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Induction of an Epithelial Integrin αvβ6 in Human Cytomegalovirus-Infected Endothelial Cells Leads to Activation of Transforming Growth Factor-β1 and Increased Collagen Production

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Human cytomegalovirus (CMV) infection is a major cause of morbidity in immunosuppressed individuals, and congenital CMV infection is a leading cause of birth defects in newborns. Infection with pathogenic viral strains alters cell-cell and cell-matrix interactions, affecting extracellular matrix remodeling and endothelial cell migration. The multifunctional cytokine transforming growth factor (TGF)-β1 regulates cell proliferation, differentiation, and extracellular matrix remodeling. Secreted as a latent protein complex, TGF-β1 requires activation before binding to receptors that phosphorylate intracellular effectors. TGF-β1 is activated by integrin αvβ6, which is strongly induced in the epithelium by injury and inflammation but has not previously been found in endothelial cells. Here, we report that CMV infection induces integrin αvβ6 expression in endothelial cells, leading to activation of TGF-β1, signaling through its receptor ALK5, and phosphorylation of its intracellular effector Smad3. Infection of endothelial cells was also found to stimulate collagen synthesis through a mechanism dependent on both TGF-β1 and integrin αvβ6. Immunohistochemical analysis showed integrin αvβ6 up-regulation in capillaries proximal to foci of CMV infection in lungs, salivary glands, uterine decidua, and injured chorionic villi of the placenta, demonstrating both its induction in endothelium and up-regulation in epithelium *in vivo*. Our results suggest that activation of TGF-β1 by integrin αvβ6 contributes to pathological changes and may impair endothelial cell functions in tissues that are chronically infected with CMV. ***(Am J Pathol 2008, 172:1127–1140; DOI: 10.2353/ajpath.2008.070448)***

Human cytomegalovirus (CMV) is a ubiquitous pathogen that infects more than half the population of the United States. On primary infection, latency is established in granulocyte-macrophage progenitors.[1,2] High levels of $CD8^+$ antigen-specific cytotoxic T lymphocytes reactive with CMV peptides indicate that reactivated infections require sustained immunosurveillance.[3,4] CMV replicates in epithelial and endothelial cells in salivary glands, lungs, kidney, uterus, and placenta.[5–10] Because cytopathic alterations, characteristic of a late stage of infection, were found in these cells, they are likely to reflect biologically relevant mechanisms operative during *in vivo* infection. Clinical studies indicate that chronic subclinical CMV infection and immune rejection in organ transplant recipients are risk factors for graft failure.[11–14]

Transforming growth factor-β1 (TGF-β1), a multifunctional cytokine, plays a central role in cell proliferation, migration, and synthesis of extracellular matrix (ECM) in the endothelium.[15] In most cell types, TGF-β1 signals through the type I receptor activin receptor-like kinase 5 (ALK5). In addition to expressing ALK5, endothelial cells express a second TGF-β1 receptor, the type I receptor ALK1. When activated, ALK1 induces phosphorylation of

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T.T. and H.K. contributed equally to this study.

We dedicate this article to Dr. Hisaaki Kawakatsu who died during this study.

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the nuclear effectors Smad1 and Smad5, which promote endothelial cell proliferation and migration.[16] In contrast, activated ALK5 induces Smad2 and Smad3 phosphorylation, leading to the inhibition of endothelial cell proliferation. TGF-β1 is secreted as an inactive, noncovalent complex with latency-associated peptide and requires activation before it can bind to its receptors. Reported mechanisms of TGF-β1 activation include cleavage by metalloproteinases or plasmin and binding to thrombospondin 1 or either of the integrins αvβ6 and αvβ8.[15,17–21] One of the *in vivo* activators of TGF-β1 is integrin αvβ6.[17,18] This activation model is particularly interesting because integrin αvβ6 is expressed principally on epithelial cells, which are very sensitive to TGF-β1-mediated growth inhibition. Integrin αvβ6 is strongly up-regulated at sites of epithelial repair and inflammation in lung and kidney,[22] and also because of the overlap of the phenotypes of TGF-β1- and integrin β6 subunit-deficient mice. Mice lacking the β6 subunit show increased inflammation and decreased fibrosis, both of which processes are strongly regulated by TGF-β1.[18,23,24]

Recent work has provided evidence for the induction of TGF-β1 in a variety of cells and tissues on CMV infection. TGF-β1 was released in increasing amounts from splenocytes infected with rat CMV *in vitro*.[25] TGF-β1 protein was increased in alveoli and stromal cells in rat lungs, spleen, and liver after radiation-induced immune suppression of CMV-infected rats.[25] Furthermore, CMV-infected murine astrocytes increased TGF-β1 transcription and protein levels.[26] In human kidney allografts, CMV proteins and DNA were associated with locally increased TGF-β1 in tubuli and arterial endothelium long after viral clearance from the blood.[27] Brain biopsy specimens from AIDS patients with CMV encephalitis were found to contain viral inclusions that co-localized with TGF-β1 protein in cells with astrocyte-specific glial filaments.[26] In addition, TGF-β1 induction in human fibroblasts has been shown to involve the transactivation of its promoter by immediate-early 2 protein through an Egr-1 consensus site by binding the zinc finger domain of Egr-1.[28,29] Although the evidence suggests that TGF-β1 may be directly involved in CMV pathogenesis, little is known about the cellular proteins involved in virus-mediated TGF-β1 activation, or what specific functional role it plays *in vivo*. In recent experiments, we found that a subpopulation of freshly isolated human cytotrophoblasts from term placentas expressed integrin αvβ6, which activates TGF-β1 *in vitro*.[30] We hypothesized that CMV infection could increase not only the production of latent TGF-β1 but also its activation, causing TGF-β1-mediated cellular responses.

Here we report that CMV-infected endothelial cells from pulmonary, uterine, and placental blood vessels activate TGF-β1 through the induction of the epithelial integrin αvβ6, promoting signaling through ALK5 and Smad3. This signaling pathway plays a fundamental role in mediating profibrotic responses at later times after infection. In our studies, immunohistochemical analysis of CMV-infected tissues showed integrin αvβ6 expression in both epithelial and endothelial cells proximal to infected foci and sites of injury. These results suggest that integrin αvβ6-mediated TGF-β1 activation could be relevant to the development of fibrosis in persistent infection.

Materials and Methods

Cell Culture, Virus, and Infection

Human microvascular endothelial cells-lung (HMVEC-L) and uterine (UtMVECs) from two donors and human umbilical vein endothelial cells (HUVECs) from two donors were maintained in EBM-2 medium supplemented with EGM-2-MV and EGM-2 Singlequots (Cambrex Bio-Science Walkersville, Inc., Walkersville, MD). Endothelial cells from different sources, 98% positive by immunofluorescence staining with a rabbit antiserum to von Willebrand factor complex (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), were used between passages 4 and 6. Cells were infected with the pathogenic clinical CMV strain VR1814[31] at 1 PFU/cell and cultured up to 15 days.

Serological and Other Reagents

The following antibodies to integrins were purified from hybridoma supernatants: mouse anti-human β5 mAb ALULA[32]; mouse anti-human αv mAb L 230,[33] mouse anti-αvβ6 CSβ6,[34] and rabbit anti-αvβ6 mAb 4B5.[35] Mouse anti-human α5 mAb P3D10 and mouse anti-human β1 monoclonal antibody (mAb) P5D2[36] were generous gifts of Dr. Elizabeth Wayner (Fred Hutchinson Cancer Center, Seattle, WA). Mouse anti-αvβ6 mAbs 3G9 and 2A1 were gifts from Drs. Shelia Violette and Paul Weinreb (Biogen Idec Inc., Cambridge, MA),[37] and mouse anti-human αvβ8 mAb 14E5 was a gift from Dr. Stephen Nishimura.[19] The following antibodies were purchased: mouse anti-human β3 mAb (clone VI-PL2; BD Biosciences, San Diego, CA), mouse anti-TGF-β1, -β2, and -β3 mAb (clone 1D11), chicken anti-human-TGF-β polyclonal antibody, anti-human ALK1 goat polyclonal antibody and mouse anti-human endoglin (CD105) mAb (R&D Systems, Minneapolis, MN); rabbit polyclonal antiserum to von Willebrand factor complex (Novocastra Laboratories; and DakoCytomation, Carpinteria, CA); mouse anti-Smad2/3 mAb, and mouse anti-human Grb2 mAb (BD Biosciences); rabbit anti-TGF-β mAb (clone 56E4), rabbit polyclonal anti-Smad1, anti-Smad5, anti-pSmad1/5/8, which specifically recognizes phosphorylated Smad1 (Ser463/465), Smad5 (Ser163/465), and Smad8 (Ser426/428), rabbit anti-pSmad3 mAb, which specifically recognizes phosphorylated Smad3 (Ser423/425) (clone C25A9) (Cell Signaling Technology, Beverly, MA); anti-TGF-β receptor I (TGF-β RI, H-100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-human type IV collagen goat polyclonal antibody (Southern Biotechnology, Birmingham, AL), mouse monoclonal anti-human CMV-infected-cell protein p76 and DNA binding protein UL44 (clone DDG9 and CCH2) (DakoCytomation), and mouse anti-actin mAb (Sigma-Aldrich, St. Louis, MO). Mouse mAbs to CMV glycoprotein B (gB) (CH112-2, UL55) and immediate-early (IE1 and IE2) nuclear proteins (CH160,

UL122 and UL123), produced in the Pereira laboratory, were used as described.[10,30] CH112-2 was conjugated with fluorescein isothiocyanate (eBioscience, San Diego, CA). TGF-β receptor I ALK5 kinase inhibitor SB431542 was purchased from Tocris Bioscience (Ellisville, MO).

