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ORIGINAL ARTICLE

Active cytomegalovirus infection in aortic smooth muscle cells from patients with abdominal aortic aneurysm

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Abstract Cytomegalovirus (CMV) is associated with atherosclerosis and transplant vascular sclerosis. The aim of this study was to explore the hypothesis that active CMV infection in the vessel wall could be associated with abdominal aortic aneurysm (AAA). We examined the prevalence of CMV in AAA specimens from 22 patients undergoing surgery and, in five cases, characterized the function of smooth muscle cells (SMCs) from the aneurysm in vitro. Twenty-one (95%) of the 22 AAA specimens were CMV positive by a polymerase chain reaction assay, in situ hybridization, or a highly sensitive immunohistochemical staining technique. No positive cells were found in aortas from three CMV-seronegative organ donor cadavers. CMV immediate-early and late antigens were expressed in SMCs in the lesions and were associated with 5-lipoxygenase (5-LO) expression. CMV-positive intimal SMCs migrated 6.6±1.5 times more efficiently than CMV-negative medial SMCs ($p<0.05$). In vitro CMV infection of medial SMCs resulted in a 3.2±1.2 times increase in migration ($p<0.05$). The intimal migration was significantly inhibited by antibodies against basic fibroblast growth factor (bFGF; $p<0.05$) in a dose-dependent fashion. Antibodies against platelet-derived growth factor (PDGF)-AB, insulin-like growth factor 1, vascular endothelial growth factor (VEGF), RANTES, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, or interleukin-1β did not significantly affect intimal SMC migration. However, intimal and medial SMCs secreted similar amounts of bFGF, MCP-1, MIP-1α, RANTES, PDGF-AB, PDGF-BB, epidermal growth factor, and VEGF. CMV infection in vitro of intimal and medial cells did not result in significant changes of bFGF or MCP-1 secretion. Since CMV infection can affect several functional parameters in SMCs, including several key factors in infected SMCs, our findings provide support for the hypothesis that CMV contributes to the pathogenesis of abdominal aortic aneurysm.

Keywords Abdominal aortic aneurysm · Cytomegalovirus · Atherosclerosis · Smooth muscle cell

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Introduction

Aortic aneurysm is a major disease that most frequently involves the abdominal aorta. Aneurysms generally expand

without causing symptoms until rupture or dissection occurs, resulting in high morbidity and mortality [1]. Abdominal aortic aneurysm (AAA) is associated with atherosclerosis in a complex manner. Some risk factors for atherosclerosis are also risk factors for AAA, such as smoking, hypercholesterolemia, and age, whereas others, such as diabetes, are not [2, 3].

Microorganisms such as *Treponema pallidum*, *Chlamydia pneumoniae*, and cytomegalovirus (CMV) have also been implicated in the pathogenesis of aortic aneurysm. In particular, *C. pneumoniae* has been identified in abdominal aortic aneurysms in several studies using a variety of techniques, and increased titers of antibodies against *C. pneumoniae* have been correlated with aneurysmal expansion [4]. However, the role of *C. pneumonia* in abdominal aortic aneurysms is controversial, and intervention trials with macrolides have shown limited benefit [4, 5].

CMV, a herpesvirus that establishes latency after primary infection, generally causes a subclinical infection but may cause severe disease in immunocompromised patients. Increasing evidence suggests that CMV is associated with the development of atherosclerosis, restenosis after coronary angioplasty in patients with chronic rejection, and increased risk of myocardial infarction in organ transplant recipients [6–8]. CMV infection has also been linked to abdominal aortic aneurysms [9, 10], more often with inflammatory than with atherosclerotic aneurysms. CMV establishes latency in myeloid lineage cells and reactivates by inflammation in macrophages [6, 11, 12]. In tissues, CMV primarily infects fibroblasts and endothelial cells [10], but CMV nucleic acids have been identified in atheromatous plaques from the abdominal aorta [9, 13, 14]. In particular, CMV DNA and immediate-early antigens appear to be present in different layers of the aorta, predominantly in the early stages of atherogenesis [15].

The evidence for a pathogenetic role of CMV in vascular diseases is strongest in transplant patients in whom epidemiological studies show that CMV increases the risk of chronic rejection and cardiovascular disease [16]. Prophylaxis with ganciclovir, a potent inhibitor of CMV replication, and CMV hyperimmune globulin reduces the risk of transplant vascular stenosis and delays its onset in heart transplant patients. Since CMV has been difficult to detect in affected organs, its effects are generally referred to as “indirect” [17].

A number of molecular mechanisms for the indirect effects of CMV have been proposed [8]. The profound impact on cellular functions in CMV-infected smooth muscle cells (SMCs), macrophages, and endothelial cells may explain how CMV can contribute to the development of vascular diseases. CMV can produce over 250 proteins in infected cells, but only 50–60 are essential for viral replication. Thus, the vast majority of CMV proteins are devoted to controlling cellular and immunological functions that could contribute to disease pathogenesis. For example, CMV can induce SMC migration, affect lipid metabolism, induce the expression of adhesion molecules, control the production of cytokines and chemokines, and induce inflammation by inducing expression of COX-2 and 5-lipoxygenase (5-LO) [18]. In vitro, CMV-infected endothelial cells are extremely thrombogenic [19] and might precipitate cardiovascular events. Consistent with this hypothesis, we found a higher percentage of blood cells positive for CMV RNA in patients with acute myocardial infarction than in controls (15% vs 2%) [20].

