Human cytomegalovirus-induced reduction of extracellular matrix proteins in vascular smooth muscle cell cultures: a pathomechanism in vasculopathies?

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Human cytomegalovirus (HCMV) infection appears to be linked to the pathogenesis of atherosclerosis. An association between HCMV infection and an enhanced restenosis rate as well as the induction of vasculopathies after solid organ transplantation has been documented. Knowledge of the cellular and molecular basis of these findings is limited, however. By Northern blot and RT-PCR analysis of human foreskin fibroblasts (HFF) and human coronary artery smooth muscle cells (SMC), we identified extracellular matrix (ECM) genes that were downregulated after HCMV infection, including collagen type I and fibronectin. Quantitative immunoassays showed a significant reduction of soluble collagen type I and fibronectin proteins in supernatants of both cell types. This was shown to be a direct effect of HCMV infection and not due to a response to interferons released from infected cells, since neutralization of alpha and beta interferon activity could not block virus-induced downregulation of matrix proteins. As the amount of ECM depends on both synthesis and degradation, we also assessed the influence of HCMV on the activity of matrix metalloproteinases (MMP). Interestingly, a significant difference in virus-induced matrix degradation could be shown between the two cell types. HCMV upregulated MMP-2 protein and activity in SMC but not in HFF. Thus, HCMV infection of SMC reduces ECM dramatically by inducing two independent mechanisms that influence synthesis as well as degradation of ECM. These may represent molecular mechanisms for HCMV-induced pathogenesis of inflammatory vasculopathies and may facilitate dissemination of HCMV by promoting the detachment of infected cells in vivo.

INTRODUCTION

Many patients suffering from cardiovascular disease lack known genetically determined or environmental risk factors. Attention has therefore been focused on the potential role of infectious agents in the pathogenesis of vascular lesions. The human cytomegalovirus (HCMV) has been shown to predispose to enhanced restenosis (Zhou et al., 1996) and vasculopathy following solid organ transplantation (Srivastava et al., 1999). A possible role for HCMV in the formation of atherosclerotic lesions is supported by the detection of viral antigen and nucleic acids in smooth muscle cells (SMC) cultured from coronary artery plaque material (Melnick et al., 1983). Potential atherogenic pathomechanisms induced by HCMV have been postulated by several studies (Reinhardt et al., 2002, 2005a, b; Speir et al., 1994; Streblow et al., 1999; Zhu et al., 1998).

Over the last decade, it has been realized that fatal complications of atherosclerosis are not necessarily linked to the presence of large plaques but are related to the rupture of a so-called vulnerable plaque (Maseri & Fuster, 2003). In traditional terms, a vulnerable plaque consists of a lipid-rich core covered by a thin and fragile fibrous cap. Since the net amount of extracellular matrix (ECM) is determined by two countercurrent metabolic processes, two potential mechanisms for the rupture of the fibrous cap have been proposed: reduced synthesis and enhanced degradation of ECM by proteolytic enzymes (Libby, 1995; Cullen et al., 2003). Proteinases that digest collagens, such as matrix metalloproteinases (MMP) 2 and 9, are known to be important. Within a plaque, besides macrophages, SMC are considered to be the main producer of MMP-2 (Aimes & Quigley, 1995).

Infection with the laboratory-adapted HCMV strain Towne (Pande et al., 1990) led to a reduction of fibronectin mRNA
in human foreskin fibroblasts (HFF), the standard cell type used in vitro in HCMV studies. Also, in HFF infected with a laboratory-adapted HCMV strain (AD169), the results of DNA array analysis suggested an HCMV-associated reduction of collagen type I gene expression (Zhu et al., 1998). To our knowledge, neither confirmation of these data by Northern blot analysis nor data concerning collagen protein synthesis under the influence of HCMV infection is available. Furthermore, nothing is known about virus-induced modulation of ECM genes and protein expression in SMC. Since SMC are thought to be the major producer of the ECM scaffold of the vessel wall (Brooke et al., 2003) and are important cells in atherogenesis, we analysed HCMV-induced effects on synthesis and degradation of ECM proteins in primary SMC obtained from human coronary arteries.

In the present study, we demonstrate a substantial HCMV-induced reduction of ECM in HFF as well as in SMC. The loss of intercellular ECM fibres is linked to direct virus-induced suppression of matrix gene expression, resulting in decreased protein production. This effect was observed in HFF and also in SMC, which are major target cells for HCMV in vivo (Sinzger et al., 1995). An additional matrix-degrading mechanism was identified in SMC, where increased amounts and activity of MMP-2 were detected. HCMV-induced modulation of ECM expression in SMC could trigger pathogenetic processes in inflammatory vasculopathies.