Flow Cytometry

Cells were harvested using cell dissociation buffer (Invitrogen, Carlsbad, CA). For analysis of surface antigen expression, cells were blocked with normal goat or donkey serum, washed with phosphate-buffered saline (PBS), incubated with primary antibodies or isotype-matched control IgG for 1 hour at 4°C, and detected with phycoerythrin-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained cells were analyzed by FACScan and CellQuest software (BD Biosciences). Propidium iodide was used to gate out contributions from dead cells. Intracellular expression was analyzed after fixation with 4% formaldehyde for 10 minutes at room temperature and permeabilization with 0.01% Triton X-100 for 5 minutes at 4°C.

Immunoblot Analysis

Cells were lysed in buffer [50 mmol/L Tris-HCl, pH 7.6, 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 20 mmol/L NaF, 1 mmol/L Na_3VO_4, and protease inhibitor cocktail (Sigma-Aldrich)] and then clarified by centrifugation. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and blocked for 1 hour in PBS containing 5% skim milk and 0.05% Tween 20. After incubation with primary antibody for 16 hours at 4°C, and then with peroxidase-conjugated secondary antibody for 1 hour, blots were developed with the ECL Western blotting detection system (GE Health Care, Piscataway, NJ).

TGF-β Reporter Cell Assay

Mink lung epithelial cells (TMLCs), which are TGF-β reporter cells stably expressing a truncated portion of the plasminogen activator inhibitor-1 promoter fused to the luciferase gene (a gift from Daniel Rifkin, New York University, New York, NY), were used to quantify TGF-β activation.[38] Exposure to active TGF-β, either produced by activating cell types or present in cell-free supernatants to TMLCs, resulted in a dose-dependent increase in luciferase activity in the cell lysates.[18,19,38] Briefly, TMLCs were co-cultured with uninfected control HUVECs and VR1814-infected HUVECs (5×10^4 cells) at 3, 7, or 10 days for 16 to 24 hours; cell lysates were then analyzed for luciferase activity.[18,38] Cells infected for 10 days were co-cultured with TMLCs with or without anti-pan-TGF-β blocking antibody (1D11, mouse IgG1) or anti-αvβ6 function-blocking antibody (3G9, mouse IgG1). As negative controls, mouse IgG1 isotype control antibody and isotype-matched non-function-blocking anti-αvβ6 antibody (CSβ6, mouse IgG1) were used in the same concentrations as the neutralizing antibodies. All assays were performed in duplicate.

Quantification of TGF-β1 by Enzyme-Linked Immunosorbent Assay

Levels of secreted TGF-β1 were measured by determining its concentration in conditioned medium using a commercial sandwich enzyme-linked immunosorbent assay (Quantikine TGF-β1 immunoassay; R&D Systems). Cells were grown in six-well plates; conditioned medium was harvested on day 1 and then at 2-day intervals thereafter, cleared by centrifugation, and stored at −80°C. Conditioned medium was acid-activated and directly assayed by visualization with tetramethylbenzidine on an enzyme-linked immunosorbent assay plate reader at 450 nm according to the manufacturer's instructions. Protein concentrations were calculated from a standard curve with twofold serial dilutions and a high standard of 2000 pg/ml.

Immunohistochemical Analysis

Lung and Salivary Gland Specimens

Samples from 11 lungs and 1 salivary gland were obtained from 12 patients with CMV infection with histological evidence of nuclear inclusion bodies. These were obtained from families at autopsy with informed consent from the Tokyo Women's Medical University, Tokyo, Japan. Tissue sections were stained with hematoxylin and eosin and immunostained using the avidin-biotin complex method on formalin-fixed and paraffin-embedded tissues. Tissue sections were deparaffinized, and antigen retrieval was performed by incubating slides in a pepsin solution at 37°C or by microwave treatment in a 10 mmol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation in a solution with 3% hydrogen peroxide for 10 minutes. Nonspecific binding was blocked with PBS containing 5% normal donkey serum (1 hour) before incubation with a primary antibody overnight (4°C). Biotinylated anti-rabbit or anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) was applied to the slides, which were subsequently treated using a Vectastain ABC kit (Vector Laboratories). Slides were visualized with a solution containing 0.01% (w/v) 3,3′-diaminobenzidine tetrahydrochloride, 0.02% (v/v) hydrogen peroxide, and 50 mmol/L Tris-HCl (pH 7.6) counterstained with hematoxylin, dehydrated, cleared, and mounted.

Decidual and Placental Biopsy Specimens

Approval for the use of human patients was obtained from the institutional review board of the University of California, San Francisco, San Francisco, CA. Detection of CMV replication and virion structural proteins in the biopsy specimens was reported.[10,39] Tissue sections were processed for immunohistochemistry as described.[9] Briefly, tissues were fixed in 3% paraformaldehyde, infiltrated with 5 to 15% sucrose followed by embedding in optimal-cutting-temperature compound, and frozen in liquid nitrogen. For double staining, tissue sec-

tions were simultaneously incubated with primary antibodies from different species and secondary antibodies labeled with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate. Nuclei were counterstained with TO-PRO-3 iodide (Invitrogen).

Statistical Analysis

Data are expressed as mean ± SE. Student's *t*-test was used to analyze the difference in expression between control and CMV-infected cells. A P value of <0.05 was considered significant.

Results

CMV-Infected HUVECs Express αvβ6

Previous investigators reported that human fibroblasts infected with a laboratory CMV strain expressed TGF-β1 transcripts and protein, but they did not examine activation of the latent protein.[28,29] The propeptide of TGF-β1, latency-associated peptide-β1, contains an Arg-Gly-Asp (RGD) motif that is recognized by a subset of integrins having in common the integrin αv subunit[18,19,40–42] and α5β1.[43] Furthermore, the integrins αvβ6 and αvβ8 have been shown to activate TGF-β1 *in vivo*.[18,19] To examine whether CMV infection alters the expression level of αv integrin β subunit partners and integrin α5, we infected HUVECs with VR1814, a pathogenic clinical CMV strain, and quantified the surface expression of integrins β1, β3, β5, β6, β8, and α5 by flow cytometry at 10 days after infection. Level of infectivity was evaluated by immunofluorescence staining and flow cytometric analysis of CMV gB expression at the cell surface. The results showed nuclear immunofluorescence of CMV IE1 and IE2 proteins and cytoplasmic gB staining in >90% of infected cells. Flow cytometry detected surface expression of gB in 60.8 ± 6.3% of infected cells. In control uninfected HUVECs, integrin subunits β1, β3, β5, and α5 were expressed abundantly, but there was no expression of β6 and only minimal expression of integrin β8 (Figure 1A). Integrin β6, whose expression is considered restricted to epithelial cells, was strongly induced in CMV-infected HUVECs, whereas levels of integrins β1, β3, β5, β8, and α5, as well as αv (data not shown), were unchanged. An analysis of the kinetics of integrin β6 induction in infected HUVECs showed that the protein was increasingly detected from 5 to 10 days after infection (Figure 1B). Expression of integrin β6 was confirmed at 10 days by immunoblot analysis (Figure 1C). These data suggested that integrin αvβ6, aberrantly expressed in infected HUVECs, participates in TGF-β1 activation. Our subsequent investigations focused on assessing integrin αvβ6 function in infected HUVECs.

