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STUDIES OF THE ROLE OF CYTOMEGALOVIRUS INFECTION IN INFLAMMATION AND CANCER

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Abstract

Human cytomegalovirus (HCMV) is a widespread disease-causing agent belonging to the herpesvirus family. After a primary infection HCMV establishes latency within its host, from which the virus may reactivate throughout life. When healthy individuals become infected by HCMV, they rarely show any symptoms, but in some cases individuals may experience symptoms such as headache, fever, a sore throat and muscle pain. In contrast, individuals with a suppressed immune system, due to medical treatment or disease, may experience severe disease or even death. HCMV-infection of fetuses, acquired during the development in the uterus, is the world leading infectious cause of birth defects. This type of infection might cause damage to visual and hearing senses as well as damages to the central nervous system with manifestations such as mental disorder and hearing loss. Furthermore, HCMV has been suggested to play a role in certain types of cancer. HCMV thrives and reactivates in parts of the body where there is an ongoing inflammation. The site of inflammation offers an environment rich in molecules, which attract latently infected blood cells into inflammatory tissues and reactivates latent HCMV. Because of that, we were interested to study the role of HCMV-infection in connection to inflammation and cancer.

I focused on understanding molecular mechanisms of HCMV-pathogenesis and found that: i) HCMV decreases the expression of PDGFR's, which may play an important biological role in congenital HCMV-infection and embryonic development. ii) HCMV induces 5-LO mRNA and protein expression in vascular SMC's, enabling these cells to synthesize LTB_4, which offers a molecular mechanism to HCMV-mediated pathogenesis in inflammatory diseases. iii) HCMV alters the balance between MMP-9 and TIMP-1 in macrophages, which may affect atherosclerotic plaque development and stability. iiii) HCMV induces telomerase activity in human fibroblasts and glioma cell lines. This phenomenon is mediated by the presence of IEA at the hTERT proximal promoter, the recruitment of Sp1, decreased HDAC-1 and -2 promoter binding and H3 acetylation. Our findings provide a novel mechanism that may explain how HCMV induces oncogenesis, a mechanism that may be critical in the understanding of the relationship between HCMV and cancer.

List of publications

I. Gredmark S[*], **Strååt K[*]**, Homman-Loudiyi M, Kannisto K, Söderberg-Nauclér C.
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Erratum in: J Virol. 2007 Jul;81(14):7822.
Copyright © American Society for Microbiology, The Journal of Virology, Volume number 81(10), Page numbers: 5112-5120, 2007.

II. Qiu H[*], **Strååt K[*]**, Rahbar A, Wan M, Söderberg-Nauclér C[*], Haeggström JZ[*].
Human CMV infection induces 5-lipoxygenase expression and leukotriene B4 production in vascular smooth muscle cells.
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III. **Strååt K**, de Klark R, Gredmark-Russ S, Eriksson P[*], Söderberg-Nauclér C[*].
Infection with human cytomegalovirus alters the MMP-9/TIMP-1 balance in human macrophages.
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IV. **Strååt K[*]**, Liu C[*], Rahbar A, Zhu Q, Liu L, Wolmer-Solberg N**,** Lou F, Liu Z, Shen J, Jia J, Kyo S, Björkholm M, Sjöberg J, Söderberg-Nauclér C and Xu D.
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[*] These authors contributed equally.

List of abbreviations

5-LO	Five-lipoxygenase
AA	Arachidonic acid
APC	Antigen presenting cell
Ca^{2+}	$Calcium^{2+}$
CD	Cluster of differentiation
cDNA	Complementary DNA
CIITA	Class II major histocompatibility complex transactivator
CMV	Cytomegalovirus
CNS	Central nervous system
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
CVD	Cardiovascular disease
DAPI	4', 6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase
ds	Double stranded
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
FLAP	Five-lipoxygenase activating protein
g(x)	Glycoprotein (x)
GBM	Glioblastoma multiforme
HAT	Histone acetyltransferase
HCASMC	Human coronary arterial smooth muscle cell
HCMV	Human cytomegalovirus
HDAC	Histone deacetylases
HDF	Human diploid fibroblasts
HHV	Human herpes virus
HPASMC	Human pulmonary arterial smooth muscle cell
HSPG	Heparan sulphate proteoglycans
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
hTR/TERC	Human telomerase mRNA component
IBD	Inflammatory bowel disease
IEA	Immediate early antigen

IE	Immediate early
IL	Interleukin
INF	Interferon
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
kbp	Kilo base pair
kDa	Kilo Dalton
L	Late
LTA_4H	Leukotriene A_4 hydrolase
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTC_4S	Leukotriene C_4 synthase
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
miR	Micro RNA
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MG	Malignant glioma
NF-κB	Nuclear factor kappa-B
NK-cell	Natural killer cell
nm	Nano meter
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI(3)K	Phosphoinositide-3-kinase
pp	Phospho protein
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SS	Sjögrens syndrome
TAP	Transporter of antigenic peptides
T-cell	Thymus cell
T_C-cell	Cytotoxic T-cell
T_H-cell	T-helper cell
TIMP	Tissue inhibitor of metalloproteinase
TSA	Trichostatin A
TVS	Transplant vascular sclerosis

UL	Unique long
US	Unique short
VZV	Varsiella zoster virus

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1. Introduction

1.1 Herpes viruses

Members of the herpes virus family, *Herpesviridae*, have an icosadeltahedral capsid structure, built up by 162 capsomeres, with a diameter of approximately 100-110 nm. The herpes virus genome, which consists of double stranded linear DNA (dsDNA), is located within the capsid structure. A tegument layer described to be asymmetric and amorphous like surrounds the capsid itself. Finally, a lipid layer referred to as the envelope encapsulates the capsid and the tegument. The envelope has protruding proteins that are constituted by both viral and host derived proteins. *Herpesviridae* has been classified into three different subfamilies, the *Alphaherpesvirinae*, *Betaherpesvirinae* and the *Gammaherpesvirinae* subfamilies. All herpes viruses seem to share four properties of biological importance: 1. They all have enzymes important in the metabolism of nucleic acid; however, the number and kind of enzymes may differ between the different herpes viruses. 2. Viral DNA synthesis and capsid assembly takes place in the nucleus. 3. The formation of new infectious viral particles is consistently accompanied by the destruction of the host cell. 4. They all share the property to establish latency within its host, and also share the ability to reactivate from latency. During latency, there are no infectious viral progeny produced.