METHODS

Cells and infection. SMC (Clonetics) were cultured in SMC basal medium supplemented with 5% fetal calf serum (FCS) and growth factors (SmGM BulletKit; BioWhittaker). SMC were used in passage 5 or 6. HFF were prepared in our laboratory and were used in passages 13–20. HCMV strain AD169 was obtained from the ATCC. The low-passage, more ‘wild-type’-like HCMV strain TB40E was provided by Dr Ch. Sinzger, University of Tübingen. Cells were normally infected 2 (HFF) or 3 (SMC) days after seeding at an m.o.i. of 1. To analyse cultures with different cell densities, HFF were also normally infected 2 (HFF) or 3 (SMC) days after seeding at an m.o.i. of 2 (HFF) or 3 (SMC) days after seeding at an m.o.i. of 1. To analyse cultures with different cell densities, HFF were also normally infected 2 (HFF) or 3 (SMC) days after seeding at an m.o.i. of 1. To analyse cultures with different cell densities, HFF were also normally infected 2 (HFF) or 3 (SMC) days after seeding at an m.o.i. of 1. 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HCMV infection induces a decline in ECM mRNAs

Using differential display RT-PCR analysis, we had previously identified several host cell genes encoding ECM proteins that were modulated by HCMV in infected HFF (Schaarschmidt et al., 1999). We confirmed these results by Northern blot analysis. In HFF, a dramatic virus-induced decrease in mRNA was observed within the first 24 h p.i. In infected HFF compared with mock-infected HFF after 24 h p.i., the strongest reduction in mRNA was observed for tenasin, with a complete loss of specific mRNA. Thrombospondin, fibronectin, decorin and collagen type I mRNAs were reduced by more than 3-fold and lectin and fibulin mRNAs were reduced 2-fold. Compared with mock-infected HFF 48 h p.i., collagen type I, lectin and fibulin specific mRNAs were reduced by more than 3-fold. For fibronectin, decorin, tenasin and thrombospondin, a complete loss of mRNA expression was observed in HCMV-infected HFF (Fig. 1a).

In subsequent experiments, we focused on the HCMV-induced modulation of collagen and fibronectin, two major components of the ECM that form the fibrous cap of atherosclerotic plaques. To exclude the possibility that the HCMV-induced reduction of collagen and fibronectin gene expression was confounded by differences in cell proliferative activity, we infected HFF cultures 2 and 5 days after seeding of cells, resulting in different cell densities. However,
cell density did not influence the HCMV-induced reduction of matrix gene expression (not shown).

In HCMV-infected compared with mock-infected SMC, a decrease in collagen type I mRNA to approximately 6% was demonstrated 24 h p.i. using quantitative PCR (Fig. 1b). The virus-induced downmodulation at 48 h p.i. was comparable after infection with the 'wild-type'-like strain TB40E and the laboratory strain AD169. Down-regulation of fibronectin mRNA was somewhat delayed and less pronounced compared with the reduction of collagen type I gene expression. Following infection of SMC with TB40E, the level of fibronectin mRNA was reduced to approximately 50, 12 and 2%, respectively, at 24, 48 and 72 h p.i.

Fibrillar collagen type I and extracellular fibronectin are reduced in infected HFF and SMC

Indirect immunofluorescence studies of HCMV-infected and mock-infected cells showed that the decreased gene expression in virus-infected cells correlated well with a decrease of matrix protein expression. A clear reduction of extracellular, insoluble collagen type I fibres was seen in HFF and SMC. Fig. 2(a) demonstrates the fluorescence intensities of a representative experiment of five. Visible changes were observed as early as 24 h p.i. (not shown) and were fully developed at 48 h p.i. Compatible with the Northern blot and RT-PCR results, the HCMV-induced down-regulation of collagen type I was more pronounced than...
that of fibronectin. To exclude virus strain-specific effects, we performed cross-over experiments, where similar results were obtained following infection of HFF with the TB40E strain as well as following infection of SMC with the AD169 strain. No HCMV strain-specific modulation was found (data not shown).

Electron microscopy confirmed the results of the immunofluorescence studies, since a dramatic HCMV-induced loss of fibrillar matrix was demonstrated in HFF and SMC. Hardly any fibres were detectable between adjacent HCMV-infected cells, whereas, in the mock-infected control, matrix fibres interlaced the intercellular gaps (Fig. 2b).