Recently, we developed a highly sensitive immunohistochemical staining (HSIS) technique that can identify active, low-grade CMV infection that is otherwise undetectable in tissue specimens [12]. Using this technique, we confirmed the presence of CMV in a majority of heart and kidney grafts in patients with chronic rejection [8]. The aim of this study was to explore the hypothesis that active CMV infection in the vessel wall could be associated with AAA. We used HSIS to assess the prevalence of CMV infection in aortic biopsies from patients undergoing surgery for abdominal aneurysm. We also performed functional analyses of SMCs in cultures established from different layers of the lesions of five patients. Our findings provide further support for a pathogenetic role of CMV in vascular diseases.

Materials and methods

Patient materials

Biopsies of abdominal aortic aneurysms were obtained from 22 patients undergoing surgery at Karolinska University Hospital, Uppsala University Hospital and Xavier Bichat Hospital. Fresh specimens were obtained from patients 1–7; paraffin-embedded tissue was available from patients 8–22. The paraffin-embedded samples from Xavier Bichat Hospital were collected by INSERM U698, 75018 Paris, France and declared to the French Ministry of Research as a human tissue collection. Biopsy specimens from three CMV-seronegative organ donor cadavers served as negative controls. The study was approved by the ethical committees at Karolinska University Hospital (98/414, 08/518-31), Uppsala University Hospital (2005:182), and Xavier Bichat Hospital (CPP Paris-Cochin no. 2095, September 2003).

Cells

SMCs were obtained from the aneurysm specimens from patients 1–7 and from the aortas of the three CMV-seronegative controls. SMCs from patients 1–7 were

evaluated for the presence of CMV DNA by polymerase chain reaction (PCR). In situ hybridization and migration experiments were preformed using SMCs from patients 1–5. Endothelial cells were removed by scraping, and the samples were cut into 1-mm pieces. In each specimen, the intima and media were gently separated with tweezers to provide matched intimal and medial SMCs from the same patient for functional experiments. The inner surface of an AAA is covered with thrombosis which was removed from the specimen already in the operating room. Thus, the innermost layer of the vessel wall studied represented a pseudointima, potentially somewhat different from the normal vessel intima which is in contact with the arterial blood-flow. The tissues were then cultured in cell culture dishes (Falcon, Becton Dickinson) containing SmGM 2 medium (Clonetics, Cambrex Bio Science) supplemented with SmGM-2 Single Quot (Clonetics) for approximately 2 weeks until the SMCs migrated from the explants. SMCs from the control specimens were obtained by collagenase-1 treatment (Worthington Biochemical Corporation). The cells were trypsinized (Gibco BRL) and cultured in complete SmGM-2 medium in cell culture flasks (Costar, Corning). Experiments were performed on cells at passages 5–8.

The purity of SMC cultures was confirmed by staining for the SMC-specific marker α-actin (Sigma) and with antibodies against von Willebrand's factor (vWf, Roche Diagnostics), an endothelial cell-specific marker. Examination by fluorescence microscopy showed that all cultures were positive for α-actin and negative for vWf (not shown), indicating that all cells were of pure SMC origin.

Detection of CMV DNA replication in SMCs

DNA was prepared from SMCs at passages 4–8, from patients 1–7, and analyzed by PCR assay with primers specific for the CMV major immediate-early (MIE) and pp150 genes as described [21]. Primers specific for glucose-6-phosphatase dehydrogenase (G6PD) were used as a positive control. DNA samples from CMV-infected and uninfected fibroblasts were included as positive and negative controls, respectively, as were frequent water controls without DNA. PCR products were visualized on 1.5% agarose gels. To assess the sensitivity of the PCR assay semi-quantitatively, a known DNA concentration of a vector containing the MIE gene (Bluescript/MIE, kindly provided by Dr. Jay A. Nelson, Portland, OR, USA) was serially diluted and used as a template in the PCR assay. The lowest concentration resulting in a positive PCR result gave the cutoff value for the sensitivity of this assay, which allowed for a detection of as few as one to ten copies of MIE DNA. All samples were run in triplicate and were considered positive if at least one was positive for MIE or pp150 in the PCR assay.

In situ hybridization

In situ hybridization was performed on SMC from patients 1–5. CMV-DNA was purified from fibroblasts infected with human CMV strain AD169, and the PCR assay was performed with primers targeting the fourth exon of the IE1 J fragment of AD169 (CMV-DNA and primers were kindly provided by Dr. Maria Brytting, Swedish Institute for Infectious Disease Control). A single-round PCR assay was performed as described [22]. The 616-bp amplimers were labeled with biotin-16-dUTP (Roche Diagnostics) by random priming according to the manufacturer's protocol.