Herpes viruses are extensively distributed in nature and most animal species have experienced a herpes virus infection at some point during their life. There are approximately 130 herpes viruses identified to date, of which nine herpes viruses have been isolated from humans: the herpes simplex virus-1 (HSV-1), and -2 (HSV-2), human cytomegalovirus (HCMV), varciella zoster virus (VZV), Epstein-Barr virus (EBV) and human herpesviruses (HHV)-6A, -6B, HHV-7 and HHV-8.

Herpes viruses differ in their biological properties in a number of aspects, for example: their time to replicate, their respective host cell tropism, and their different clinical manifestations. The size of the mature virion could also differ among the *Herpesviridae* members, ranging from 120-300 nm in diameter. Also, the lengths of their

respective DNA strands differ, from approximately 120 to 250 kbp. Herpes viruses generally have the capacity to encode for 70-200 genes, (reviewed in Roizman, 2001), and HCMV has been described to encode 252 ORF's (Murphy et al., 2003). Herpesvirus-encoded microRNAs are not included in that estimation; however, herpesviruses express microRNAs. The function of these microRNAs is not fully investigated, but some microRNAs has been described to prevent apoptosis and NK cell-mediated killing or to coordinate viral gene expression, possibly during viral latency, (reviewed in Grey et al., 2008).

1.2 Human cytomegalovirus (HCMV)

1.3 History of HCMV

As early as 1904, Jesionek made a report about abnormal "protozoan-like" cells located in lungs, liver and kidney of a premature dead fetus (Jesionek, 1904). The same year, Ribbert reported that he had observed similar cells in the kidneys of a stillborn infant as early as 1881 (Ribbert, 1904). Ribbert´s report, about his findings in 1881, is probably the first documentation of a disease caused by human cytomegalovirus (HCMV). During the following years these abnormal "protozoan-like" cells was described in various organs, and the similarity between the cytopathology with other herpesviruses and these abnormal cells was noted, but was still believed to be caused by *protozoa*. Löwenstein, at that time working in Ribbert´s laboratory, described that these "protozoan-like" cells had cytoplasmic and intranuclear inclusions surrounded by a clear zone (Löwenstein, 1907). In 1921, Goodpasture and Talbot used the term *cytomegalia* to describe the features of these abnormal cells in lesions of infancy (Goodpasture EW, 1921). Goodpasture and Talbot did not agree with the current opinion that the inclusions were caused by a protozoan. Later on, Mueller suggested that the cytopathology observed in stillbirth implied prenatal insult (Mueller, 1922). In 1925 Von Glahn and Pappenheimer reported about the similarities between the cytomegalic cells and herpesvirus infected cells, and drew the conclusion that these cells were infected by a closely related or identical herpesvirus member (Von Glahn WC, 1925). In the early 1950s electron microscopy revealed viral particles in the clear zone surrounding the intranuclear inclusions and also in the cytoplasm of

cytomegalic cells from the pancreas (Minder, 1953). In the mid 1950s three different laboratories reported that they had been able to isolate HCMV from tissue cultures (Rowe WP, 1956; Smith, 1956; Weller TH 1957). These isolates were given the names: Smith, Davis and AD169. Later on, they were given the name cytomegalovirus (Weller TH 1960).

1.4 Epidemiology and transmission

HCMV-infections are common worldwide and the percentage of infected individuals varies among different populations, ranging between 60-100%, depending on geographical location and socioeconomic situation. During and up to years after primary infection the virus is present in bodily fluids, which probably is an important source for the spread of HCMV to new hosts, (reviewed in Pass, 2001). The percentage of infected individuals increases with age in all groups. HCMV-infection is usually acquired during childhood through breast-feeding or close contact with other children and the incidence is 30-40% during the first year of life (Grillner and Strangert, 1986; Mocarski, 2001; Onorato et al., 1985).

A primary infection is followed by a persistent, latent infection from which the virus may reactivate. During latency HCMV is mainly harbored in monocytes (Taylor-Wiedeman et al., 1991). Inflammation seems to be an important factor in the reactivation of latent HCMV (Soderberg-Naucler et al., 1997) and epidemiological evidence indicate that active HCMV is present in many different types of diseases characterized by inflammation such as atherosclerosis, (reviewed in Bruggeman et al., 1999), inflammatory bowel disease (IBD) (Rahbar et al., 2003), different types of cancer (Cobbs et al., 2002; Harkins et al., 2002; Samanta et al., 2003) and autoimmune diseases (Einsele et al., 1992; Newkirk et al., 1994; Sekigawa et al., 2002). During inflammation, infected monocytes are recruited to the site of inflammation; a milieu with inflammatory cells secreting molecules that may reactivate the virus in latently infected monocytes.

1.5 Clinical features and diagnostics

HCMV-infection in the immunocompetent host is usually asymptomatic but may result in mononucleosis malaise with symptoms such as headache, fewer, sore through, rash and enlarged

spleen and/or liver. A primary or recurring HCMV-infection in pregnant women may result in transplacental transmission of virus to the fetus and cause congenital HCMV-infection. Congenital HCMV-infection is today the leading cause of birth defects with mild to severe symptoms like mental retardation, hearing loss and visual impairment. In the immunocompromised host symptomatic HCMV-infection is rather common. The severity of the infection is parallel to the degree of immunosuppression. The most complicated infections are seen in bone marrow transplant recipients and AIDS patients, followed by patients receiving solid organ transplants and patients on immunosuppressive chemotherapy. The infection might be limited and relatively mild to systemic and life threatening, (reviewed in Pass, 2001).

For unknown reasons, the spectrum of CMV disease varies different patient populations. In bone marrow transplant recipients the most common complication due to HCMV-infection is pneumonitis followed by gastrointestinal disease, pneumonia, hepatitis, encephalitis and retinitis (Ljungman, 1996). The most common complications due to HCMV-infection in solid organ transplant recipients are rather mild with manifestations such as fewer, rash, malaise, leucopenia and pain in joints. But some of these patients may aquire life threatening disease like pneumonitis, gastrointestinal ulceration, liver dysfunction, opportunistic fungal infections, and impaired function of the graft (Hebart and Einsele, 1998; Ljungman, 1996; Prentice and Kho, 1997). In AIDS patients with HCMV-infection, retinitis is the most commonly observed clinical manifestation followed by esophagitis, colitis, encephalitis, periferal neuropathy, polyradiculoneuritis, pneumonitis, gastritis and hepatitis (Cheung and Teich, 1999).