CMV Induces Integrin β6-Dependent TGF-β1 Activation

In the next series of experiments, we assessed whether integrin αvβ6 in HUVECs induced by CMV activates TGF-

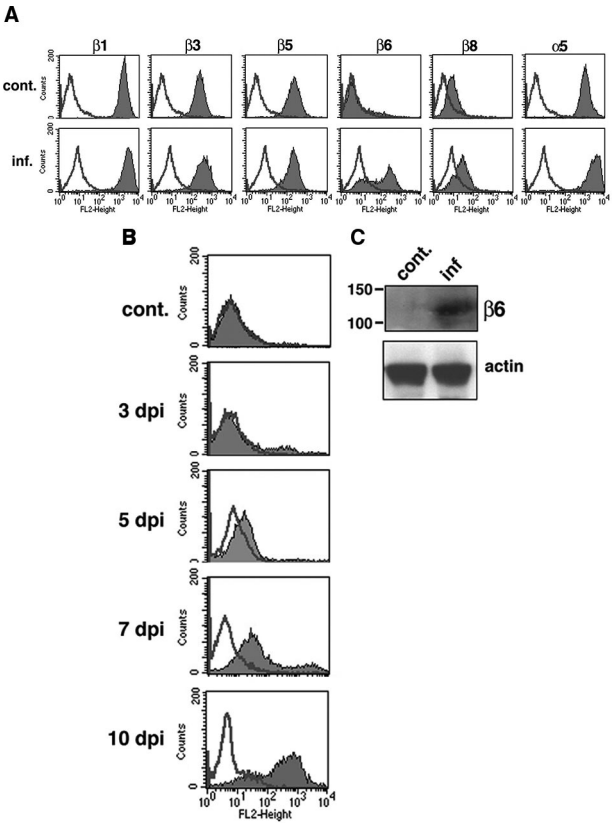

Figure 1. CMV strain VR1814-infected HUVECs induce integrin αvβ6 expression at late times. **A:** Flow cytometric analysis of integrin subunits β1, β3, β5, β6, β8, and α5 in HUVECs at 10 days after infection. Experiments were repeated at least five times. Typical histograms from control (cont.) and infected (inf.) HUVECs are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. **B:** Flow cytometric analysis of integrin αvβ6 in HUVECs at 3, 5, 7, and 10 days after infection (dpi) and control (cont). Typical histograms are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. Experiments were repeated at least four times. **C:** Cell lysates (100 μg) from control or infected HUVECs at 10 days after infection were immunoblotted with an anti-integrin αvβ6 (2A1) and anti-actin antibodies as a loading control. Molecular mass (kDa) is shown on the **left**.

β1. First, we quantified the level of TGF-β1 released into the medium from CMV-infected HUVECs and uninfected control cells. After day 1, conditioned medium from infected and control cells was collected on alternate days and frozen. To quantify TGF-β1 by enzyme-linked immunosorbent assay, conditioned medium was acid-treated to convert the latent TGF-β1 to the immunoreactive form. Increasing amounts of TGF-β1 were secreted from HUVECs as early as 3 days after infection (Figure 2A). Significantly more TGF-β1 was released from cells at 5 to 7 days after infection. In contrast, control cells did not show any increase in the amounts of soluble TGF-β1 in a comparable culture period (Figure 2A).

Because secretion of TGF-β1 is increased by infection, we then asked how much surface (ie, bound) and total cellular TGF-β1 was present by flow cytometry. Surface expression of TGF-β1 on infected cells was increased at 7 to 10 days after infection, whereas no change was observed in uninfected control cells (Figure 2B). Expression of total TGF-β1 in infected cells was significantly increased at 10 days after infection (Figure 2C).

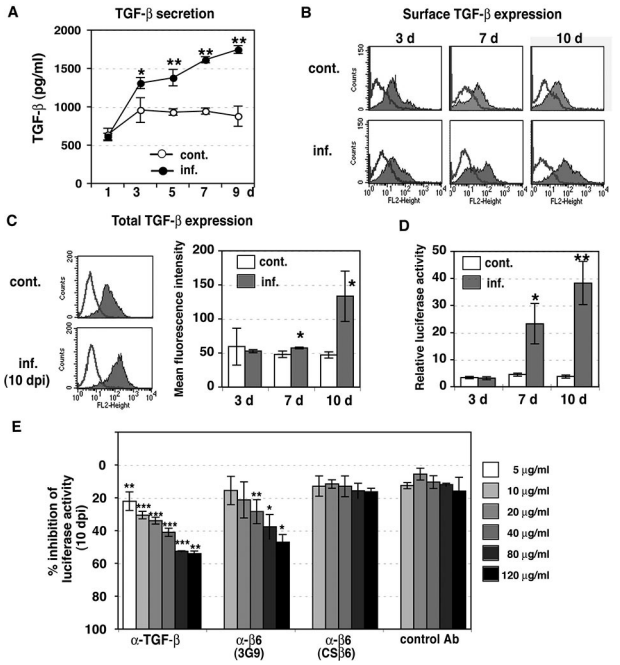

Figure 2. Integrin αvβ6-dependent TGF-β1 activation in CMV-infected HUVECs. **A:** TGF-β1 production by infected HUVECs. Conditioned medium was collected from control (**open circles**) and infected (**filled circles**) HUVECs at 1 to 9 days, and TGF-β1 was quantified by enzyme-linked immunosorbent assay. Results are the mean (±SE) of three experiments done in duplicate. **Asterisks** indicate the amount of TGF-β1 in infected HUVECs as compared with uninfected controls (*$P < 0.05$, **$P < 0.01$). **B:** Surface expression of TGF-β1 in HUVECs was analyzed by flow cytometry at 3, 7, and 10 days after infection and controls (cont). Typical histograms are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. Experiments were repeated at least three times. **C:** Total TGF-β1 was analyzed by flow cytometry using permeabilized cells at 3, 7, and 10 days after infection (inf.) and controls (cont). **Left:** Typical histograms at 10 days are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. **Right:** Results are the mean fluorescence intensity (±SE) of three experiments. **Asterisks** indicate expression in infected HUVECs as compared with uninfected controls (*$P < 0.05$). **D:** TGF-β bioassay of active TGF-β produced by infected HUVECs. Equal numbers of TMLC TGF-β reporter cells, and control (cont.) or infected HUVECs (inf.) were cultured for 16 to 24 hours at 3, 7, and 10 days after infection. Relative luciferase activity in cell lysates was defined as the measured activity divided by TMLC baseline activity. Results are the mean (±SE) from 6 to 11 experiments done in duplicate. **Asterisks** indicate the TGF-β1 activity in infected HUVECs as compared with uninfected controls (*$P < 0.05$, **$P < 0.001$). **E:** Inhibition of luciferase activity in TGF-β bioassay by anti-integrin αvβ6. HUVECs infected for 10 days were co-cultured with TMLCs with anti-TGF-β neutralizing antibody (1D11); function-blocking anti-αvβ6 antibody (3G9); isotype-matched, non-function-blocking anti-αvβ6 antibody (CSβ6); or mouse IgG1 isotype control antibody (control Ab). Results are the mean (±SE) from three to five experiments done in duplicate. **Asterisks** indicate inhibition of TGF-β1 activation relative to untreated infected HUVECs (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

To determine whether CMV activates TGF-β1, we co-cultured HUVECs with TMLCs. At 3, 7, and 10 days after infection, control HUVECs or infected cells were trypsinized and then co-cultured with TMLCs for 16 to 24 hours before measurement of luciferase activity in cell lysates. We found a dramatic increase in luciferase activity, indicating TGF-β1 activation, in 7- to 10-day-infected HUVECs co-cultured with TMLCs (Figure 2D). Little luciferase activity was observed in control HUVECs co-cultured with TMLCs (Figure 2D). We then tested whether the increased luciferase activity is dependent on TGF-β1 or integrin αvβ6. HUVECs infected for 10 days were co-cultured with TMLCs, with or without function-blocking antibodies against either TGF-β (1D11) or αvβ6 (3G9). Negative controls included isotype-matched, non-function-blocking antibodies with either unrelated specificity or non-function-blocking specificity against αvβ6 (CSβ6). The increase in luciferase activity was partly abrogated by function-blocking anti-TGF-β (1D11) and anti-β6 (3G9) but not by control antibodies (CSβ6 or isotype control) (Figure 2E), indicating that TGF-β1 activation after CMV infection is at least integrin αvβ6-dependent. Although the inhibition of luciferase activity by neutralizing antibodies was dose-dependent, even very high concentrations of anti-TGF-β were able to reduce luciferase activity by only ~50% compared with untreated cells, suggesting that CMV may also activate the plasminogen activator-1 promoter through a mechanism not dependent on TGF-β1.

CMV-Infected HUVECs Undergo ALK5/Smad3 Signaling

Activated TGF-β1 can bind the type I receptors ALK1 and ALK5, which then phosphorylate the transcriptional activators Smad1/5 and Smad2/3, respectively.[44] To determine which of these TGF-β1 signaling pathways is activated in CMV-infected HUVECs, we analyzed Smad1/5 and Smad3 phosphorylation by immunoblotting with antibodies specific to Smads and their phosphorylated forms (Figure 3A). Smad3 phosphorylation was strongly detected in 7- and 10-day-infected cells. In contrast, only weak staining for phosphorylated Smad1/5 was observed, and this level either did not change or was decreased at 10 days after infection. Phosphorylated Smad1/5 was also weakly detected in the control. Protein levels of Smad1, Smad5, and Smad2/3 were the same in both infected and control cells.

