Downregulation of the cellular adhesion molecule Thy-1 (CD90) by cytomegalovirus infection of human fibroblasts

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The deregulation of cellular adhesion molecules by human cytomegalovirus (HCMV) appears to be correlated with the development of vascular disease. In this study, it was investigated whether the expression of Thy-1 (CD90), a member of the immunoglobulin superfamily of adhesion molecules with constitutive expression on fibroblast cells, is modulated following infection with HCMV. It was observed that Thy-1 cell surface expression decreased significantly during the course of infection. Addition of neutralizing antibodies, as well as UV inactivation of virus, prevented Thy-1 downregulation. In contrast, inhibition of virus replication by cidofovir did not alter Thy-1 regulation by HCMV, indicating that immediate-early (IE) and/or early (E) gene products are responsible. Interestingly, after infection of fibroblasts with a recombinant GFP-expressing virus, infected as well as non-infected cells showed a reduced Thy-1 cell surface expression. From these findings, it is concluded that IE or E gene products of HCMV induce a so far unidentified soluble factor that mediates Thy-1 downregulation.

Human cytomegalovirus (HCMV) is a widespread opportunistic herpesvirus that causes severe and fatal diseases in immunocompromised patients, including organ transplant recipients or individuals with AIDS (Alford et al., 1990). It is also a leading cause of virus-induced birth defects and is associated with vascular alterations in immunocompetent and immunocompromised individuals, such as coronary artery disease (Muhlestein et al., 2000), restenosis after angioplasty (Speir et al., 1994; Zhou et al., 1996) and severe transplant vasculopathy ultimately leading to chronic allograft rejection (Grattan et al., 1989; Borchers et al., 1999).

The ability of HCMV to perturb normal cellular control mechanisms has been studied intensively. Changes in cellular gene expression occur immediately after binding of virions to the cell (Boldogh et al., 1990; Simmen et al., 2001; Yurochko & Huang, 1999). Similarly, expression of the viral immediate-early (IE) and early (E) genes also results in physical and functional interactions between the viral gene products and cellular factors, resulting in perturbation of cellular transcription, cell cycle control and the secretion of chemokines and cytokines (Fortunato et al., 2000).

The expression pattern of various cell surface proteins is also modulated during HCMV infection. It has been shown that HCMV leads to the upregulation of cellular adhesion molecules on endothelial cells as well as fibroblasts, such as the intercellular adhesion molecule 1 (ICAM-1). The increased expression of these adhesion molecules augments the adherence and infiltration of inflammatory cells that are capable of promoting vascular disease (Lemstrom et al., 1995; Steinhoff et al., 1995; Yilmaz et al., 1996; Martelius et al., 1998; Waldman et al., 1998; Lautenschlager et al., 1999; Kronschnabl & Stamminger, 2003). In contrast, killing of infected cells by cytotoxic T cells is inhibited by downregulating the cell surface expression of MHC class I receptors by a variety of specific mechanisms (Alcami & Koszinowski, 2000; Warren et al., 1994). Other cell surface proteins associated with peptide processing have also been reported to be downregulated during HCMV infection (Phillips et al., 1998). Furthermore, a negative regulation of the epidermal growth factor receptor (EGFR) could be demonstrated. Interestingly, EGFR can serve as both a specific receptor for HCMV attachment and a mediator of HCMV-induced signal transduction (Fairley et al., 2002; Wang et al., 2003). Thus, downregulation of cell receptors, resulting in the abrogation of receptor-mediated cell signaling, may be a common occurrence during HCMV infection.