A number of previous studies suggest an association of HCMV seropositivity and the presence of atherosclerosis, transplant vascular sclerosis (TVS) and restenosis following coronary angioplasty and TVS (Melnick et al., 1990; Melnick et al., 1993; Speir et al., 1994; Streblow et al., 2001; Zhou et al., 1996; Zhu et al., 1999). HCMV nucleic acids and proteins have been detected in the human atherosclerotic lesions and in the aorta (Hendrix et al., 1991; Hendrix et al., 1989; Hendrix et al., 1990). Furthermore, HCMV-infected heart transplant recipients have been shown to have a twofold

increased risk for graft failure, and prophylactic HCMV treatment significantly reduced the risk for graft failure, (reviewed in Soderberg-Naucler, 2006).

Experimental animal studies support the idea that HCMV-infection plays a role in vascular diseases. Mice as well as rat studies have shown that CMV-infection negatively affects atherosclerosis and TVS following solid organ transplantation (Bruggeman et al., 1999; Lemstrom et al., 1993), atherosclerosis (Berencsi et al., 1998) and restenosis (Zhou et al., 1999a). Moreover, HCMV-infection has been detected in the bowel of patients suffering from IBD (Rahbar et al., 2003) and different autoimmune diseases such as RA (Einsele et al., 1992), SLE (Sekigawa et al., 2002) and SS (Newkirk et al., 1994).

Recent findings also suggest that HCMV have clinical importance in certain types of cancer such as colon cancer, malignant glioma, prostate carcinoma, and breast cancer, see section 1.12.

1.6 The HCMV genome

The human CMV genome consists of a linear double stranded DNA, which in size has been described to be approximately 200 to 240-kbp (Chee et al., 1990; Geelen et al., 1978; Lakeman and Osborn, 1979; Stinski et al., 1979). Recent studies of CMV's coding potential in some clinical isolates suggest that there are 252 open reading frames (ORF) (Murphy et al., 2003). However, only about 50 of the 252 possible ORF's are essential for CMV replication (Dunn et al., 2003; Yu et al., 2003). The vast majority of the remaining ORF's encodes for proteins predominantly involved in immune evasion strategies, piracy and manipulation of the host cellular machinery.

HCMV is estimated to encode for 11 microRNA (miR). One of these microRNAs, miR-UL112-1, has been shown to attenuate HCMV replication up to fivefold and might have an important function in the control of latency. miR-UL112-1 has also been described to target a cellular gene encoding the major histocompatibility complex class-I related chain B (MICB), a stress induced ligand of the natural killer (NK)-cell receptor NKG2D. Targeting MICB with miR-112-1 is most likely a strategy to avoid NK-cell mediated killing of the infected cell, as is the expression of

the HCMV encoded UL16 protein which inhibits MICB signaling, (reviewed in Grey et al., 2008).

The CMV genome has internal repeats and undergoes inversion of two genome components, which is unique among the so far characterized betaherpesviruses. The CMV genome has a unique long U_L and a unique short U_S region flanked by inverted repeats referred to as the TR_L/IR_L and the IR_S/TR_S. At the genome termini there is a repeated sequence that can also be found, but inverted, in the junction between the U_L and U_S region. This arrangement of repeats makes inversion of the genome possible and also puts CMV in the E class of genome structures. The E class genome structure consisting of two components with direct and inverted repeats isomerizes to give four sequence arrangements. The CMV genome is G+C rich and contains more direct and inverted repeats than other herpesviruses. Three specific regions within the CMV genome has a particular high quantity of repeats in close proximity to each other, which are the DNA replication origin, *ori*Lyt, the transcriptional enhancers, *ie*1 and *ie*2, and US3, (reviewed in Mocarski, 2001).

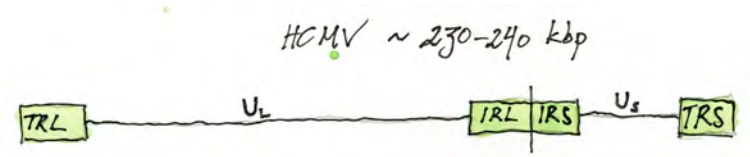

Figure 1. Illustration of HCMV genome organization.

1.7 Structure

The structure of CMV is based on an icosadeltahedral capsid structure. The genome is kept inside the capsid that appears in three different forms, the A-, B- and C-capsid. The three different forms of capsid structures represent three different stages of virion maturation. The A capsid lacks viral DNA due to improper packaging of the DNA. B capsid structures contains viral assembly and scaffolding proteins but still lacks viral DNA and are mainly found in the host cell nucleus. Type C capsids are fully mature nucleocapsids, (reviewed in Mocarski, 2001). The capsid is built-up by virally encoded proteins with different properties important for the shape and assembly of the capsid (Britt and Boppana, 2004). A tegument layer consisting of 25 or more virally encoded proteins surrounds the

capsid structure. Many of the tegument proteins are phosphorylated and ready to act immediately after entry of the host cell. Some of the tegument proteins are denoted to regulate viral gene expression while others interferes with host cell responses against viral infection. The most abundant tegument proteins: pp150, pp65 and pp28 have been shown to evoke an immunological response in the host cell. Furthermore, pp71 has been reported to transactivate the IE-promoter. A host cell derived lipid bilayer with viral and host derived proteins surrounds the tegument. Several major viral glycoprotein (g) complexes can be found in CMV's envelope, these are the; gB, gM/gN, and gH/gL/gO complexes, (reviewed in Mocarski, 2001) and the gH/gL/UL131-128 complex, known to mediate entry into epithelial and endothelial cells (Wang and Shenk, 2005).

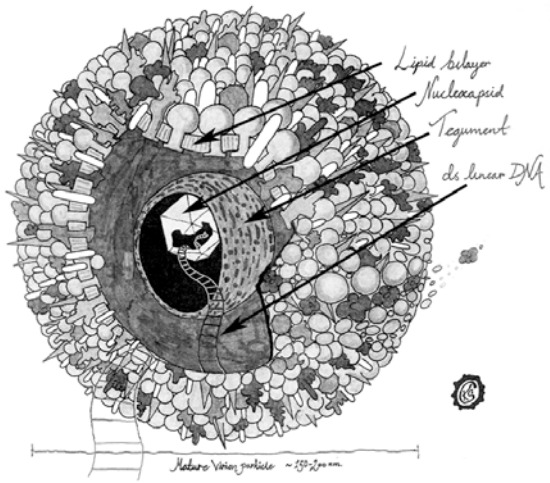

Figure 2. Illustrates the structure of HCMV.