Next, the fixed cells were denatured at 80°C for 10 min in the presence of 5 ng/μl biotin-labeled probe (616 bp) for immediate-early (IE) gene, diluted in hybridization solution consisting of 50% formamide, 5% salmon sperm DNA (10 mg/ml), 10% 20× saline–sodium citrate (SSC), 2% 50× Denhardt's solution (Sigma), and 33% Tris–EDTA, pH 7.4 (1× TE) and hybridized at 37°C overnight under sealed coverslips. After hybridization, the slides were washed twice for 15 min each in 4× SSC at 42°C, twice in 2× SSC at room temperature, and twice in 0.2× SSC at 50°C. The slides were then incubated in 100 mM Tris, pH 7.5/ 50 mM NaCl containing 1% blocking reagents (Roche Diagnostics) at room temperature for 30 min and then in streptavidin–alkaline phosphatase diluted in dilution buffer containing 100 mM Tris–HCl, pH 7.5, 1% bovine serum albumin, and 0.5% blocking reagent (Roche Diagnostics) for 1 h at room temperature. After washing, the cells were incubated for 1 h in substrate solution consisting of 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide/0.075 mg/ml 4-nitroblue tetrazolium in 70% dimethylformamide in 100 mM Tris, pH 9.5/100 mM NaCl/50 mM $MgCl_2$. This reaction was stopped by rinsing cells in distilled water three times for 5 min each. The cells were counterstained for 10 min in eosin and mounted in Kaisers glycerol gelatin (Merck).

CMV-infected SMCs served as a positive control. A beta-actin DNA probe (Panomics) was used as an internal control for DNA integrity and yielded a positive signal in all cells. A human papilloma virus DNA probe (Dakopatts) was used as a negative control.

HSIS of tissues

HSIS was performed on abdominal aortic aneurysm biopsy specimens from patients 8–22 [23]. Briefly, paraffin sections (4 μm) were dewaxed, hydrated, and digested with pepsin. After antigen retrieval in citrate

buffer, the sections were treated with hydrogen peroxide (Innovex Sciences), avidin, and biotin (Dako) and then with an Fc receptor blocker (Innovex Sciences). The sections were then incubated with the following antibodies: mouse anti-human CMV immediate early antigen (IEA) (Chemicon), mouse anti-human CMV late antigen (LA) (Chemicon), mouse anti-human SMC α-actin (Dako), rabbit anti-human 5-LO (Abcam), mouse anti-human platelet-derived growth factor receptor (PDGFR)-β (R&D Systems), and mouse anti-human vWf (Dako). Seven of the biopsies were also incubated with mouse anti-human MCP-1 (Dako) and goat anti-human RANTES (R&D Systems). As a negative control, slides were incubated without primary antibodies. Visualization was achieved using horseradish peroxidase detection system (BioGenex) with the chromogen diaminobenzidine (Innovex Sciences). Finally, the slides were counterstained with hematoxylin (Sigma-Aldrich) and mounted in permanent mounting medium (Dako).

Migration assay

The migration assay was performed with SMCs from patients 1–5. For migration assays, a transwell system with an insert filter (12-mm diameter, 3.0-μm pore size; Costar, Corning, USA) was used as described [24]. Briefly, 10^5 SMCs were seeded on top of the filter and cultured for 3 days, the filters were transferred to a new well, and the cells were used for experiments. At this point, a group of medial SMC isolated from the patients were also infected with the CMV strain Towne at a multiplicity of infection (MOI) 0.3. Four to five days after antibody treatment or infection, the filters were removed, and the cells in the lower compartment were counted in five fields at 4× magnification by light microscopy.

Antibody blocking studies of SMC migration

To analyze intimal SMC migration, we performed blocking studies with antibodies against basic fibroblast growth factor (bFGF), PDGF-AB, insulin-like growth factor (IGF)-1, vascular endothelial cell growth factor (VEGF), RANTES, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, interleukin (IL)-1β, and isotype control goat IgG (R&D Systems) at concentrations of 0.5–50 μg/ml. The maximum antibody concentrations (μg/ml) were 0.5 for IL-1β, 5 for insulin-like growth factor 1 (IGF-1), 10 for VEGF, 20 for bFGF, 40 for PDGF-AB, and 50 for RANTES, MCP-1, and MIP-1α. The antibodies were added to both the upper and lower chamber wells when the inserts were transferred to new plates. Migration was evaluated after 4 days. All the experiments were performed in duplicate with aneurysm samples from three patients. Results are presented as percent of control.

Evaluation of cytokines and chemokines produced by SMCs

Cytokine and chemokine secretion was analyzed with an enzyme-linked immunosorbent assay (ELISA). To obtain cell-free supernatants for the assay, intimal and medial SMCs were grown to 80% confluency. Supernatants were collected after 6, 24, and 48 h and 4 and 7 days and used in SMC cultures. The cells were counted on days 1, 2, 3, and 7. Proliferation was greatest in supernatants from 24-h cultures (data not shown). Therefore, supernatants were collected at 24 h, centrifuged for 5 min at 1,500 rpm, and stored at −70°C. A group of SMCs isolated from the patients were also infected with the CMV strain Towne at an MOI of 0.3. ELISA kits for MCP-1, MIP-1α, RANTES, PDGF-AB, PDGF-BB, bFGF, epidermal growth factor (EGF), VEGF (R&D Systems), and IGF-1 (Diagnostic Systems Laboratories) were used according to the manufacturer's protocol.