In this study, we investigated whether Thy-1 (CD90), a member of the group of adhesion molecules in the immunoglobulin superfamily, is similarly deregulated by HCMV infection. Thy-1 is a cell surface glycoprotein with a molecular mass of 35 kDa. In humans, constitutive Thy-1 expression is restricted to neuronal cells, fibroblasts and a subset of CD34+ blood stem cells (Craig et al., 1993). Fibroblasts possess a very high basal level of Thy-1 surface expression and therefore Thy-1 can be considered as a marker protein for this cell type. It is constitutively expressed in lipid rafts (Sharom & Lehto, 2002) and is induced during the early phase of wound healing.
suggesting an activation of Thy-1 expression by inflammatory mediators (Saalbach et al., 1996). Moreover, Thy-1 shows an inducible expression on endothelial cells and is detectable on microvascular endothelial cells during neoangiogenesis (Lee et al., 1998). A so far unknown ligand of human Thy-1 has been postulated on monocytes and polymorphonuclear leukocytes and is suggested to mediate the binding to activated Thy-1-positive microvascular endothelial cells and fibroblasts (Saalbach et al., 2000, 2002). Additionally, the Thy-1 protein has been shown to be involved in the attachment of fibroblasts to extracellular matrix components, such as fibronectin and collagen I (Saalbach et al., 1998).

In this report, we have demonstrated that HCMV leads to downregulation of Thy-1 protein levels after infection of human foreskin fibroblasts (HFFs). HFFs were infected with HCMV, strain AD169 (m.o.i. of 2) or mock infected. The cells were harvested at various time points post-infection (p.i.) for determination of Thy-1 cell surface expression by flow cytometry (FACS) analysis. In parallel, HCMV-mediated induction of ICAM-1 cell surface expression was monitored as a control. For FACS analysis, cells were fixed with formaldehyde (3 %, v/v, in PBS) and incubated with the Cohn-II fraction of human immunoglobulin (1 mg ml\(^{-1}\)) to avoid non-specific antibody binding. Thereafter, cells were stained with specific antibodies [fluorescein isothiocyanate (FITC)-conjugated anti-human ICAM-1 (Calbiochem); phycoerythrin (PE)-conjugated anti-human Thy-1; mouse IgG1 PE-conjugated and mouse IgG1 FITC-conjugated isotype control antibodies] and analysed with a Becton Dickinson FACScalibur with CELLQUEST software (BD Pharmingen). As a central finding, HCMV infection drastically decreased the expression of Thy-1 on the surface of HFFs (Fig. 1A). After 72 h, 77-3 % (Fig. 1A; sum of cells present in lower-left and lower-right quadrants) of the infected cells showed a reduced level of Thy-1 surface protein. In contrast, the ICAM-1 cell surface expression was increased in 30-1 % of the cells at 72 h p.i. (Fig. 1A; sum of upper-right and lower-right quadrants). Similarly, Thy-1 downregulation was also observed after infection with other HCMV strains, such as TB40E, Toledo and a clinical isolate (data not shown).

Western blot analysis was then performed to address the question of whether a decreased cell surface presentation of Thy-1 resulted from downregulation at the protein level (Fig. 1B). Indeed, we could observe an overall reduction in the steady-state levels of Thy-1 protein as measured by Western blot analysis using whole-cell lysates. This was already detectable by 24 h p.i. and was very obvious at 72 h p.i. (Fig. 1B, upper panel, lanes 2, 4 and 6). The staining of cellular β-actin served as a loading control (Fig. 1B, lower panel). Next, Thy-1 mRNA levels were determined by semi-quantitative RT-PCR. As shown in Fig. 1(C), there was only a slight decrease in Thy-1 mRNA levels at 72 h p.i. with HCMV. We assume that this minor decline cannot be fully responsible for the massive change in Thy-1 protein levels after HCMV infection. This suggests that Thy-1 protein levels are mainly regulated by HCMV in a post-transcriptional manner.