1.8 Entry, replication and viral assembly

Viral attachment to host cell membranes is established through interactions between HCMV glycoproteins and host cell heparan sulphate proteoglycans (HSPG), integrins (Compton et al., 1993) and different cell surface receptors. Numerous studies have tried to identify cellular receptors important during HCMV-entry. Among the suggested receptors is a 30-34 kDa protein (Nowlin et al., 1991; Taylor and Cooper, 1990) later identified as annexin II, a calcium and phospholipid binding protein with membrane bridging function and the capacity to bind HCMV virions (Wright et al., 1995; Wright et al., 1994) through direct interaction with HCMVs gB-protein

(Pietropaolo and Compton, 1997). Aminopeptidase N (cluster of differentiation 13 [CD13]) is another receptor that has been suggested to be important during HCMV entry (Soderberg et al., 1993a; Soderberg et al., 1993b). Further studies, based on the idea that only the CD13-positive peripheral blood mononuclear cells (PBMCs) supported productive viral infection, showed that CD13 neutralizing antibodies (ab) and chemical inhibitors of CD13 repressed entry of the host cell (Soderberg et al., 1993a). The epidermal growth factor receptor (EGFR) was previously described to be an HCMV receptor required for HCMV-mediated signaling and viral entry (Wang et al., 2003). Later studies proposed that HCMV also uses integrin $\alpha 2\beta 1$, $\alpha 6\beta 1$, and $\alpha V\beta 3$ as HCMV entry receptors and signaling mediators during entrance (Feire et al., 2004) and that $\alpha V\beta 3$ acts as a coreceptor for EGFR (Wang et al., 2005). A recent study by Soroceanu et al., presented data demonstrating that PDGFR-α is a receptor for HCMV, more specifically, they showed that HCMV's gB protein interacted with PDGFR-α with following receptor phosphorylation and activation of the phosphoinositide-3-kinase (PI(3)K) signaling pathway, and that cells lacking this receptor was non-permissive for HCMV entry (Soroceanu et al., 2008).

The penetration per se, in fibroblasts, is governed through a pH-independent fusion mechanism occurring at the cellular membrane (Compton et al., 1992). The HCMV-encoded protein complex: gH/gL/UL131-128 mediates entry into epithelial and endothelial cells cells (Wang and Shenk, 2005). However, expression of the gH/gL/UL131-128 complex in epithelial cells made the cells resistant to HCMV-infection, providing information that there probably is a receptor or molecule for the gH/gL/UL131-128 complex, which is blocked, masked or down-regulated when the gH/gL/UL131-128 complex is expressed in epithelial cells (Ryckman et al., 2008). It's known that HCMV virions activate several cell-signaling pathways upon cell-contact, the Ca^{2+} homeostasis is altered, phospholipase A_2 (PLA_2) is activated via the mitogen-activated protein kinase (MAPK) -signaling pathway and an increased release of arachidonic acid (AA) and some of its metabolites has been noted (Fortunato et al., 2000b). Various transcription factors are also activated upon cell-contact such as nuclear factor kappa-B (NF-κB), Sp1, myc, and also activation of

MAPK extracellular signal-regulated kinases (ERK)1/2 and p38 has been described (Boldogh et al., 1991; Boyle et al., 1999; Kowalik et al., 1993; Yurochko et al., 1995). Still, the actual fusion process remains unknown. The HCMV envelope glycoproteins gB and gH might have an important role during the fusion process and it has been suggested that gB and gH contain regions possibly forming coil-coil structures important for fusion by driving the energetic folding of membranes together (Lopper and Compton, 2004). Since PLA_2 and AA has been described to exhibit fusogenic properties (Blackwood et al., 1996; Karli et al., 1990) it is interesting to note that HCMV carries host cell derived PLA_2 in its envelope (Allal et al., 2004) and that it also activates PLA_2 and increase AA release during viral attachment to the cell (Fortunato et al., 2000b). These phenomena's might have important functions both in entry and egress of HCMV.

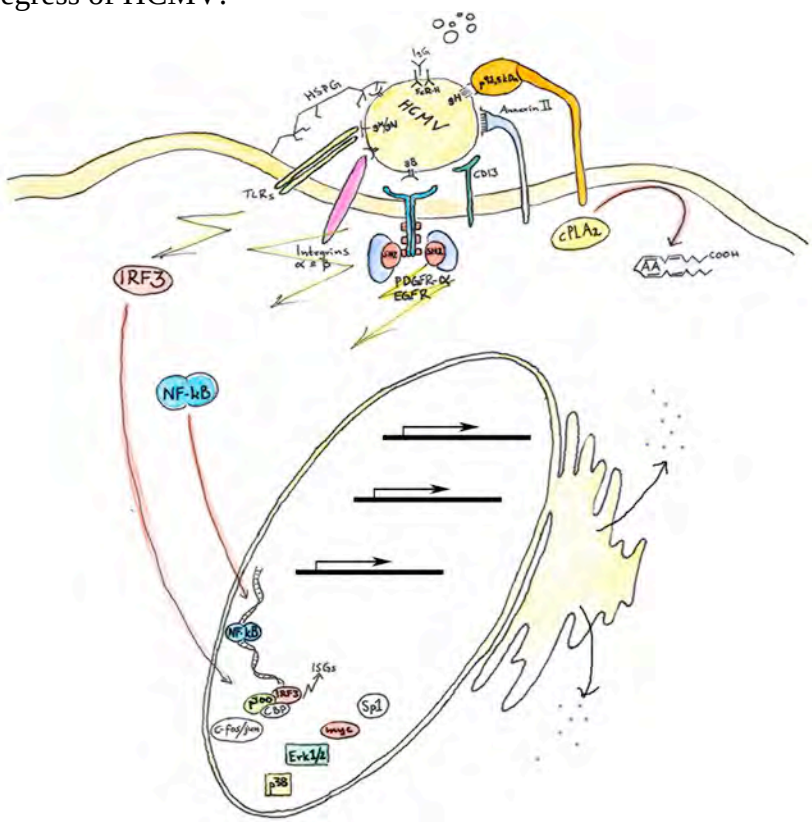

Figure 3. HCMV cellular attachment involves interactions with numerous cellular receptors and subsequent intracellular signaling events followed by transcriptional activity.