Statistics

The results are shown as average ± standard error of mean. Unpaired Student's *t* test was used for evaluating significance, and a p value of <0.05 was regarded as statistically significant. Statistical significance is indicated by an asterisk in the figures.

Results

Abdominal aortic aneurysms are CMV-infected

Twenty-one of the 22 specimens of abdominal aortic aneurysm were CMV-positive by PCR, in situ hybridization, or HSIS. In 14 of the 15 AAA specimens analyzed by HSIS, CMV IEA and LA were widely expressed in inflammatory cells and SMC-like cells (Figs. 1 and 6a,b, e,f). Stored serum samples were available from ten of the 22 patients. Nine of the samples were positive for CMV IgG in serology tests, and one was negative; the aortic biopsy specimen from the latter patient was CMV-positive.

SMCs from abdominal aortic aneurysms are CMV-infected

Intimal and medial aortic SMCs from the aneurysm specimens of patients 1–7 and from seronegative control aortas were cultured. All isolated cells were positive for α-actin and negative for vWf (not shown), indicating pure SMC

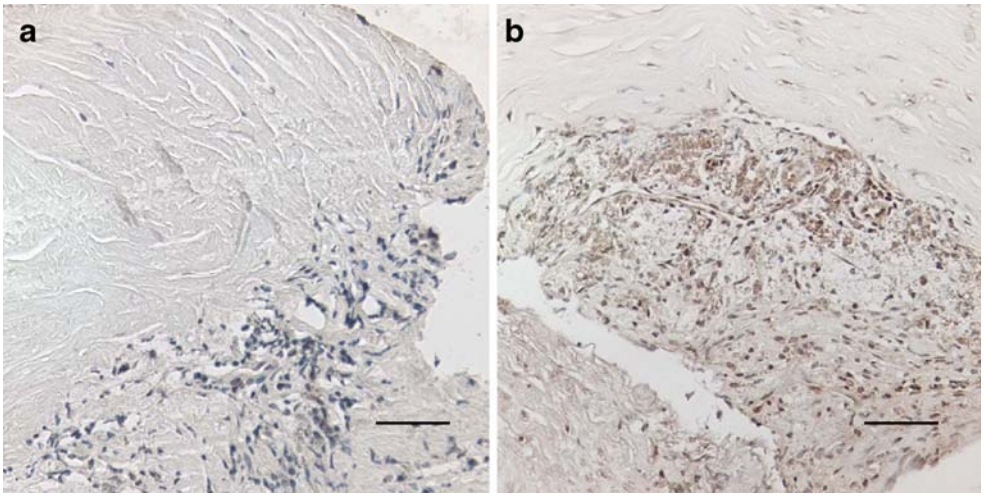

Fig. 1 HSIS reveals CMV antigens in tissue sections of abdominal aortic aneurysm specimens. Abdominal aortic aneurysm sections from two different patients stained for CMV IEA reveal one CMV IEA-negative specimen (**a**) and one representative CMV IEA-positive specimen out of 14 (**b**). The *size bars* indicate 100 μm

origin. A CMV-specific PCR assay that detects as few as one to ten copies of the viral genome showed CMV DNA (pp150 or MIE) in SMC cultures from all seven patients, but not in cultures of control SMCs (Fig. 2).

Increased migration of CMV-infected intimal SMCs

Since CMV can induce SMC migration, we compared the migration of intimal and medial SMCs from patients 1–5. Analysis after 7 days of culture in a transwell system showed that the intimal SMCs migrated 6.6±1.5 times more efficiently ($p<0.05$) than medial SMCs (Fig. 3). To investigate the cause of the increased migration, we determined the percentage of SMCs that contained CMV DNA by in situ hybridization. CMV-infected SMCs served as positive control. As an internal control of DNA integrity, a beta-actin DNA probe was used and yielded a positive signal in all cells. A human papilloma virus DNA probe was used as negative control and did not yield any positive signals (Fig. 4). In situ hybridization demonstrated CMV DNA in 42.0±10.2% of intimal SMCs, but not in medial SMCs in cultures established from the five patient samples (Fig. 4). CMV DNA was not found in control SMCs.

To determine if CMV infection results in increased migration, we infected the medial SMCs with CMV. The SMCs from patients were more sensitive to CMV infection than in our previous experience using SMCs from diseased healthy donors [24]. Here, we used a multiplicity of infection of only 0.3. Infection of the medial SMCs resulted in a 3.2±1.2 times more efficient migration when compared to uninfected medial SMCs ($p<0.05$; Fig. 3).

