected cell lines the expression of IE1 led to prolonged changes in cellular gene expression. This ability of IE1 to induce a prolonged profibrotic response in fibroblasts seems to depend

on the properties of the individual cell lines, which were obtained from different donors. While the nature of the cellular factors responsible for this persistent profibrotic response is not known, our recent studies show that CTGF upregulates ECM synthesis in fibroblasts in a cell context dependent manner (24). Importantly, recent studies have demonstrated that CMV infection enhances the development of interstitial fibrosis in chronic renal allograft rejection in the rat (25).

In conclusion, this study demonstrates that IE1 induces CTGF and other cellular genes associated with ECM remodeling. Local HCMV reactivation even without active HCMV infection may be directly involved in excessive ECM deposition in diseases characterized by tissue remodeling.

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