**HCMV influences the equilibrium of synthesis and degradation of ECM**

Immunofluorescence assays do not allow exact quantification of the proteins studied. Therefore, we performed quantitative immunoassays for collagen type I and fibronectin proteins in the supernatants of HCMV-infected and mock-infected HFF and SMC. Furthermore, by adding EDTA, as an inhibitor of MMPs, we wanted to determine whether HCMV, besides influencing synthesis, also modulates degradation of matrix proteins. Therefore, soluble collagen type I and fibronectin were quantified by ELISA with and without added EDTA. A reduction of mean fibronectin concentrations to 65 and 48% was observed in HCMV-infected cultures compared with uninfected HFF and SMC, respectively. Although some further decrease in fibronectin concentrations was observed, addition of EDTA to the cultures did not alter levels of soluble fibronectin significantly in HCMV-infected HFF and SMC (Fig. 3a, b).

It is reasonable to assume that proteases uninfluenced by EDTA are involved in the reduction of fibronectin following HCMV infection, since an increased casein-digesting activity was observed in supernatants of HCMV-infected SMC (see Fig. 6a).

The loss of free collagen was most notable ($P < 0.0001$) in infected HFF, where a reduction to 3% of the mean collagen concentration of the uninfected control was observed within 24 h p.i. (Fig. 3c). A significant ($P = 0.0006$; results from three independent experiments, using different SMC donors) reduction of collagen to 38% compared with the uninfected control could also be demonstrated in HCMV-infected SMC. Addition to the cell cultures of antibodies that neutralize IFN-$\alpha$ and -$\beta$ activity did not alter the extent of HCMV-induced reduction of fibronectin and collagen expression in the supernatants of infected cells (Fig. 4). Thus, the observed downmodulation appears to be a direct effect of HCMV.

With respect to matrix degradation, we observed an interesting difference between the two cell types (Fig. 3c, d). In contrast to HFF, inhibition of MMPs by addition of EDTA led to a significantly ($P < 0.0009$) diminished reduction of soluble collagen, indicating that matrix-degrading phenomena are involved (d). In infected HFF, this effect was not observed (c).

**Fig. 3.** Quantification of soluble matrix proteins in the supernatants of HCMV-infected and mock-infected HFF and SMC. Concentrations of soluble fibronectin (a, b) and collagen type I (c, d) in the supernatants of mock-infected and HCMV-infected HFF (AD169) (a, c) and SMC (TB40E) (b, d) cultures were quantified by immunoassay 48 h p.i. To inhibit enzymic ECM degradation, EDTA was added to parallel cultures. Results are shown as percentages (means ± SEM) of the mock-infected HFF or SMC cultures. Absolute concentrations (ng ml$^{-1}$; means ± SEM) for mock-infected cells are given above the columns. The data summarize two (HFF) and three (SMC) independent experiments with triplicate determinations. SMC from three different donors were used. The extent of collagen type I downmodulation following infection with the laboratory strain AD169 (HFF) or TB40E (SMC) was significant compared with mock-infected cells (Student’s $t$-test; $P < 0.0006$). Adding EDTA to the HCMV-infected SMC cultures led to a significantly (Student’s $t$-test; $P = 0.0009$) diminished reduction of soluble collagen type I, indicating that matrix-degrading phenomena are involved (d). In infected HFF, this effect was not observed (c).
EDTA antagonized the reduction of soluble collagen in HCMV-infected SMC cultures partially, but significantly ($P=0.0009$). This indicates that, in SMC, HCMV infection modulates matrix expression by influencing synthesis as well as degradation.

To analyse these findings further, we first performed indirect immunofluorescence on HCMV-infected SMC and HFF with and without added EDTA during infection. In HCMV-infected SMC cultivated with EDTA, the specific red fluorescence signal for insoluble extracellular collagen type I fibres (Fig. 5a) was significantly stronger compared with infected SMC cultures without inhibition of degradation ($P=0.01$) (Fig. 5b). In contrast, addition of EDTA to HCMV-infected HFF did not alter extracellular collagen type I staining noticeably (Fig. 5a). Comparing the numbers of cells positive for nuclear staining with the anti-viral IE1 antibody and with bisbenzimide demonstrated that, following infection with an m.o.i. of 1, all cells of the cultures were infected with HCMV (Fig. 5a) (Reinhardt et al., 2005a).