To determine the relative contributions of TGF-β1 and αvβ6 to the observed ALK5 and Smad3 signaling, we performed function-blocking experiments using anti-

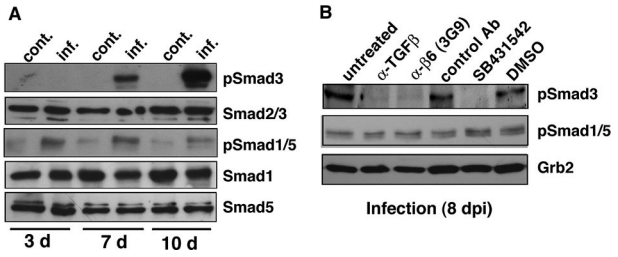

Figure 3. A: CMV-infected HUVECs induce Smad3 phosphorylation. Cell lysates from control (cont.) or infected (inf.) HUVECs at 3, 7, and 10 days after infection were fractionated by 10% SDS-PAGE and blotted on nitrocellulose. Phosphorylation of Smad3 (pSmad3) and Smad1/5 (pSmad1/5) was analyzed by immunoblotting using phospho-specific Smad3 and Smad1/5/8 (pSmad1/5) antibodies. Equal loading of the gels was confirmed using Smad2/3, Smad1, and Smad5 protein levels. **B:** Effects of anti-TGF-β antibody, anti-αvβ6 antibody (3G9), and ALK5 kinase inhibitor on Smad3 phosphorylation. Infected HUVECs were cultured without antibody (untreated) or with anti-TGF-β neutralizing antibody (1D11, 40 μg/ml), function-blocking anti-αvβ6 antibody (3G9, 80 μg/ml), mouse IgG1 isotype control antibody (control Ab, 80 μg/ml), the ALK5 kinase inhibitor SB431542 (2.5 μmol/L), or the vehicle DMSO for 8 days. Lysates were fractionated by 10% SDS-PAGE and blotted. Filters were incubated with antibodies to phosphorylated Smad3 (pSmad3), phosphorylated Smad1/5/8 (pSmad1/5), and Grb2 (loading control). Results are representative of at least four independent experiments.

TGF-β (1D11) and anti-$\alpha v\beta 6$ (3G9) antibodies in 8-day-infected HUVECs. Both neutralizing antibodies blocked Smad3 phosphorylation, whereas the isotype control antibody had little effect (Figure 3B). Treatment of infected cells with the ALK5 kinase inhibitor SB431542 also prevented Smad3 phosphorylation (Figure 3B). Phosphorylation of Smad1/5 was not blocked by treatment with these neutralizing antibodies, suggesting that the activation of Smad1/5 depends on a separate pathway. Together the results of these experiments show that CMV-infected HUVECs release increasing amounts of TGF-β1 and activate TGF-β1 through an integrin $\alpha v\beta 6$-mediated mechanism that stimulates ALK5 signaling and downstream Smad3 phosphorylation.

Induction of Integrin β6 Requires TGF-β/ALK5 Signaling and Viral DNA Replication

We next assessed how integrin β6 was induced on CMV infection in HUVECs. It has been reported that TGF-β1 induces *de novo* synthesis of integrin β6 in normal human keratinocytes[45] and strongly up-regulates its expression in primary cultures of human airway epithelial cells.[46] Having found increased secretion of TGF-β1 in infected cells as early as 3 days after infection (Figure 2A), we then investigated the effect of TGF-β1 on induction of integrin β6. As expected, expression of integrin β6 was greatly reduced (by ~70%) by treatment with the anti-TGF-β neutralizing antibody (Figure 4A). In addition, the ALK5 kinase inhibitor SB431542 (0.1 μmol/L to 1 μmol/L) was able to increasingly block the induction of integrin β6 with increasing inhibitor concentrations and nearly abolish it at high concentrations, whereas the control solution, containing the same concentration of the solvent dimethyl sulfoxide had no effect (Figure 4A). Next, we investigated whether soluble factors participate in the induction of integrin β6. After day 1, conditioned medium from infected cells was collected on alternate days and frozen. HUVECs were cultured with the filtered conditioned medium for 8 days, and expression of integrin β6 was analyzed. No integrin β6 expression was observed in cells cultured with conditioned medium from any time point (data not shown), even though the secretion of TGF-β1, which could be mostly present in an inactive form, from infected cells increased throughout time. We then asked whether viral late gene expression is required for the up-regulation of integrin β6 in infected cells because the expression was observed only at late times after infection. HUVECs were infected and cultured in the presence of the viral polymerase inhibitors Foscarnet (400 μmol/L) or phosphonoacetic acid (100 μg/ml). Both viral polymerase inhibitors blocked induction of integrin β6 (Figure 4B) and strongly suppressed induction of TMLC luciferase activity (Figure 4C). The remaining luciferase activity was further reduced by the addition of an anti-TGF-β antibody, but not by an anti-integrin β6 neutralizing antibody (3G9) (data not shown), indicating that increased luciferase activity was not attributable to integrin $\alpha v\beta 6$-mediated TGF-β1 activation. Together, these results indicate that TGF-β1/ALK5 signaling and viral DNA replication are important factors for the induction of integrin β6 in HUVECs.

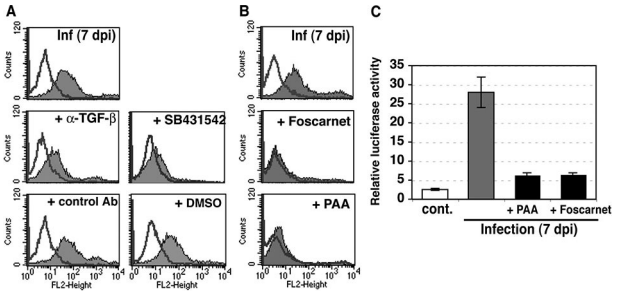

Figure 4. Induction of integrin $\alpha v\beta 6$ expression requires TGF-β signaling and viral DNA replication **A:** Infected HUVECs were cultured with or without chicken anti-TGF-β polyclonal antibody (20 μg/ml), chicken IgY isotype control antibody (control Ab, 20 μg/ml), the ALK5 kinase inhibitor SB431542 (0.5 μmol/L), or the vehicle DMSO for 7 days, and surface expression of integrin $\alpha v\beta 6$ was analyzed by flow cytometric analysis. Typical histograms are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. Experiments were repeated at least two times. **B:** Surface expression of integrin $\alpha v\beta 6$ was analyzed by flow cytometric analysis at 7 days after infection with or without viral DNA polymerase inhibitors, Foscarnet, and phosphonoacetic acid (PAA). Typical histograms are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. Experiments were repeated six times. **C:** Active TGF-β was not produced by infected HUVECs in the presence of viral DNA polymerase inhibitors. Equal numbers of TMLC TGF-β reporter cells and control (cont.) or infected HUVECs were cultured for 16 to 24 hours at 7 days after infection. Relative luciferase activity in cell lysates was defined as the measured activity divided by TMLC baseline activity. Representative data (mean ± SE) are from four experiments done in triplicate.

CMV-Infected HUVECs Dysregulate ALK1 and ALK5 Protein Levels

Endothelial cells express ALK1, which stimulates Smad1/5 phosphorylation during angiogenesis and counterbalances TGF-β1/ALK5 signaling.[47,48] The ALK1 signaling pathway involves an accessory receptor, endoglin, which is highly expressed in endothelial cells, and indirectly inhibits TGF-β1/ALK5 signaling. Preferential phosphorylation of Smad3 in CMV-infected HUVECs suggested that the ratio of ALK1 and ALK5 receptors on the cell surface might be altered. By flow cytometry, we found that uninfected HUVECs expressed ALK1, endoglin, and ALK5 (Figure 5A). Intensities of both ALK1 and ALK5 changed appreciably in infected HUVECs at late time points, with a significant decrease in ALK1 and endoglin expression and a significant increase in ALK5 expression as compared with uninfected cells (Figure 5A, Table 1). Immunoblot analysis revealed the same pattern of changes in expression levels (Figure 5B). Interestingly, the shift in receptor expression occurred even when cells were treated with anti-integrin $\alpha v\beta 6$, anti-TGF-β neutralizing antibody, or the ALK5 kinase inhibitor (data not shown), indicating that this change was independent of $\alpha v\beta 6$-mediated TGF-β1 activation. Then we investigated the possibility that soluble factors mediate the observed changes in the expression of ALK1, endoglin, and ALK5. After day 1, conditioned medium from infected cells was collected on alternate days and frozen. HUVECs were cultured with the filtered conditioned medium for 8 days, and the surface expression