The binding of HCMV virions to cellular receptors is sufficient for the activation of signalling cascades and the rapid upregulation of cellular transcription factors, such as Sp1 and NF-κB (Yurochko et al., 1997). Thus, the activated state of transcription factors is a hallmark of numerous regulatory effects induced by HCMV attachment. Therefore, we investigated whether the observed Thy-1 modulation was already affected by the physical interaction between the virus and the target cell. First, the human anti-HCMV monoclonal antibody (mAb) C23 was used to interfere with penetration, but not attachment of HCMV (Ohizumi et al., 1992). Infection of HFFs with neutralized virions did not alter the Thy-1 cell surface expression indicating that virus attachment is not sufficient for Thy-1 modulation by HCMV (Fig. 2A). To analyse whether penetration of the virions is able to induce Thy-1 downregulation, experiments with UV-inactivated virus were performed. Inoculation of HFFs with UV-inactivated HCMV resulted in a small decrease in Thy-1 cell surface expression; however, the effect was minor in comparison with infection with untreated virus (Fig. 2B). Taken together, these data indicated that neither adsorption nor penetration of HCMV virions is sufficient for the downregulation of Thy-1 expression.

Since we observed the strongest decrease in Thy-1 levels at 72 h p.i., we investigated next whether viral late gene expression is required for this effect. For this, HFFs were infected in the absence or presence of cidofovir (CDV, 10 μM). CDV is a potent inhibitor of viral DNA synthesis and late gene expression. Despite the presence of CDV, Thy-1 protein levels were strongly reduced after infection with HCMV. In FACS analysis, the Thy-1 mean fluorescence intensity (m.f.i.) decreased to 21 % (40 m.f.i. versus 186 m.f.i.) of the normal level at 72 h p.i. (Fig. 2C). This indicated that IE and/or E gene products are responsible for Thy-1 downregulation.

In the next step, HFFs were infected with a recombinant GFP-expressing HCMV (AD169–GFP) to distinguish between infected and non-infected cells by FACS analysis (Fig. 3A). Again, infected GFP-positive cells showed a clear decrease in Thy-1 cell surface expression. Interestingly, GFP-negative, non-infected cells also showed a reduced cell surface expression compared with mock infection. This finding suggested that a soluble factor might be induced after HCMV infection mediating Thy-1 downregulation. To confirm this result, co-cultivation experiments were performed. HFFs were infected with HCMV (AD169–GFP) and thereafter co-cultivated with untreated cells in Transwell chambers. After 72 h, cells were harvested and the Thy-1 surface expression of both cell populations (infected and non-infected, co-cultivated cells) was determined by FACS analysis. Infected (Fig. 3B, a and c) as well as co-cultivated cells (Fig. 3B, b and d) showed a significantly
decreased level of Thy-1 cell surface expression. Thus, these results excluded the influence of cell-to-cell contact on HCMV-mediated Thy-1 regulation and supported the conclusion that a soluble factor is involved.

HCMV encodes several cytokine and chemokine homologues, such as viral IL10 or IL8. As a consequence, the MHC class I protein is downregulated in part by the action of viral IL10 (Spencer et al., 2002). On the other hand, cellular interleukins, such as IL6 and IL8, are upregulated during HCMV infection (Almeida et al., 1994; Murayama et al., 1997). It is tempting to speculate that one of these soluble factors may be involved in Thy-1 downregulation. In this respect, we analysed the possible effects of TNF-α.