**HCMV infection induces MMP-2 activity in SMC**

To identify the activity responsible for the collagen degradation present in the supernatants of HCMV-infected SMC, zymographic assays were performed. An increase in gelatin-digesting activity corresponding to the molecular size of MMP-2 and activated MMP-2 was detected. HCMV-induced elevation of MMP-2 activity could be demonstrated in five independent experiments using SMC from different donors and two distinct HCMV isolates. Zymographs from representative experiments are shown (Fig. 6a). The activated form of the MMP-2 protein was quantified in supernatants from the same experiment using a commercial assay. A 2- to 3-fold increase compared with the uninfected control was observed within 72 h of HCMV infection (Table 1). These results corresponded well with the zymographic pattern (Fig. 6a; right panel). Interestingly, in supernatants from HCMV-infected HFF, a decrease rather than an increase in MMP-2 activity was detected by zymography, independent of the HCMV isolate used (Fig. 6b). Using an ELISA, we quantified MMP-2 protein in HFF and SMC following infection with AD169 and TB40E. In HCMV-infected SMC, we could show a 1.5- to 2-fold increase in MMP-2 protein concentration within 72 h p.i., whereas the amount of MMP-2 protein in HFF decreased to 60–80% when compared with mock-infected cells (Fig. 6c). Again, these results are in good accordance with the quantitative results obtained by immunoassay (Fig. 3). Thus, these data support the notion that, depending on the cell type, HCMV modulates different mechanisms that lead to a reduction of ECM proteins.

**DISCUSSION**

HCMV is a strictly species-specific virus, and animal CMVs differ in their biological behaviour and sometimes even in

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**Fig. 4.** Quantification of soluble matrix proteins in the supernatants of HCMV-infected and mock-infected HFF and SMC after neutralization of IFN-α and -β activity. Concentrations of soluble fibronectin (a, b) and collagen type I (c, d) in supernatants of mock-infected and HCMV-infected HFF (AD169) (a, c) and SMC (TB40E) (b, d) cultures were quantified by immunoassay 48 h p.i. The biological activity of IFN-α and -β was neutralized by addition of antibodies; irrelevant (irrel. Ab) as well as neutralizing antibodies were added at 1 μg ml$^{-1}$. Results are shown as percentages (means ± SEM) of mock-infected HFF or SMC cultures. The data summarize two independent experiments with triplicate determinations. SMC from different donors were used.
the function of homologous genes (Wagner et al., 2000). Thus, animal models using animal CMVs are not completely suitable to verify findings observed in vitro with HCMV. A wide spectrum of cell types, such as fibroblasts, endothelial cells and SMC, can be infected by HCMV in vivo (Sinzger et al., 1995). However, there are differences in susceptibility towards distinct HCMV strains and HCMV might interfere with different pathways of the cell machinery depending on the infected cell type. In experimental models, it is therefore of particular importance to use target cells that are relevant for pathogenesis in vivo. In order to meet this criterion, we included coronary artery SMC in our studies. Further, the HCMV strain used is important, since the commonly used laboratory strain AD169 carries large gene deletions and has a restricted cell tropism relative to clinical strains, which call into question its de facto role as a prototype (Gerna et al., 2003). Therefore, other, more ‘wild-type’-like HCMV strains, such as TB40E, have to be used in parallel when studying HCMV-induced modulations of cellular genes.

Our study shows that, early after HCMV infection, ECM gene expression and the amount of the respective proteins is reduced significantly, independent of the cell type and HCMV strain used. Neutralization of IFN-α and -β activity did not influence the HCMV-induced modulation of ECM protein expression, suggesting that the downregulation is due directly to the HCMV infection and not a response to IFN (Fig. 4) (Zimmermann et al., 2005). The data demonstrate that HCMV affects both synthesis and degradation of ECM only in SMC, which in fact proves differential gene modulations in different cell types by the same virus. To our knowledge, this is the first study to demonstrate an HCMV-induced effect on fibronectin and collagen gene expression and protein synthesis in SMC, a cell
type highly relevant for vascular disease. The reduction of ECM proteins is not just a uniform effect due to a generalized virus-induced shutdown of the host cell machinery. On the contrary, the expression of several cellular genes in SMC is even induced by HCMV, such as the functionally active vascular endothelial growth factor (VEGF) (Reinhardt et al., 2005b) or the platelet-derived growth factor receptor-β (PDGFR) (Reinhardt et al., 2005a). Further, proving the viability of HCMV-infected SMC, a greater responsiveness to PDGF stimulation (Reinhardt et al., 2005a) as well as an increase in chemotaxis (Streblow et al., 1999) could be shown in HCMV-infected SMC.