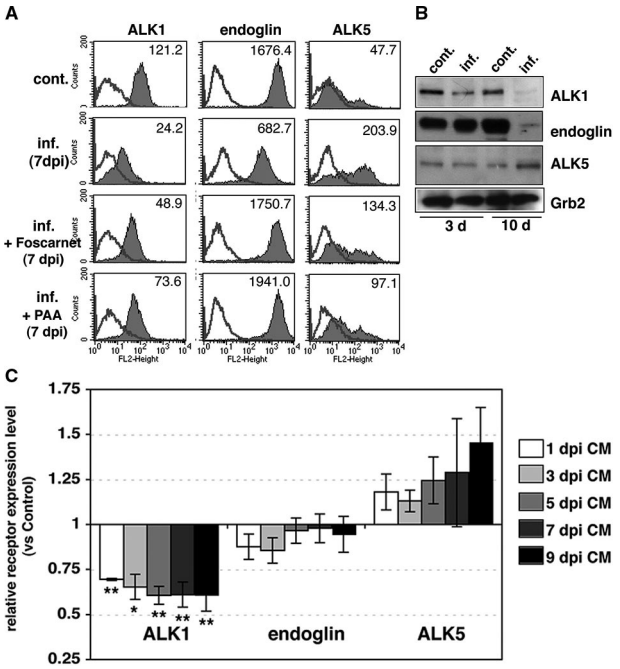

Figure 5. CMV-infected HUVECs increase expression of ALK5 and reduce ALK1. **A:** Surface expression of ALK1, endoglin, and ALK5 was analyzed by flow cytometric analysis at 7 days after infection in the absence or presence of viral DNA polymerase inhibitors. Typical histograms from control (cont.) and infected (inf.) HUVECs are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. Numbers represent mean fluorescence intensity. The experiments were repeated at least four times. **B:** Cell lysates from control (cont.) or infected (inf.) HUVECs at 3 and 10 days after infection were fractionated by 10% SDS-PAGE and blotted on nitrocellulose. Filters were incubated with antibodies to ALK1, endoglin, ALK5, and Grb2 (loading control). **C:** Surface expression of ALK1, endoglin, and ALK5 was analyzed by flow cytometric analysis at 7 days of culture with conditioned medium (CM) from infected HUVECs. Relative surface expression as expressed by mean fluorescence intensity was normalized for control HUVECs in the same experiment. Results are the mean (±SE) from three to seven experiments. **Asterisks** indicate relative expression level of receptors in HUVECs cultured with conditioned medium as compared with controls ($^*P < 0.05$, $^{**}P < 0.01$).

of the receptors was analyzed by flow cytometry. Expression of ALK1 was decreased in cells cultured with conditioned medium from all time points. Expression of ALK5 increased in cells cultured with the conditioned medium from 5, 7, and 9 days after infection. Expression of endoglin was not much affected by conditioned medium from any time point (Figure 5C).

Finally, viral polymerase inhibitors were able to partially block the change in ALK1 and ALK5 expression (Figure 5A), suggesting that a part of those changes may be mediated through immediate-early or early genes. In contrast, expression of endoglin was not changed by infection while in the presence of a viral DNA polymerase inhibitor (Figure 5A), indicating that viral replication is required for change in endoglin expression. Together, these results indicate that there are both direct effects of viral infection on receptor expression and indirect effects that depend on secreted molecules. Our results confirmed that CMV-infected HUVECs reduce ALK1 and endoglin expression, whereas they increase ALK5 expression. Increased availability of ALK5 for TGF-β1 binding, in conjunction with reduced levels of ALK1 and endoglin in infected HUVECs, could explain preferential Smad3 phosphorylation and possible downstream signaling events.

Integrin αvβ6-Mediated TGF-β Activation Increases ECM Production in CMV-Infected Cell Cultures

TGF-β1 is a potent fibrotic factor responsible for the synthesis of ECM, and profibrotic TGF-β1 responses are induced primarily via ALK5/Smad3 signal transduction in normal fibroblasts.[49] TGF-β1 also potently promotes the synthesis and deposition of ECM in endothelial cells.[50] In microarray analysis, HUVECs infected with recombinant adenovirus carrying a constitutively active form of ALK5 up-regulate ECM genes, whereas ALK1 either does not exhibit a significant effect or causes down-regulation of these genes.[51] Therefore, we investigated whether CMV-activated TGF-β1 could increase ECM production and whether blocking TGF-β1 activation could prevent the effect. Surface expression of type IV collagen, analyzed by flow cytometry, was significantly increased in infected HUVECs at late time points (Figure 6, A and B). Immunoblot analysis also showed an increased production of type IV collagen in infected cells (Figure 6C). To evaluate the effect of inhibition of activation of TGF-β1 on CMV-induced profibrotic response, we treated infected cells with anti-TGF-β (1D11) and anti-αvβ6 (3G9) antibodies for 7 days. The results showed that these neutralizing antibodies prevented CMV-induced elevation of type IV collagen expression and that 40 μg/ml of either antibody almost completely abolished the effect (Figure 6D). Immunoblot analysis revealed that neutralizing antibodies reduced the production of type IV collagen in infected cells and had no effect on uninfected control cells (Figure 6E). Furthermore, the ALK5 kinase inhibitor SB431542 had an inhibitory effect on surface expression of type IV

Table 1. CMV-Infected Endothelial Cells Increase Expression of ALK5 and Reduce ALK1/Endoglin

	ALK1		Endoglin		ALK5	
	Control	Infected	Control	Infected	Control	Infected
HMVEC-L	84.6 ± 21.6	14.9 ± 13.9*	2399.0 ± 376.3	1190.7 ± 137.8†	23.7 ± 9.4	180.5 ± 83.1*
UtMVECs	516.9 ± 76.1	297.0 ± 61.5*	4640.9 ± 465.0	2564.3 ± 523.2†	216.4 ± 56.3	338.9 ± 90.8
HUVECs	138.8 ± 20.0	49.2 ± 9.2‡	2479.9 ± 355.2	426.8 ± 87.2‡	81.8 ± 23.7	547.0 ± 107.5‡

Surface expression of ALK1, endoglin, and ALK5 was analyzed by flow cytometry late in infection. Numbers represent mean fluorescence intensity (MFI) (mean ± SE) of 3 to 11 experiments. Asterisks and symbols indicate significantly changed MFI in infected cells compared with uninfected control cells ($^*P < 0.05$; $^†P < 0.01$; $^‡P < 0.001$). HMVEC-L: ALK1 ($n = 3$), endoglin ($n = 5$), ALK5 ($n = 4$); UtMVECs: ALK1 ($n = 3$), endoglin ($n = 5$), ALK5 ($n = 6$); HUVECs: ALK1 ($n = 10$), endoglin ($n = 5$), ALK5 ($n = 11$).

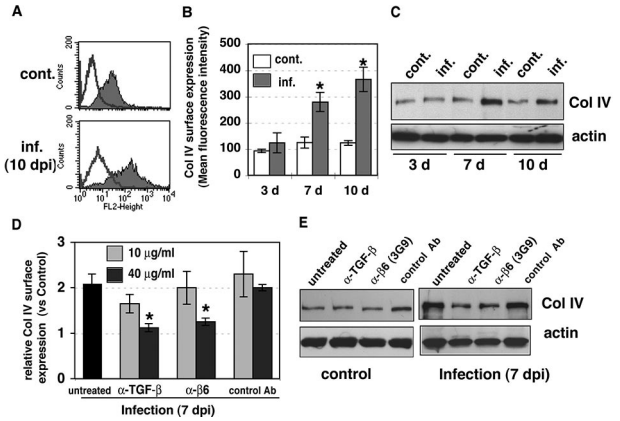

Figure 6. Increased type IV collagen synthesis by CMV infection was blocked by anti-TGF-β and anti-$\alpha v\beta 6$ neutralizing antibodies. **A:** Surface expression of type IV collagen was analyzed by flow cytometric analysis at 10 days after infection. Typical histograms from control (cont.) and infected (inf.) HUVECs are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. **B:** The results represent the mean fluorescence intensity of type IV collagen (mean ± SE) from three to seven experiments. **Asterisks** indicate surface expression in infected HUVECs as compared with uninfected controls ($^*P < 0.01$). **C:** Cell lysates from control (cont.) or infected (inf.) HUVECs at 3, 7, and 10 days after infection were fractionated by 8% SDS-PAGE and blotted on nitrocellulose. Filters were incubated with anti-type IV collagen (Col IV) and anti-actin (loading control) antibodies. **D:** Surface expression of type IV collagen was analyzed by flow cytometric analysis at 7 days without antibody (untreated) or with anti-TGF-β neutralizing antibody (1D11), function-blocking anti-$\alpha v\beta 6$ antibody (3G9), or mouse IgG1 isotype control antibody (control Ab). Relative surface expression as expressed by mean fluorescence intensity was normalized for control HUVECs in the same experiment. Results are the mean (±SE) from three experiments. Treatment with neutralizing antibodies significantly decreased surface expression of type IV collagen compared with infected cells ($^*P < 0.01$). **E:** Effects of anti-TGF-β antibody and anti-$\alpha v\beta 6$ antibody on type IV collagen production. Control and infected HUVECs were cultured without antibody (untreated) or with anti-TGF-β neutralizing antibody (1D11, 40 μg/ml), function-blocking anti-$\alpha v\beta 6$ antibody (3G9, 40 μg/ml), or mouse IgG1 isotype control antibody (control Ab, 40 μg/ml) for 7 days. Lysates were fractionated by 8% SDS-PAGE and blotted. Filters were incubated with specific antibodies. Results are representative of at least four independent experiments.

collagen in infected cells in a dose-dependent manner (data not shown). A similar effect was seen in control cells, indicating that the ALK5 kinase inhibitor blocked the basal level of TGF-β more efficiently than blocking antibodies and had a greater effect on inhibition of type IV collagen synthesis. In addition, surface expression of fibronectin was increased at late times after infection, and was reduced by the ALK5 kinase inhibitor (data not shown). Taken together, these results indicate that ECM production is increased by integrin $\alpha v\beta 6$-mediated TGF-$\beta 1$ activation in infected HUVECs.