**Fig. 1.** (A) Downregulation of Thy-1 cell surface expression on HFFs after infection with HCMV. HFFs were either infected with HCMV strain AD169 (+HCMV) or mock infected (–HCMV) and harvested at the indicated time points. HCMV infection was performed with an m.o.i. of 2 resulting in approximately 95% infected cells. Cell surface expression of Thy-1 (PE-conjugated Thy-1 mAb) and ICAM-1 (FITC-conjugated ICAM-1 mAb) was determined by FACS analysis. Note that ICAM-1 cell surface expression increased during the course of viral infection (24, 48 and 72 h) in contrast to Thy-1, which decreased. (B) HCMV infection leads to a decrease in Thy-1 protein levels. Cell lysates of non-infected (–) or infected (+) HFFs were fractionated by SDS-PAGE followed by Western blot analysis using a polyclonal antiserum directed against Thy-1 (upper panel). Restaining of the Western blot was performed with a mAb against human β-actin (lower panel; Sigma). (C) Semi-quantitative RT-PCR analysis showing no significant change in Thy-1 mRNA expression after HCMV infection of HFFs. The reverse transcription reaction was performed using the Titan One Tube RT-PCR System (Roche) and a specific primer pair for the amplification of the Thy-1 mRNA (ThyRNA5, 5'-ATGAACCTGGCACCACCATCGC-3'; ThyRNA3, 5'-TCACAGGGACATGAAATCCGTGG-3'). PCR cycling parameters were as follows: 10 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 45 s, followed by an additional 20 and 30 cycles, respectively, with 5 s elongation of each polymerization step at 68°C per cycle. GAPDH mRNA was amplified as an internal control using primers 698GAP5 (5'-GTACGTCGTGGAGTCCACTG-3') and 698GAP3 (5'-TCCACCACCTGTGCTGTA-3'). The conditions of the RT-PCRs and the amount of template RNA are indicated in the figure.
IFN-γ and IL6, but so far we have been unable to detect any influence of these cytokines on Thy-1 cell surface expression (data not shown).

Perturbation of cellular functions appears to be essential for viruses to optimize the cell for productive infection. Thus, a number of cellular genes involved in immune modulation as well as chemokine and cytokine production have been described as being regulated by HCMV. The upregulation of cellular adhesion molecules such as ICAM-1, which augments the adherence and infiltration of leukocytes, directly contributes to the dissemination of the virus via the peripheral blood, thus playing an important role in the pathogenesis of acute infection (Grundy et al., 1998). In contrast, other cell surface markers that are involved in immune surveillance are counter-regulated by HCMV. The downregulation of MHC class I and other peptide-processing cell surface molecules has been studied in detail (Ahn et al., 1996; Jun et al., 2000; Phillips et al., 1998). Here, we demonstrated that virus infection of fibroblasts, which constitutively express high amounts of Thy-1, led to a downregulation of this cellular adhesion molecule. Whether Thy-1 expression is also modulated in endothelial cells after HCMV infection, where Thy-1 has been described as a marker of neoangio genesis (Lee et al., 1998), needs further investigation. We have shown that virus attachment is not sufficient, and viral IE and/or E gene expression is necessary for this effect. Furthermore, evidence has been presented that Thy-1 was significantly downregulated in non-infected HFFs by a soluble factor induced after HCMV infection. Despite extensive investigation, the exact function of Thy-1 in various cell types remains unknown. It has been shown that Thy-1 acts as a cell adhesion molecule, which triggers the adhesion of polymorphonuclear leukocytes to activated endothelial cells and fibroblasts (Saalbach et al., 2000). Furthermore, it has been demonstrated that Thy-1 mediates the adherence of fibroblasts to extracellular matrix components (Saalbach et al., 1998). This might be in causal connection with the finding that Thy-1 is abundantly expressed in non-tumorigenic cells while expression could not be detected in several ovarian cancer cell lines with high migration capacity (Abeyesinghe et al., 2003). Besides Thy-1, several extracellular matrix transcripts are also downregulated during HCMV infection (Schaarschmidt et al., 1999). Therefore, one molecular mechanism contributing
to the cytopathic effects of HCMV infection may be the downregulation of proteins involved in cell structure and intercellular connection. Thus, we hypothesize that decreased Thy-1 surface expression after HCMV infection may promote the detachment of fibroblasts from extracellular matrix components, which may disturb the integrity of the vessel wall layer. Our findings suggest the possibility that Thy-1 is a regulated cellular factor possibly contributing to these HCMV-induced pathologies.

Acknowledgements

We thank Regina Kupfer, Karin Gross and Martina Freitag for excellent technical assistance, P. Lischka for helpful discussions and B. Fleckenstein for continuous support. This work was supported by the Bundesministerium für Forschung und Technologie (IZKF Erlangen) and the Deutsche Forschungsgemeinschaft (SF8473).

References