Increased migration of intimal SMCs is mediated by bFGF

To determine if a specific growth factor, cytokine or chemokine, mediated the increased migration of intimal SMCs, we performed antibody blocking studies. Neutralizing antibodies against bFGF, PDGF-AB, IGF-1, VEGF, RANTES, MCP-1,

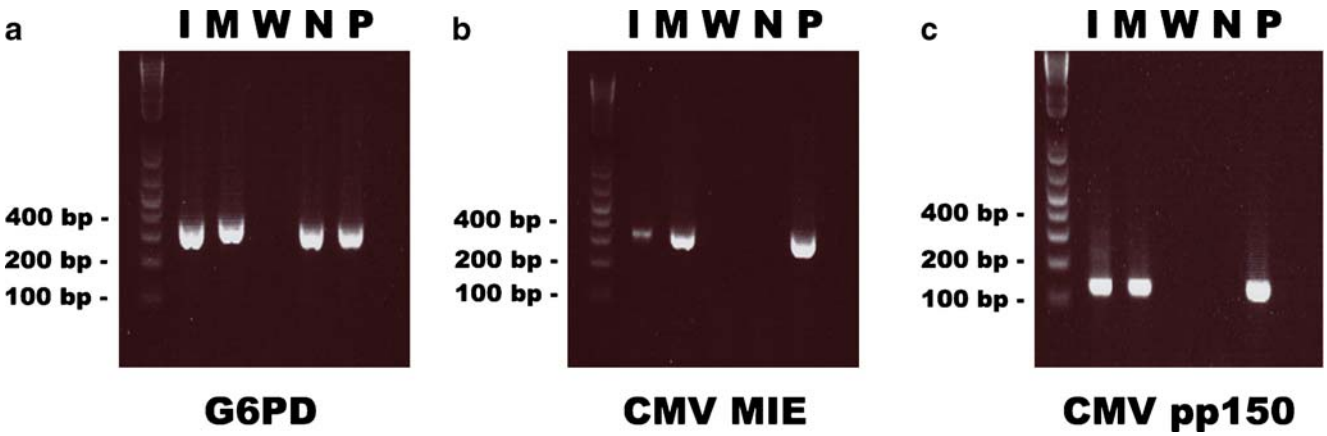

Fig. 2 CMV infection in SMCs from a representative abdominal aortic aneurysm sample. This sample was run in triplicate, and one positive reaction is shown in the figure. Results of PCR assay using DNA as a template and primers specific for G6PD (272 bp) (**a**), MIE (332 bp) (**b**), and pp150 (150 bp) (**c**). A 1-kb Plus DNA ladder is shown on the *left of each panel* as a size marker. *I* intimal SMCs, *M* medial SMCs, *W* water control, *N* negative control (uninfected fibroblasts), *P* positive control (CMV-infected fibroblasts)

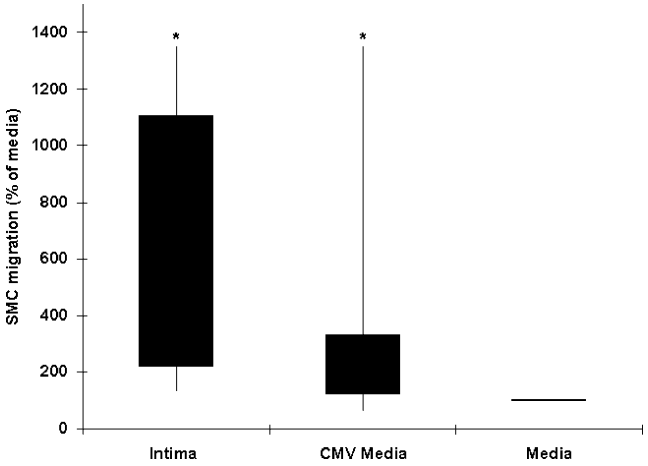

Fig. 3 CMV-positive intimal SMCs migrate 6.6±1.5 times more efficiently ($p<0.05$) than uninfected medial SMCs in vitro. When the medial SMCs were CMV-infected in vitro, they migrated 3.2±1.2 times more efficiently as compared to uninfected medial cells ($p<0.05$). SMCs isolated from the intima and media of abdominal aortic aneurysms were allowed to migrate in a transwell system. Cells that migrated through the filter were counted on day 7 after seeding. All the experiments were performed in duplicate with aneurysm samples from five patients. Statistically significant differences ($p<0.05$) are marked by an *asterisk*

MIP-1α, or IL-1β were added to the culture medium when the filters were transferred to a new well and were present in the culture medium throughout the experiment (Fig. 5). Anti-bFGF (20 μg/ml) reduced the migration of intimal SMCs to 38.9±5.6% of control ($p<0.05$) in a dose-dependent fashion (Fig. 5). None of the other observed changes in migration were statistically significant.

No significant changes were observed in the pattern of chemokine and cytokine production

Previous studies suggested that CMV changes the pattern of chemokine and cytokine production (e.g., MCP-1, MIP-1α, and RANTES) [25–27]. We therefore measured, by ELISA, the production of MCP-1, MIP-1α, RANTES, PDGF-AB, PDGF-BB, bFGF, EGF, and VEGF by intimal and medial SMCs of patients 1–5. Individual differences were observed in the secretion of MCP-1 (see Electronic supplementary material), which is involved in monocyte recruitment and SMC migration and is believed to be one of the major chemokines expressed in atherosclerotic plaques [28, 29]. However, based on the whole population ($n=5$), there were no statistically significant differences in MCP-1 secretion. No differences in the secretion of the other growth factors or chemokines were observed (Table 1).

In order to further elucidate the effects of CMV on MCP-1 and bFGF secretion, we infected intimal and medial SMCs and subsequently measured MCP-1 and bFGF secretion. CMV infection did not result in statistically significant differences in secretion of MCP-1 (see Electronic supplementary material) or bFGF (data not shown).