CMV-Infected Microvascular Endothelial Cell Types Induce Integrin $\alpha v\beta 6$ and Switch TGF-β Receptor Expression

To determine whether CMV infection altered integrin $\alpha v\beta 6$ expression in other endothelial cell types, we analyzed VR1814-infected HMVEC-L and UtMVECs for surface expression of integrin $\alpha v\beta 6$ at 10 days after infection and compared it with surface expression in infected HUVECs. Integrin $\alpha v\beta 6$ was induced in both microvascular endothelial cell types after infection

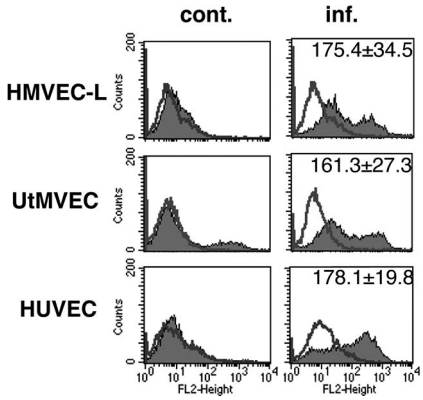

Figure 7. Different CMV-infected endothelial cell types induce different levels of integrin $\alpha v\beta 6$. Flow cytometric analysis of integrin $\alpha v\beta 6$ in HMVEC-L, UtMVECs, and HUVECs at 10 days after infection with VR1814. Typical histograms from control (cont.) and infected (inf.) cells are shown. Shaded areas represent expression of specific proteins. Lines represent isotype control. Numbers represent mean fluorescence intensity (mean ± SE). The experiments were repeated at least three times.

(Figure 7). Interestingly, integrin $\alpha v\beta 6$ was present in uninfected UtMVECs, but the induction level at late times after infection was not different from that of infected HUVECs. In addition, we compared the levels of the repertoire of TGF-β receptors expressed by HMVEC-L and UtMVECs (Table 1). All endothelial cells expressed high levels of ALK1 and endoglin and lower levels of ALK5. After infection, ALK1 and endoglin expression were significantly decreased, and ALK5 was significantly increased, as was observed in infected HUVECs. Interestingly, levels of TGF-β receptor expression on the surface of infected cells differed according to the vascular beds from which the endothelial cells were obtained.

Up-Regulated Integrin $\alpha v\beta 6$ in Blood Vessels of CMV-Infected Organs

Having found that the pathogenic CMV strain VR1814 induces integrin $\alpha v\beta 6$, which initiates TGF-$\beta 1$/ALK5 signaling in infected endothelial cells *in vitro*, we examined specimens from salivary gland, lung, uterus, and placenta with natural infection to ascertain whether expression occurs *in vivo*. We performed immunohistochemical analysis on tissues with confirmed histological evidence of cytomegalic cells (ie, sites of viral replication and active infection). In submandibular glands, islands of integrin $\alpha v\beta 6$-positive cells were detected among much larger areas of nonexpressing cells (Figure 8, A, C, and D). Expression of integrin $\alpha v\beta 6$ was found in infected cytomegalic cells (owl's eye appearance) (Figure 8, B and C) and was up-regulated in nearby epithelium (Figure 8D). In infected lungs, strong integrin $\alpha v\beta 6$ induction was seen in endothelial cells (Figure 8E). However, induction was infrequent (2 of 11 lung samples), and only focal expression of integrin $\alpha v\beta 6$ was found. Analysis of serial sections from infected lungs showed a vascular staining pattern for von Willebrand factor (Figure 8F) proximal to infected endothelial cells (Figure 8G) that induced integrin $\alpha v\beta 6$ expression (Figure 8H). Interest-

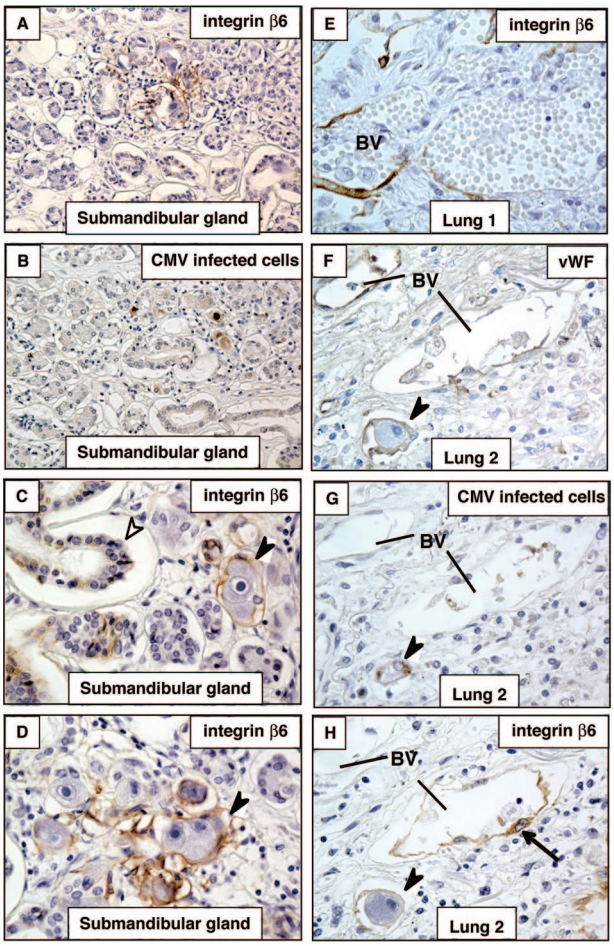

Figure 8. CMV-infected tissues induce integrin αvβ6 expression in epithelium and vascular endothelium *in vivo*. Samples (1 submandibular gland and 11 lung) obtained from 12 patients with CMV infection with histological evidence of nuclear inclusion bodies were evaluated for integrin β6 expression **A–D:** Integrin αvβ6 immunostaining in CMV-infected cells and gland epithelium in submandibular gland. **A** and **B:** Serial sections of infected submandibular gland immunostained with antibodies to integrin αvβ6 (**A**) and CMV replication proteins in infected cells (**B**). **C** and **D:** Integrin αvβ6 was strongly up-regulated in epithelial cells of submandibular glands proximal to cytomegalic cells (foci of viral replication). **E:** Integrin αvβ6 immunostaining in vascular endothelium of CMV-infected lung. Expression of integrin αvβ6 in blood vessels was found in two samples. **F–H:** Serial sections of infected lung immunostained with antibodies to von Willebrand factor (vWF) (**F**), CMV replication proteins in infected cells (**G**), and integrin αvβ6 induction (**H**). **Black arrowheads**, integrin αvβ6-positive cytomegalic cells; **white arrowheads**, glandular epithelium; **black arrows**, integrin αvβ6-positive endothelial cells. BV, blood vessels. Original magnifications: ×20 (**A**, **B**); ×40 (**C–H**).

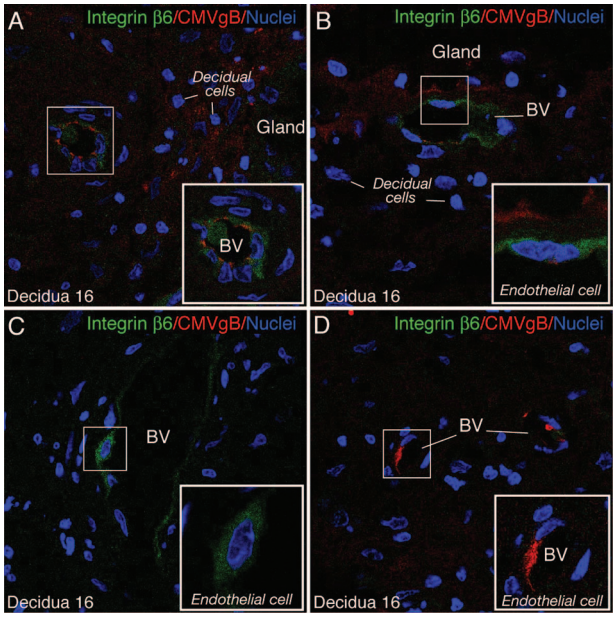

Figure 9. Integrin αvβ6 induction in blood vessels of CMV-infected decidua in early gestation. **A:** Immunostaining of integrin αvβ6 expression (green) in infected blood vessel (BV) proximal to infected decidual cells immunostained for CMV glycoprotein B (gB) (red). **B:** Integrin αvβ6 expression (green) in blood vessel proximal to infected glandular epithelium (red). **C:** Integrin αvβ6 expressed in blood vessel of the same tissue (decidua 16) in an area without viral proteins. **D:** Integrin αvβ6-negative BV. Expression of integrin αvβ6 was found in two of three decidual biopsy specimens. Original magnifications, ×400.

ingly, integrin αvβ6-specific antibodies showed that the protein was present in blood vessels immediately adjacent to CMV-infected cells, but no staining was observed in distal capillaries (Figure 8F).