Whereas ECM has traditionally been regarded as an inert scaffold that merely provides mechanical support for functional cells within an organ or tissue, in recent years it has become increasingly evident that ECM regulates pivotal processes such as growth, death, adhesion, migration, invasion, gene expression and differentiation of cells. In addition, the sponge-like architecture of the ECM may serve as a reservoir for vasoactive growth factors, such as VEGF or PDGF (Streuli, 1999). Finally, modulating the amount or composition of ECM may be relevant for the detachment and spreading of cells that harbour infectious agents and for the pathogenesis of several disease entities, among them inflammatory processes of vessels. Vascular remodelling, consisting of degradation and reorganization of ECM, allows adaptation and repair-healing of adult vessels. Incorrect remodelling or lack thereof leads to vascular disease (Ivan et al., 2001). This process can result in a clinically stable (Schoenhagen et al., 2000) or unstable angina, the latter related to plaque instability and rupture (Cullen et al., 2003; Naghavi et al., 2003). A potential pathomechanism for plaque rupture appears to be a reduction of the ECM scaffold, especially of collagens that confer the tensile strength to the cap. Influences on SMC, the

Table 1. Quantification of MMP-2 activity in supernatants of HCMV-infected and mock-infected SMC

<table>
<thead>
<tr>
<th>Infection</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
<th>72 h p.i.</th>
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<tbody>
<tr>
<td>Mock</td>
<td>1.3</td>
<td>5.1</td>
<td>8.93</td>
</tr>
<tr>
<td>TB40E</td>
<td>3.44</td>
<td>15.6</td>
<td>17.35</td>
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Fig. 6. Evaluation of matrix digestive activity in supernatants of HCMV-infected HFF and SMC. (a, b) Detection of MMP activity by gelatin or casein zymography in the supernatants of mock-infected (m) and HCMV-infected (i) SMC (a) and HFF (b). Representative zymographic results from at least five independent experiments using SMC of different donors are shown. (c) The amount of total MMP-2 protein present in infected and mock-infected culture supernatants, obtained at indicated time points p.i., was quantified by ELISA. Results are given as percentages of mock-infected cultures at the same time point. Mean values ± SD of three independent experiments are shown.
main producer of collagens within the vessel wall (Brooke et al., 2003), are therefore of particular importance. In this light, our data demonstrating a substantial HCMV-induced loss of ECM in SMC (Figs 1, 2, 3 and 4) may represent a potential HCMV-induced pathomechanism towards a high-risk phenotype of instable atherosclerotic plaques, facilitating plaque rupture. The loss of matrix fibres in SMC was based on both a reduction in matrix gene expression and an increase in matrix degradation by MMP-2 activity (Fig. 6). Degradation of the ECM scaffold by specialized enzymes is an important prerequisite for the reshaping of tissue. Regarding vascular disease in vivo, local overexpression of matrix-degrading activity has been shown in the vulnerable shoulder regions of plaques (Galis et al., 1994). Disruption of the equilibrium of the ECM content in vivo is especially associated with an increase in MMP-2 activity (Pasterkamp et al., 2000) and seems to be related to plaque instability. Endothelial cells and SMC produce MMP-2 constitutively (Galis et al., 1994). By influencing matrix production and matrix degradation, HCMV infection of SMC may alter vascular remodelling in infectious vasculitis. In an artery organ culture model, we could identify SMC in the vascular media and intima as prime target cells of HCMV infection (Reinhardt et al., 2003), underlining the potential pathogenetic relevance of HCMV-induced modulations of gene expression in SMC.

Modulating the quantity and quality of ECM may also facilitate virus spread during primary and recurrent infections in the infected organism. A small number of highly infectious, HCMV-containing cells has been observed in circulation during active HCMV infection of immuno-suppressed patients, and it was proposed that circulating infected cells may serve as a vehicle for virus dissemination (Greffet et al., 1993). Here, we present an HCMV-induced molecular mechanism that potentially promotes cell rounding and loss of contact to the underlying tissue. As a consequence, HCMV-infected intima cells could detach from the vessel wall because of virus-induced reduction of ECM proteins, enter the blood circulation and thus lead to cell-bound virus spread.

In conclusion, we have shown that HCMV infection of SMC and HFF leads to a significant loss of collagen type I and fibronectin. Two independent, directly HCMV-induced mechanisms for ECM reduction were identified in SMC, being major players in the pathogenesis of vascular diseases: namely, an HCMV-associated decrease in matrix synthesis and an increase in matrix degradation through MMP-2. As for the pathophysiological relevance of this virus-induced effect, we can only hypothesize that the reduction of the ECM scaffold might on the one hand lead potentially to plaque instability, with all its clinical consequences, and on the other hand to a promotion of cell-associated, systemic virus spread.

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