CMV infection in aortic aneurysms is associated with 5-LO expression and inflammation

We recently showed that CMV infection induces expression of 5-LO in SMCs and mediates the recruitment of inflammatory cells around infected vessels [18]. To confirm the presence of 5-LO in aortic aneurysms, 15 biopsies were

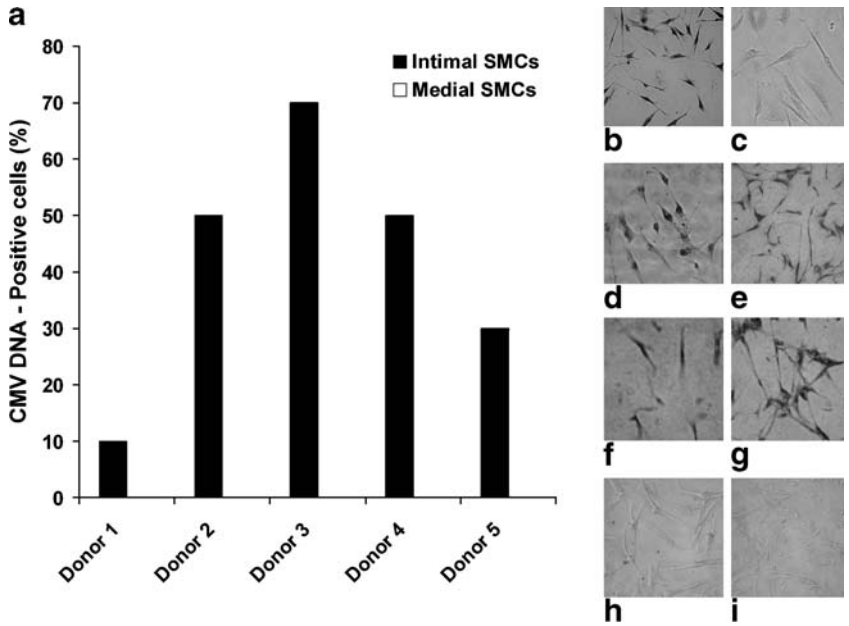

Fig. 4 Intimal but not medial SMCs are positive for CMV by in situ hybridization. Percentages of CMV-positive SMCs in patients 1–5; values are averaged from two independent experiments (**a**). Photographs of in situ hybridizations with the CMV probe show CMV DNA (**b**) in 70% of intimal SMCs and 0% of medial SMCs (**c**). In situ hybridizations with the CMV probe in CMV-infected intimal (**d**) and medial SMCs (**e**). In situ hybridizations with the beta-actin DNA probe in intimal (**f**) and medial (**g**) SMCs. In situ hybridizations with the human papilloma virus DNA probe in intimal (**h**) and medial (**i**) SMCs. All photographed SMC samples were from donor 3