After CMV has entered its host cell, the tegumented viral particle is transported to the nuclear membrane and the viral DNA is delivered from the capsid to the host cell nucleus. Following the injection of viral DNA into the host cell nucleus, a temporal expression of viral genes takes place in three stages. The first viral genes to be expressed are the immediate early (IE) genes, followed by the early (E) and late (L) viral genes. The IE expression takes place immediately after entry. The IE expression is not dependent on the expression of any other viral genes, however, some IE proteins have the capability to regulate the expression of other IE genes. The expression of E-genes is dependent on the expression of IE-genes, which are able to transactivate E-genes, and the E-gene products take part in viral DNA-replication. The expression of L-genes is in turn dependent on successful E-gene transcription, translation and protein formation. A complete replication of CMV takes approximately 72h, after which newly formed virus particles are released through cellular lysis or cell-to-cell spread, (reviewed in Mocarski, 2001).

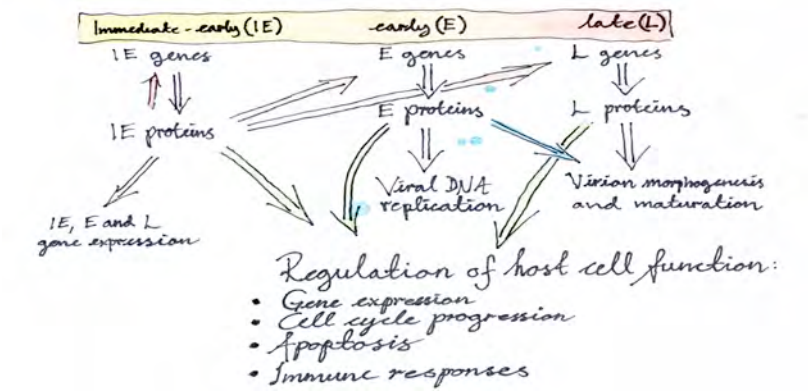

Figure 4. A simplified illustration of HCMV's temporal gene expression and their effects on viral and host gene regulation. Adapted and modified from (Landolfo et al., 2003).

Capsids are formed and packaged with newly synthesized viral DNA within the nucleus. Later, the capsid will acquire its tegument layer. After tegumentation the envelopment takes place, possibly in a two-step process, by acquiring a pre-envelope while passing through the nuclear membrane, which is lost after the passage, to later acquire

a new envelope while entering a Golgi-derived secretory vacuole (Homman-Loudiyi et al., 2003).

1.9 Tissue specificity, permissiveness and latency

Most cell types in different organ systems can be infected by HCMV. The different cell types include endothelial cells, retinal cells, fibroblasts, smooth muscle cells, hepatocytes, neuronal cells, placental cells as well as monocytes and macrophages (Sinzger et al., 2008). The ability to infect such wide range of different cell-types is in part the reason why HCMV-infection gives rise to many diseases. Cells of epithelial origin represent a route for HCMV entry into and exit from its host, and are the major target cells in the course of HCMV-infection (Britt, 2006; Landolfo et al., 2003; Sinzger et al., 1995). HCMV-infection of the endothelium in blood vessels also serves as a route for the virus to spread to monocytes/macrophages and lymphocytes, which distribute the virus throughout the body (Bentz et al., 2006; Gerna et al., 2004). Depending on cell-type and viral strain there is a difference in entry mechanism, tissue specificity, permissiveness and the establishment of latency. It has been noted that laboratory strains of HCMV, which have been extensively propagated in fibroblasts, lacks the ability to productively infect cells of epithelial origin (Cha et al., 1996; Dolan et al., 2004; Murphy et al., 2003) due to genetic alterations in the UL150-128 region (Cha et al., 1996) acquired during propagation (Sinzger et al., 1999), which is not seen in clinical isolates of HCMV. Hahn et al. showed that the HCMV encoded genes: UL131-128, were indispensable for viral replication in endothelial cells and for transfer of virus to leukocytes (Hahn et al., 2004).

In 1985, Schrier et al., were able to show by in situ hybridization that PBMCs contained HCMV-DNA (Schrier et al., 1985). This was later also confirmed by PCR by several investigators (Bevan et al., 1991; Stanier et al., 1989; Taylor-Wiedeman et al., 1993), and it was shown that it was the adherent monocytes, rather than T-cells or B-cells, that harbored HCMV-DNA (Taylor-Wiedeman et al., 1991). In 1996 Mendelson et al. showed that CD34+ progenitor cells, precursors of monocytes, isolated from bone marrow, also contained HCMV-DNA (Mendelson et al., 1996). Latent HCMV has been reported to be present in monocytes as episomes (Bolovan-Fritts et al., 1999), but no investigations have been able to show an

integration of HCMV-DNA into the human genome, as is the case for HHV-6 (reviewed in Roizman, 2001). *In vitro* experiments concluded that cultured monocytes could be infected by HCMV after differentiation into macrophages by stimulation of non-adherent cells (Ibanez et al., 1991). *In vivo*, monocytes carrying latent HCMV also require a differentiation into macrophages in order to reactivate and produce new infectious viral particles (Soderberg-Naucler et al., 1997). By using a non-characterized cocktail from allogenically stimulated T-cells, Söderberg-Naucler et al., managed to reactivate and isolate newly produced HCMV (Soderberg-Naucler et al., 1997). Dendritic cell precursors has also been reported to carry latent HCMV, and differentiation of these cells reactivates HCMV *in vitro* (Hahn et al., 1998).

Viral replication is less efficient in some cells including endothelial cells and monocytes/macrophages. In some cell types the viral replication is restricted to IE and E gene expression and different viral strains could be successful in replication while others are not. Non-permissive cells can be turned into a permissive phenotype by using HDAC-inhibitors, such as trichostatin A (TSA) (Nevels et al., 2004). It has been shown that HCMV IE-proteins interact with HDAC-1, -2 and -3 (Murphy et al., 2002; Nevels et al., 2004; Park et al., 2007; Reeves et al., 2006; Straat et al., 2009). The sequestering of HDACs presumably facilitates histone acetylation and subsequent transcriptional activation of viral and host genes. These epigenetic modifiers and their impact on HCMV replication tell us something about the importance of the cellular state of differentiation in a particular cell type for successful replication. It also gives a hint into the importance of different viral strains ability to influence epigenetic molecules such as HDACs and Dnmt's.