We reported that CMV replicates at the uterine-placental interface, transmitting virus from infected capillaries to decidual cells and cytotrophoblast progenitor cells of epithelial origin in the adjacent placenta.[10,52] In the present study, we found that infected UtMVECs induce integrin αvβ6 expression, suggesting that the same induction could occur *in utero*. We therefore examined three paired decidual and adjacent placental biopsy specimens naturally infected with CMV in early gestation, and eight placentas from healthy deliveries at term. In the decidua, immunostaining for CMV virion gB revealed areas with infected decidual cells (Figure 9A). Nearby, an infected capillary showed up-regulated integrin αvβ6 expression in an overall diffuse staining pattern (Figure 9A). When glandular epithelia were infected, integrin αvβ6 was induced in proximal blood vessels (Figure 9B). At times, marked expression was found in endothelial cells without evidence of viral proteins in surrounding tissue (Figure 9C). Occasionally, endothelial cells were infected, but capillaries showed little or no integrin αvβ6 staining (Figure 9D). In the placenta, immunostaining revealed clusters of cytotrophoblast progenitor cells with intense membrane expression of integrin αvβ6 in chorionic villi where syncytiotrophoblasts had signs of local damage (Figure 10). For example, we found intense surface membrane staining on cytotrophoblast progenitors underneath syncytial knotting (Figure 10A) and in the vicinity of blood clots adhering to villi in contact with maternal blood (Figure 10B). Occasional cytotrophoblasts contained scattered cytoplasmic vesicles with CMV gB, a pattern suggesting virion uptake in caveolar vesicles without replication.[53,54] In contrast, integrin αvβ6 was not expressed by cytotrophoblasts when CMV virion gB accumulated in villus core macrophages, and syncytiotrophoblasts were undamaged (Figure 10C). Similar patterns of expression were seen in the other placenta.

Immunostaining of a placenta at term (five of eight) revealed high integrin αvβ6 induction in cytotrophoblast progenitor cells located next to fibrinoids, which are large ECM deposits formed on the surface of chorionic villi in contact with maternal blood (Figure 10, D and E). In areas

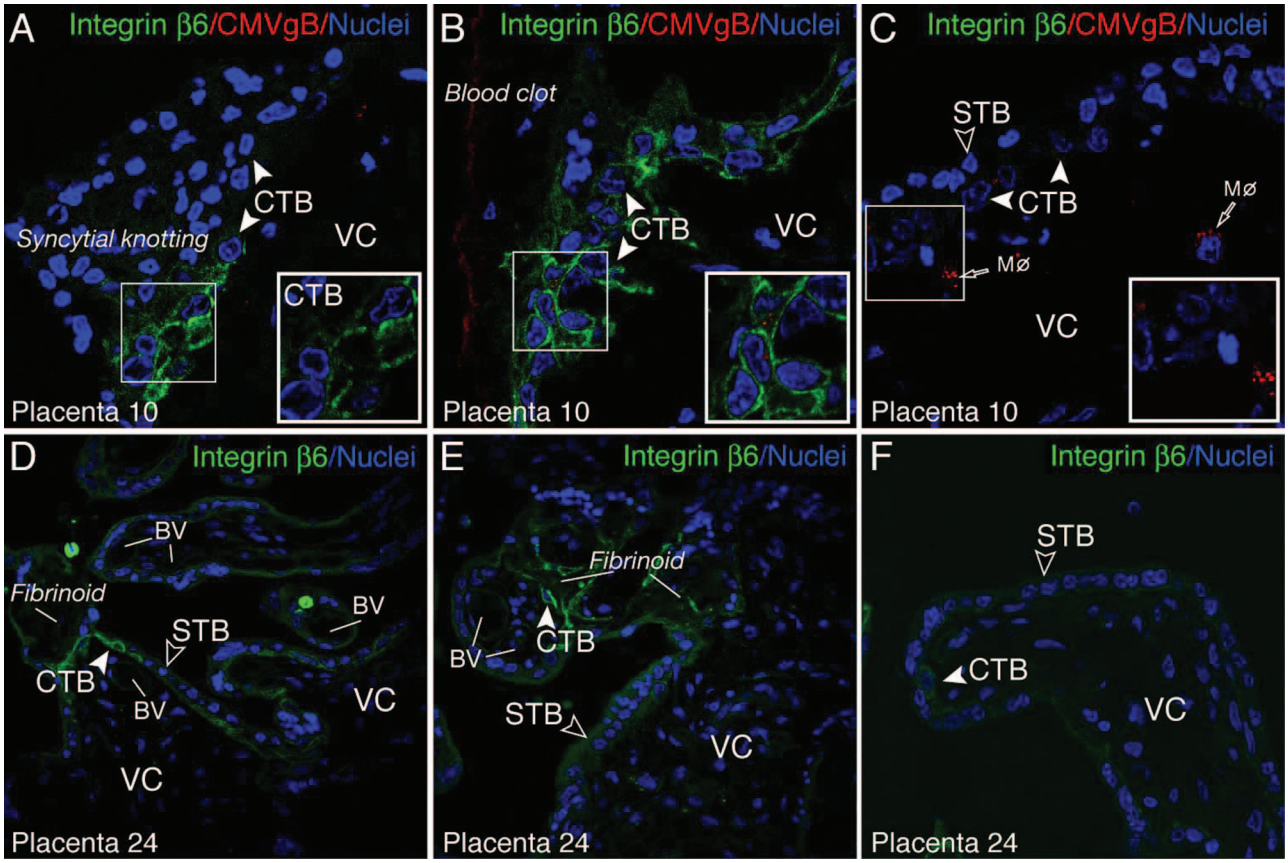

Figure 10. Up-regulated integrin $\alpha v\beta 6$ expression in villus cytotrophoblast progenitor cells, epithelial cells of the placenta. **A–C:** CMV-infected early gestation placenta. **D–F:** Uninfected placenta at term. Cytotrophoblasts broadly induced integrin $\alpha v\beta 6$ (green) proximal to sites of damage, syncytial knotting (**A**), and adherent blood clots (**B**), but not in healthy chorionic villi with macrophage (Mϕ) uptake of CMV virion gB proteins (**C**) of the same tissue (placenta 10). Expression of integrin $\alpha v\beta 6$ in cytotrophoblasts was found in two of three placental biopsy specimens. **D** and **E:** Cytotrophoblasts contiguous with fibrinotic deposits (ECM accumulation) on the villous surface strongly up-regulate integrin $\alpha v\beta 6$ (green). **F:** Integrin $\alpha v\beta 6$-negative villus in healthy villus in the same tissue (placenta 24). Similar patterns were found in five of eight term placenta. CTB, cytotrophoblast; STB, syncytiotrophoblast; VC, villus core; BV, blood vessel. Original magnifications, ×400.

with undamaged chorionic villi, cytotrophoblasts showed little or no detectable integrin $\alpha v\beta 6$ expression (Figure 10F). Together these results confirm and extend our *in vitro* findings and show that integrin $\alpha v\beta 6$ is up-regulated in diverse infected tissues. However, not all endothelial cells adjacent to the infected cells expressed integrin $\alpha v\beta 6$, suggesting a requirement for additional cellular factors or a special environment.

Discussion

To our knowledge, the data presented here are the first reported evidence for virus-induced expression of an epithelium-specific integrin, $\alpha v\beta 6$, in endothelial cells. Furthermore, although induction of TGF-$\beta 1$ in endothelial cells by CMV infection has been reported previously, these data provide evidence for a mechanism by which CMV may cause the activation of latent TGF-$\beta 1$ and downstream signaling that could alter vascular function or otherwise contribute the fibrotic and vascular components of CMV pathology. However, we cannot rule out the possibility of small contributions from other TGF-$\beta 1$ activation mechanisms, such as those involving metalloproteinases, plasmin, thrombospondin 1, and integrin $\alpha v\beta 8$.[15,17–21] Furthermore the absolute contribution of $\alpha v\beta 6$ to TGF-$\beta 1$ activation *in vivo* remains unclear. Nonetheless, our data suggest that integrin $\alpha v\beta 6$ is the main activator of TGF-$\beta 1$ in CMV-infected endothelial cells *in vitro* (Figure 2E) and further suggest that this is a major pathway for TGF-$\beta 1$ activation *in vivo.* The mechanisms underlying integrin $\alpha v\beta 6$ induction in infected vasculature are still unclear. Integrin $\alpha v\beta 6$ is a TGF-β-inducible integrin expressed at sites of epithelial inflammation and remodeling in diverse organs and binds to the RGD site in TGF-β latency-associated peptide.[18,22] This complex is tethered by a disulfide linkage to the latent TGF-β binding protein, resulting in a conformational change in the latent complex and allowing cell-associated active TGF-β to interact with TGF-β receptors on immediately adjacent cells without releasing free active TGF-β.[55,56] This indicates that cell-to-cell contact is required for integrin $\alpha v\beta 6$-mediated activation of TGF-β. Our immunohistological analysis suggested that integrin $\alpha v\beta 6$ in endothelial cells is induced by infection or up-regulated through contact with adjacent infected cells (Figures 8 and 9). Although TGF-$\beta 1$ induces the *de novo* synthesis of integrin $\alpha v\beta 6$ in normal human keratinocytes,[45] TGF-$\beta 1$ secreted by infected HUVECs (mostly in an inactive form) (Figure 2A) is less likely to play a role in the induction, because conditioned medium from infected

cells did not induce expression of integrin αvβ6. Our data indicate that viral DNA replication may be necessary for the *de novo* synthesis of integrin αvβ6 in endothelial cells, as demonstrated by the lack of induction of integrin αvβ6 when viral late gene expression is blocked (Figure 4B). In addition, TGF-β1/ALK5 signaling plays an important role in the induction of integrin αvβ6, because anti-TGF-β neutralizing antibody and the ALK5 kinase inhibitor strongly suppressed the expression of the integrin (Figure 4A). Once integrin αvβ6 is expressed, it may act to continuously process TGF-β1 into its active from. Active TGF-β1, locally bound to the ECM, could serve as an amplifier for the induction of integrin αvβ6 in adjacent cells by cell-to-cell contact. The activation of TGF-β1 and expression of αvβ6 could then be involved in a positive feedback loop[45,46] that amplifies the response within endothelial cells.