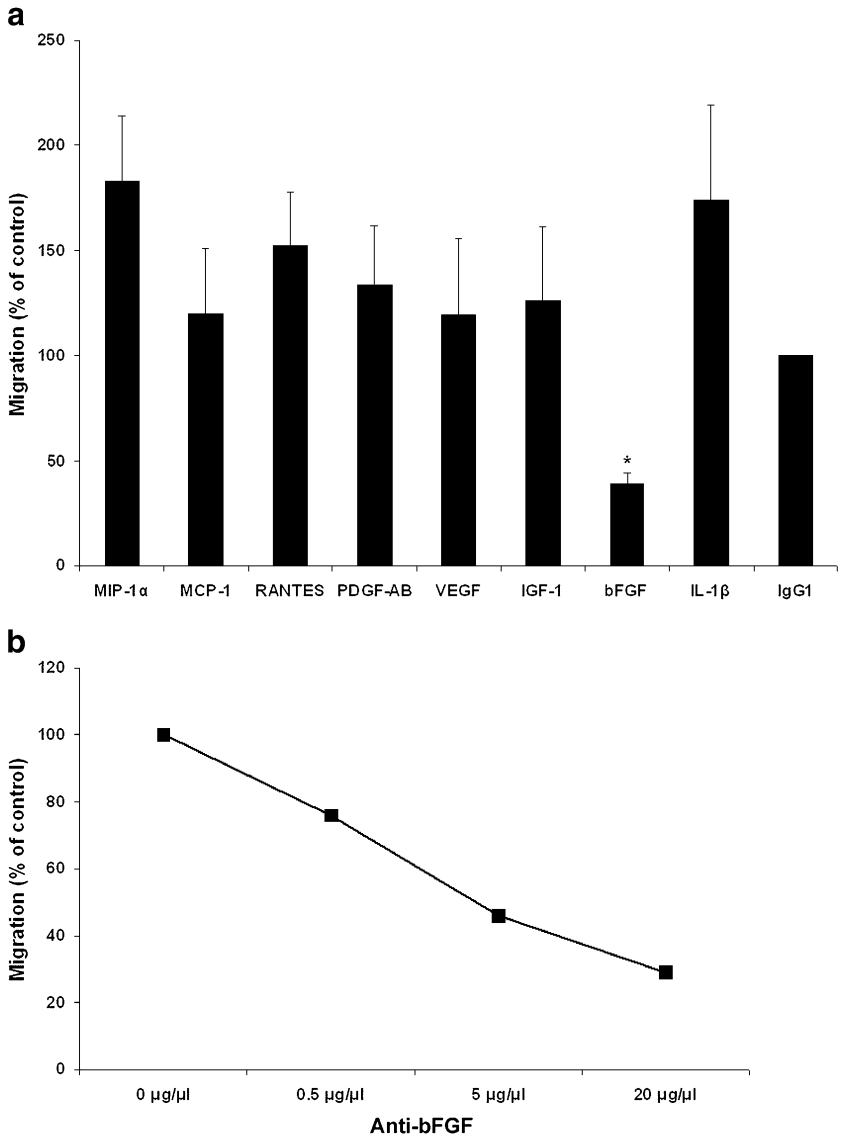

Fig. 5 bFGF-Specific antibodies inhibit SMC migration in transwell migration assay. **a** Antibodies against MIP-1α, MCP-1, RANTES, PDGF-AB, VEGF, IGF-1, IL-1β, or bFGF were added to the cultures when the filter was transferred to a new well. Antibodies against bFGF significantly reduced intimal SMC migration ($p<0.05$). None of the other observed changes were statistically significant. All the experiments were performed in duplicate with aneurysm samples from three patients. **b** Antibodies against bFGF inhibited SMC migration in a dose-dependent fashion. A representative graph from one patient is shown

stained with antibodies against 5-LO, PDGFR-β, vWf, and α-actin. Seven of the biopsies were also stained for MCP-1 and RANTES. We found high expression of 5-LO in areas with high reactivity for CMV IEA and LA that contained large inflammatory infiltrates. In contrast, only a few scattered cells expressed RANTES (not shown) MCP-1 or PDGFR-β (Fig. 6).

Table 1 Chemokine and growth factor secretion in SMC cultures from patients 1–5 as measured by ELISA

Chemokine/growth factor	Intimal SMCs	Medial SMCs
RANTES	None	None
MIP-1α (ng/ml)	0.01–0.05	0.01–0.05
PDGF-AB	None	None
PDGF-BB	Low	Low
EGF	None	None
IGF-1 (ng/ml)	10–40	10–40
VEGF (ng/ml)	1.4–1.7	1.25–2.1
bFGF (pg/ml)	<100	<100
MCP-1 (pg/ml)	430–1797	657–2062

Discussion

In this study, we observed widespread active CMV infection in areas of abdominal aortic aneurysms that contained numerous cells expressing 5-LO and in few scattered cells expressing MCP-1. These observations support the theoretical ability of CMV to induce and maintain inflammation. In addition, we found that SMCs persistently infected with CMV migrated more efficiently than uninfected SMCs from the same aortic lesion. These infected cells also exhibited individual differences in the production of MCP-1.

LTB4 and MCP-1 are suggested to be driving forces in the recruitment of inflammatory cells and SMCs into the growing plaque. In support of this hypothesis, MCP-1 is a

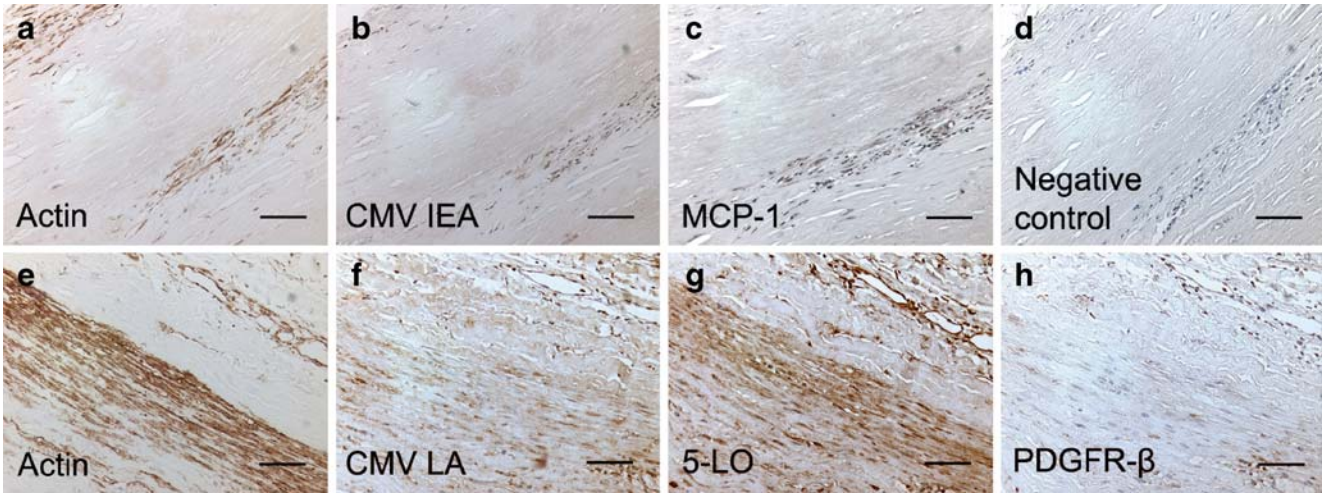

Fig. 6 HSIS reveals CMV antigens and expression of 5-LO and MCP-1 in tissue sections of abdominal aortic aneurysm specimens. **a–d** Sections from one patient stained for α-actin (SMC-marker) (**a**), CMV IEA (**b**), and MCP-1 (**c**); a negative control without primary antibody (**d**) showed no staining with diaminobenzidine. **e–h** Sections from another patient stained for α-actin (**e**), CMV LA (**f**), 5-LO (**g**), and PDGFR-β (**h**). The *size bars* indicate 100 μm