1.10 Immune responses to HCMV

Both innate and humoral immunity is important in the host defence against HCMV-infection. However, the cellular immunity seems to play a key role in the host immune system to combat HCMV-infections (Quinnan et al., 1984; Quinnan and Rook, 1984; Rasmussen, 1990).

NK-cells contributes to a protective innate immune response against various infections by the release of cytokines and perforin

(Biron et al., 1999). NK-cells have been shown to target HCMV-infected cells *in vitro* (Borysiewicz et al., 1985), and even though NK-cells are believed to be important in the defense against HCMV, only one case report shows the importance of NK-cells in the clearance of HCMV-infection *in vivo* (Biron et al., 1989).

Plasmacytoid dendritic cells (PDC), which are main producers of type I IFN's, plays a role in the innate immunity against viral infections (Jego et al., 2003), and it has been shown that HCMV-infection activates PDC's (Varani et al., 2007).

Both $CD4^+$ and $CD8^+$ T-cells play key roles in the cellular immunity against HCMV-infection. $CD4^+$ T-cells are able to secrete cytokines that activates $CD8^+$ T-cells, which in turn can activate B-cells. Upon activation the $CD8^+$ T-cells can develop into cytotoxic T-cells (CTL). The CTL's have the capacity to lyse infected cells through the release of cytolytic proteins. HCMV-specific $CD8^+$ T-cell responses are most important in protection and recovery from HCMV-infection (Reusser et al., 1991). HCMV immunogens induces a remarkably strong immune response. Up to 50% of the entire $CD8^+$ thymus (T)-cell repertoire can recognize HCMV antigens in the elderly population (Looney et al., 1999; Wikby et al., 2002).

Upon infection HCMV induces an inflammatory response in its host. The infection modulates the normal host immune response to a more favorable milieu for viral persistence and latency by affecting the expression of various chemokines, cytokines and receptors.

The HCMV genome encodes for at least four G protein-coupled receptor (GPCR) homologous referred to as US27, US28, UL33 and UL78, (reviewed in Vischer et al., 2006). The HCMV GPCR homologue US28 is activated by MCP-1, MCP-3, MIP-1α, MIP-1β, and RANTES (Billstrom et al., 1998; Gao and Murphy, 1994) and may upon activation increase intracellular Ca^{2+} levels and activate the MAPK signaling pathway (Billstrom et al., 1998). US27 and UL78 has not been described to induce any signaling activity, while UL33 has been shown to induce cAMP-responsive element (CRE) in a ligand-independent manner, (reviewed in Vischer et al., 2006).

Furthermore, HCMV activates interferon sensitive genes (ISG) through interferon regulatory factor 3 (IRF3) (DeFilippis et al., 2006) and also triggers production of inflammatory cytokines through interactions with TLR2 and CD14 (Compton et al., 2003).

1.11 HCMV immune evasion strategies

During the evolution HCMV has developed numerous sophisticated strategies to avoid recognition of the hosts immune system in order to persist within its host. During infection, the host cell induces cellular signaling pathways triggering the production of type 1 IFNs with anti-viral properties. The HCMV encoded gene product IE-86 is able to inhibit the transcription and secretion of the type 1 IFN: IFN-β (Taylor and Bresnahan, 2005). A variety of cytokines and chemokines such as IL-1, IL-6, IL-8, IL-10, IFN-β, TGF-β, MCP-1, MIP-1α, MIP-1β and RANTES are also produced upon HCMV-infection, (reviewed in Soderberg-Naucler, 2006). HCMV encodes for an IL-10 homologue, cmvIL-10, (Kotenko et al., 2000), which has the ability to decrease MHC class I and II expression as well as the expression of pro-inflammatory molecules e.g.: IL-1, IL-6, GM-CSF, and TNF-α (Spencer et al., 2002).

HCMV infection reduces the expression of major histocompatibility complex (MHC) class I on somatic antigen presenting cells (APC) and interferes with the presentation of antigens to the $CD8^+$ T-cells. HCMV also manages to avoid recognition by the $CD4^+$ T-cells by inhibiting MHC class II expression, associated with antigen presentation by professional APCs, such as DCs and macrophages. The HCMV genome encodes for four known proteins that interfere with the MHC class I antigen presentation, and those include the gp-US2, -US3, -US6 and -US11. The HCMV-encoded gp-US2 and -US11 independently facilitates the degradation of MHC class I and thereby reduces the surface expression and subsequently the antigen presentation by MHC class I molecules. Glycoprotein US3 retains MHC class I bound peptides in the endoplasmatic reticulum (ER) while gpUS6 binds the ER part of the transporter of antigenic peptides (TAP) which also retains the peptide-loaded MHC class I molecules in the ER. Furthermore, HCMV has been demonstrated to degrade MHC class II molecules and to interfere with the class II major histocompatibility complex

transactivator (CIITA)/JAK/STAT signaling pathway which controls the promoter region of the MHC class II gene, (reviewed in Loenen et al., 2001).

To avoid NK-cell mediated lysis due to decreased MHC expression, HCMV expresses an MHC class I homologue: UL18 (Reyburn et al., 1997), further the HCMV-encoded gpUL40 stimulates the expression of HLA-E that interacts with the inhibitory NK-cell receptor CD94/NKG2A (Tomasec et al., 2000; Ulbrecht et al., 2000) in addition the HCMV protein UL16 protects infected cells from NK-cell released cytolytic proteins (Odeberg et al., 2003). HCMV also expresses miR-UL112-1, described to target the MICB, a stress induced ligand of the natural killer (NK)-cell receptor NKG2D, and thereby avoid NK-cell mediated killing of the infected cell, (reviewed in Grey et al., 2008).

The UL144 gene encodes for a TNF-receptor homologue, which also shows homology to HveA, an HSV-encoded protein important for viral entry (Benedict et al., 1999). The HCMV-genes UL146, UL147 and UL152 encodes for chemokine homolougs (Cha et al., 1996).

Furthermore, the HCMV-genome encodes for two Fc-receptor homologues, UL119-118 and TRL11/IRL11, which might protect the HCMV-infected cell from complement binding to the Fc-receptor part of the IgG (Atalay et al., 2002; Lilley et al., 2001). HCMV also induces the expression of the complement inhibitory molecules CD46 and CD55 (Spiller et al., 1996).