However, we cannot exclude the possibility that endothelial cells might normally express trace amounts of integrin αvβ6 that become dramatically up-regulated by some other mechanism. Integrin αvβ6 binds an RGD motif, the most common integrin binding sequence,[57] also contained in several CMV proteins. When present in the plasma membrane of infected cells, these proteins could serve as integrin αvβ6 receptors through an RGD motif and activate signaling pathways. CMV protein UL148, and CMV-specific transmembrane proteins UL30 and US23[58,59] each contain an RGD motif. It is intriguing that UL148, encoded by pathogenic CMV strains, confers tropism for endothelial cells and leukocytes[60,61] and could theoretically mediate functional changes associated with pathogenesis.

Integrin αvβ6 is expressed at low levels on uninjured epithelia of healthy tissues but dramatically increases in response to epithelial injury and wound healing during subclinical, acute, or chronic inflammation.[22] In a mouse model of lung injury and edema induced by bleomycin treatment, integrin αvβ6-mediated activation of TGF-β1 induces early lung edema and late lung fibrosis in normal mice, whereas mice with an interruption in the β6 integrin gene show exaggerated inflammation in the lungs and skin but are protected from pulmonary fibrosis and edema.[18,23,62] This combination of effects suggests a localized deficiency of active TGF-β1 resulting from loss of β6 integrin.[23] Likewise, lack of the β6 integrin gene protects against tubulointerstitial renal fibrosis in mice with unilateral urethral obstruction[63] and blockade of integrin αvβ6 protects against renal fibrosis in a murine model of Alport's syndrome.[24] In transgenic mice overexpressing human integrin β6 in the epithelium, chronic ulcers with areas of severe fibrosis develop, and TGF-β1 expression significantly increases in lesions as compared with normal skin.[64] These various lines of evidence suggest that integrin-αvβ6-mediated TGF-β activation is of general importance in the development of fibrosis in multiple epithelial organs, and the same mechanism may well contribute to fibrosis and other pathological outcomes in CMV disease.

Episodes of immune rejection, ischemia, and CMV infection are among the risk factors for chronic allograft nephropathy with interstitial inflammation, glomerular lesions, and interstitial fibrosis.[13,65] In a rat CMV model of chronic renal allograft rejection, remarkable fibrosis, combined with glomerular and tubular damage, was found within weeks after graft transplantation.[66] In human kidney allografts, persistent CMV infection without acute rejection was associated with increased TGF-β1 expression in arterial endothelium and tubular epithelial cells.[67] Patients with ongoing CMV infection had significantly increased urinary excretion of TGF-β1 and developed interstitial fibrosis in the kidney 6 months after transplantation.[68] Although CMV-infected transplants were not examined in our study, integrin αvβ6-mediated TGF-β1 activation could be central to the pathology associated with viral replication and interstitial fibrosis in diverse tissues.

Another interesting and potentially relevant phenomenon is that vascular intimal thickening is increased in biopsy specimens from kidney allografts with persistent CMV infection.[67] Intimal thickening could involve an endothelial-to-mesenchymal transition caused by TGF-β, as observed in cardiac fibrosis in mouse models,[69] as well as during embryonic development of the heart.[70] In addition, *in vitro* studies have shown that embryonic endothelial cells changed to an epithelioid phenotype corresponding with the down-regulation of von Willebrand factor-related antigen.[71] In the present study, we found that CMV-infected endothelial cells express epithelial integrin αvβ6 *in vitro* (Figures 1 and 7) and *in vivo* (Figures 8 and 9), switch expression levels of TGF-β receptors (Figure 5, Table 1), and down-regulate endothelial-specific proteins, including VE-cadherin, von Willebrand factor, and PECAM-1 (T.T. and L.P., unpublished). Taken together, these results suggest that CMV-infected endothelial cells undergo a phenotypic change to a nonendothelial cell type, a transition that could be associated with CMV pathogenesis.

In pregnancies affected by congenital CMV infection, substantial evidence of virus-initiated pathology is provided by inflammation, leukocytic infiltration, edema, and fibrinotic deposits that occlude blood vessels in the villus core.[72,73] Except in cases of severe symptomatic CMV disease, evidence of ongoing viral replication in the placenta is seldom detected. Here we determined that integrin αvβ6 is up-regulated in blood vessels in early gestation decidua with focal sites of viral replication and in villus cytotrophoblasts in placentas containing viral DNA (Figures 9 and 10). Remarkably strong induction was observed in cytotrophoblasts near blood clots adhering to damaged chorionic villi and in cells contiguous with fibrinoids composed of fibronectin, laminin, and collagen IV, suggesting that integrin-mediated TGF-β1 activation contributes to pathology in the uterine and fetal compartment. Purified villus cytotrophoblasts isolated from placentas at term that contain CMV DNA, and virion proteins without active replication express integrin αvβ6 that activates TGF-β1.[30] Deposition of ECM protein by integrin αvβ6-mediated activation of TGF-β1 (Figure 6), impairment of ECM degradation by down-regulation of matrix metalloproteinase 2 activity by CMV-encoded viral interleukin-10,[74] and increased production of the tissue inhibitor of metalloproteinases 1, which is independent of TGF-β1 activation (T.T. and L.P., unpublished), could

explain the marked pathology at the uterine-placental interface in congenital infection.

In most cell types, TGF-β1 binds to the ubiquitously expressed ALK5 receptor, which activates Smad2 and Smad3. In endothelial cells, TGF-β1 can also bind to ALK-1 in the presence of functional ALK5, resulting in phosphorylation of both Smad1 and Smad5.[75] However, it has been suggested that TGF-β1 may not represent the physiological ligand for the ALK1 receptor, since Goumans and colleagues[75] investigated binding of TGF-β1 to ALK1 under specific experimental conditions. We found that phosphorylation of Smad1/5 was present during the course of infection and that the levels of phosphorylation either did not change or decreased at late times after infection (Figure 3A). In addition, phosphorylated Smad1/5 was not blocked by treatment with either anti-TGF-β or anti-αvβ6 neutralizing antibody (Figure 3B). This raises the possibility that another ligand(s) could bind to ALK1 and/or endoglin and activate Smad1/5. Bone morphogenetic protein (BMP)-9 and BMP-10 have been shown to bind to ALK1, inducing phosphorylation of Smad1/5 in human dermal microvascular endothelial cells, and could have an effect on angiogenesis.[76] In addition, overexpression of endoglin increases the BMP-9 response.[76] Endoglin has been shown to interact with TGF-β1, TGF-β3, activin A, BMP-2, and BMP-7 in the presence of type I or type II receptor.[77] Although the function of endoglin in endothelial cells is still unclear, it has been shown to antagonize TGF-β1-induced ALK5/Smad3 signaling and enhance the BMP-7/Smad1/5 pathway.[78] Phosphorylation of Smad1/5 could be attributable to a response to BMP(s). It will be interesting to find out whether CMV infection induces BMP/Smad1/5 signaling. If so, it will be important to ask what specific functional role it plays *in vivo*.

Although our results provide a better understanding of the regulation of TGF-β1 in CMV-infected endothelial cells, much remains to be learned. For one, TGF-β1 activation could occur through other mechanisms not dependent on integrin αvβ6, and these could be important contributors to TGF-β effects *in vivo*. In addition, analysis of infected tissues suggests that up-regulation of αvβ6 in the endothelium could be limited and require additional cellular factor(s) and/or a special environment, suggested by the patchy distribution of integrin αvβ6 in blood vessels (Figures 8 and 9). Although animal models for the study of human CMV pathogenesis do not exist because of strict species specificity, studies of integrin αvβ6 knockout mice infected with murine CMV may provide clues about the physiological role of integrin αvβ6 induction in infected tissues and vascular beds.

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