strong factor for recruitment of inflammatory cells into the vascular wall [28, 29] and is crucial for CMV-induced SMC migration in vitro. Human and murine CMV has also been shown to induce the production of MCP-1 [27, 30]. In the present study, we found individual differences in the production of MCP-1 in CMV-infected SMCs. In the aortic biopsies, inflammatory cells in particular expressed MCP-1. These cells were not maintained in the SMC cultures, but may play a pivotal role in CMV-induced inflammation in vivo. Consistent with this hypothesis, we identified MCP-1 as a crucial factor in the rejection of human and animal heart grafts (submitted manuscript). Thus, MCP-1 and LTB4 could contribute to the inflammatory process of atherosclerosis [31, 32] and aneurysm formation and at the same time help establish a microenvironment conducive for efficient replication of CMV in tissue macrophages. This situation would most likely further exacerbate the inflammatory process. AAA tissue is characterized by inflammation with abundance of activated macrophages [33], which is consistent with our findings.

Another critical factor in the development of vasculopathy is the infiltration and proliferation of SMCs in the vascular intima. Previously, we demonstrated that CMV infection induces SMC migration in vitro that is dependent on expression of the viral chemokine receptor homologue US28 [24], which can bind to MCP-1, RANTES, and MIP 1-α. In our in vitro functional studies, intimal SMCs from the abdominal aortic aneurysms were infected with CMV and migrated 6.6±1.5 times more efficiently than medial SMCs from the same vessel, which were CMV-negative by in situ hybridization. In contrast, in the PCR assay, both intimal and medial SMCs were positive for CMV. This is likely due to the higher sensitivity of the PCR assay which is able to detect as few as one to ten CMV copies. However, it is unlikely that a CMV infection of SMCs that is only detectable by nested PCR has any biological significance.

Since SMCs generally do not migrate without a specific stimulus, we speculate that the intimal SMCs underwent a phenotypic change, possibly induced by CMV infection, providing the requirements for the induced SMC migration of infected intimal SMCs as compared to the non-infected medial SMCs. Indeed, CMV infection of medial SMCs resulted in a 3.2±1.2 times increased migration. These experiments were performed with cells from five AAA patients with high inter-individual variability in migration, and therefore, it is difficult to predict the relevance of CMV in the pathogenesis of AAA. It is plausible that the migration differences observed between intimal and medial SMCs also were affected by other factors in the environment in vivo, and thereby, the migration induction observed may not be mainly dependent on CMV. Unfortunately, we did not have access to the pivotal control of intimal and medial SMCs from CMV-negative AAA patients.

We previously reported that US28-mediated SMC migration is dependent on endogenous MCP-1 production. Therefore, we investigated whether the increased secretion of MCP-1 in some of the persistently infected SMC cultures might explain the enhanced migration of intimal SMCs. However, neutralizing antibodies against MCP-1 did not affect SMC migration, which was instead reduced by neutralizing antibodies against bFGF ($p<0.05$) in a dose-dependent fashion. Although we did not observe induced production of bFGF or MCP-1, CMV has been described to enhance the production of both MCP-1 and bFGF [27]. It is possible that secretion of bFGF was increased and that the secreted bFGF was immediately taken up by the SMCs.

Consistent with this possibility, neutralizing antibodies against bFGF blocked SMC migration.

In our previous experience, the effect of CMV infection on SMC migration varies tremendously between SMCs obtained from different vessels from deceased otherwise healthy donors [24]. The CMV-induced SMC migration levels observed in the AAA patients in our study are much lower than those we previously observed using SMCs from healthy donors [24]. It is plausible that the SMCs from AAA patients have an impaired ability to migrate due to their disease. We also noted that these SMCs were more sensitive to CMV infection in vitro; a much lower multiplicity of infection (0.3 versus 10) was therefore used in the present study. This may explain the lower CMV-induced SMC migration levels observed. We were not able to block migration by adding neutralizing antibodies to MCP-1 and did not observe a consistent pattern in MCP-1 secretion from intimal and medial cells. Therefore, we did not further investigate the role of US28 expression in these patients.

The presence of CMV DNA in aortic aneurysms and in atherosclerotic lesions does not provide direct evidence for CMV as a causative agent. However, our HSIS technique for the first time provided evidence of widespread, low-grade active CMV infection in 14 of the 15 abdominal aortic aneurysms. We also observed functional differences between persistently infected SMCs and non-infected SMCs from the same aortic lesions. Thus, the potent ability of CMV to change the local environment in the vessel wall and its unique ability to control cellular and immunological functions make it a candidate in the pathogenesis of aortic aneurysms.

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