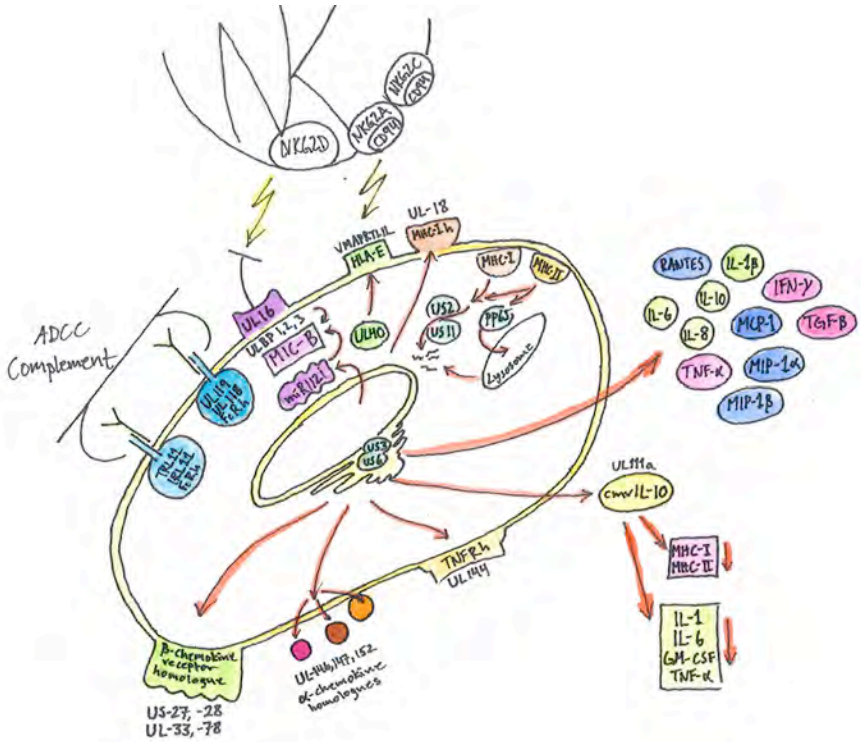

Figure 5. Simplified illustration over HCMV's immune evasion strategies.

1.12 HCMV in inflammation and cancer

Immune evasion strategies will help the virus to avoid recognition and elimination by the immune system. Thus, the virus and viral antigens will be present in tissues for extended periods of time. This prolonged persistence of the virus may result in maintained inflammation in the tissue (see section 1.11). In addition, old and new data suggest that the virus may be involved in tumor formation by providing oncogenic and oncomodulatory mechanisms. In such case the immune evasion strategies will help CMV infected tumor cells to avoid elimination by the immune system.

HCMV has for many years been suspected to contribute to the development of human cancer (Cinatl et al., 2004a; Cinatl et al., 2004b; Soderberg-Naucler, 2006). It has been reported that CMV acts as a mutagen (AbuBakar et al., 1988) and that CMV is capable of transforming certain types of mammalian cells (Boldogh et al., 1990; Clanton et al., 1983; Geder et al., 1976; Geder et al., 1977), and that the HCMV-encoded chemokine receptor US28 and the IE-

72 protein enhance proliferation, thereby promoting cellular transformation or tumor progression (Cobbs et al., 2008; Maussang et al., 2006). Further it has been shown that the HCMV-encoded protein US28 mediates increased COX-2 expression through NF-κB, which in turn drives vascular endothelial growth factor (VEGF) production and tumor transformation (Maussang et al., 2009). These observations imply a link between HCMV, inflammation and cancer development.

HCMV encodes for several different proteins that affect the cell cycle progression, which is a tightly controlled cellular process that if disrupted, may contribute to development of cancer. The HCMV-encoded proteins: IE72, IE86 and pp71 inactivate Rb family members, thereby promoting entry into the S-phase of the cell cycle. IE86 also mediates accumulation and activation of p53, which inhibits cell cycle progression, and the HCMV-protein pUL69 contributes to cell cycle arrest. The effects exerted by HCMV-regulatory proteins on cell cycle differ between normal cells and malignant cells. Some HCMV-regulatory proteins induce cellular arrest in normal cells, while this effect is absent in malignant cells with disrupted p53 signaling. The HCMV-encoded protein, US28, induces cell cycle progression in tumor cells but not in normal cells, which instead undergo apoptosis. Resistance against apoptosis is commonly observed in cancer cells. HCMV-genome encodes for proteins that interfere with apoptosis. The IE86, and UL36-38 proteins have been shown to interfere with apoptosis. IE86 binds p53 and suppresses p53-mediated apoptosis, UL36 inhibits Fas-mediated apoptosis by binding caspase-8, UL37 inhibits Bcl-2 protein recruitment to mitochondria, thereby inhibiting apoptosis and UL38 disrupt regulation of the rapamycin complex, (reviewed in Michaelis et al., 2009). In theory, these mechanisms would render HCMV infected tumor cells less sensitive to chemotherapy. In support of this hypothesis expression of UL37 and viral induction of Bcl-2 in HeLa and neuroblastoma cell lines protects cells from chemotherapy-induced apoptosis in vitro (Cinatl et al., 1998; Goldmacher et al., 1999).

Even though many years has past by since CMV was described as a virus with oncogenic properties, the HCMV-mediated mechanism

for transformation or tumor progression is still not known. Instead of being regarded as a virus with oncogenic properties, HCMV has been described to exert oncomodulation e.g. influencing cellular mechanisms that lead to tumor initiation and/or progression (reviewed in Michaelis et al., 2009).

2. Aims of the thesis

The overall aims of this thesis were to explore the role of HCMV-infection in inflammation and cancer.

More specifically:

In paper one I focused on examine the expression of growth factor receptors for platelet derived growth factor (PDGF) -α and -β on human vascular smooth muscle cells and fibroblasts challenged by HCMV.

In the second paper I explored HCMV's possible effect on the expression of molecules involved in leukotriene (LT) -biosynthesis in human smooth muscle cells.

In paper three I investigated HCMV's influence on the expression of different matrix metalloproteinases (MMP) and their inhibitors; tissue inhibitor of MMP (TIMP) in primary human macrophages.

In paper four I investigated if HCMV-infection could affect hTERT expression and telomerase activity in human diploid fibroblasts and malignant glioma cell lines.

3. Paper I

In the first study we aimed to study the expression of growth factor receptors for platelet derived growth factor (PDGF) -α and -β on human vascular smooth muscle cells (SMCs) and fibroblasts challenged by HCMV. PDGF plays major roles during embryonic development and also participates in wound heeling processes, cancer development and arteriosclerosis. We wanted to investigate if HCMV could affect the receptor expression at protein level and/or mRNA level, and if so, is viral replication needed to alter the expression.

3.1 Platelet derived growth factors, an introduction

Platelet-derived growth factor (PDGF) is an important mitogen and chemoattractant for a variety of different types of cells. PDGF acts through receptors with signaling pathways involved in embryonic development, wound healing, carcinogenesis, and atherosclerosis. PDGF interacts with the PDGF receptor (PDGFR), which is a member of the tyrosine kinase family of receptors. Two human PDGFRs have been identified; PDGFR-α, which binds PDGF-A and -B isoforms, and the PDGFR-β, with affinity for the PDGF-B isoform, (reviewed in Claesson-Welsh, 1994; Heldin and Westermark, 1999). PDGF-C has a binding pattern similar to that of PDGF-A/B as it binds PDGFR-α/α homodimers as well as PDGFR-α/β heterodimers (Gilbertson et al., 2001; Li et al., 2000) whereas PDGF-D binds only to the PDGFR-β (Bergsten et al., 2001).

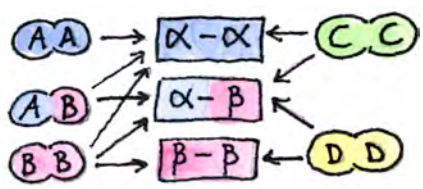

Figure 6. Illustration over different PDGF isoforms and their affinity to different PDGFR -homo or -hetero dimmers.

PDGF is secreted by a variety of cells, including platelets, smooth muscle cells (SMCs), endothelial cells, and macrophages. PDGF has been found to be mitogenic and to serve as a chemotactic factor for mesenchymal cells such as SMCs, fibroblasts, neutrophils, and

mononuclear cells (reviewed in Heldin and Westermark, 1999). PDGF stimulates dimerisation of the receptors, which in turn causes an autophosphorylation within and outside of the receptor kinase domain. The phosphorylation gives an opportunity for signal transduction molecules with SH2-domains to interact with the receptor complex and to induce signaling pathways. PI(3)K, Phospholipase C-γ (PLC-γ), Grb2/Sos, GTPase activating protein (GAP), Stat, SHP-2, and Src family members are some examples of such signal transduction molecules known to interact with the PDGFRs (reviewed in Heldin and Westermark, 1999).

PDGF and its receptors in disease such as atherosclerosis or cancer are generally associated with an increased expression of the receptors and the cognate ligands and constitutive signaling, while genetically depleted PDGF or PDGFR genes are associated to defects during embryonic development. PDGFR-α expression is found in all grades of human glioma and the expression increases in higher-grade tumors. Also, PDGF-α expression is increasing from low in low-grade to high in high-grade glioma tumors, indicating a role in tumor progression. Increased expression of PDGF and PDGFRs has been reported in atherosclerotic lesions and may play a role in the development of the atherosclerotic lesion. The enhanced PDGF expression might influence migration of SMCs from the media to the intima layer in the vessel wall, where they proliferate and produce matrix proteins, thereby contributing to intimal thickening. Studies in mice where PDGF or PDGFR genes have been inactivated implicate that PDGF and PDGFRs are important during development since all mice died either during embryogenesis or just before or just after birth, (reviewed in Heldin and Westermark, 1999). Defects observed in mice lacking PDGFR-α included cleft face, subepidermal blistering, spina bifida, impared neural crest formation, incomplete cephalic closure, cardiovascular and skeletal defects (Soriano, 1997). Mice lacking PDGFR-β have been reported to exhibit abnormal kidney glomeruli, thrombocytopenia, edema and hemorrhage (Soriano, 1994).

Previous *in vitro* studies by Zhou et al. (Zhou et al., 1999b) demonstrated an increased migration of CMV-infected human SMCs in response to PDGF stimulation as compared to non-infected SMCs.

Zhou et al. also presented data that the CMV-infected SMCs had an increased expression of PDGFR-β (Zhou et al., 1999b). However, the data are not very compelling since Zhou et al. used rat-CMV to infect human cells. CMV is a highly species-specific virus, which makes the significance of this finding unclear. A more recent study by Soroceanu et al. showed that PDGFR-α is a receptor for HCMV. More specifically Soroceanu et al. showed that HCMV's gB protein interacted directly with the PDGFR-α with following phosphorylation and activation of the PI(3)K and Act signaling pathways. Interestingly, cells with genetically depleted or blocked PDGFR-α became non-permissive to HCMV entry and viral gene expression (Soroceanu et al., 2008).

3.2 Results and discussion paper I

In the first study, we investigated the effect of HCMV-infection on the expression of PDGFR-α and -β on human SMCs and fibroblasts. Since CMV as well as PDGFRs has been associated with the development of vascular disease, the initial hypothesis was that HCMV-infection would increase the expression of PDGFRs, and would thus provide a molecular link between HCMV-infection and vascular disease. Previous studies demonstrated an increased expression of PDGFR-β after HCMV-infection (Reinhardt et al., 2005; Zhou et al., 1999b). However, Zhou et al., infected rat SMCs with HCMV at an MOI of 200 (an unusually high viral dose), further, with the notion that HCMV is a highly species-specific virus, the outcome of such an experiment is questionable. This type of infections results in modest and restricted expression of the HCMV-IE genes.

Reinhardt et al., probably conducted their study at the same time as we were doing our study. Since we didn't know about their findings, we also sought to explore the expression of PDGFRs in human vascular smooth muscle cells, rather than in SMCs from rat, as Zhou et al., did, to determine if this was the case also in human vascular smooth muscle cells. Just before submission of our manuscript, Reinhardt et al., published their article showing that HCMV-infection induced PDGFR-β expression in HCASMC.

In contrast to the previous findings we found that HCMV-infection of HPASMC (another cell type used than in Reinhardt et al.'s study) and fibroblasts caused a decreased expression of PDGFRs. In our study we analyzed the mRNA expression of PDGFR-α and -β by real-time PCR at different time points after infection and found that the mRNA levels were consistently decreased after infection in contrast to the uninfected control cells. The total cellular protein levels as well as the surface expression of PDGFR-α and -β, were also found to be decreased after infection in parallel with attenuated mRNA levels, analyzed by FACS and western blotting techniques respectively. In the western blotting experiments we used two different antibodies targeting either PDGFR-α or -β, one monoclonal and one polyclonal, both antibodies could detect weaker bands in cell lysates from infected HPASMCs and fibroblasts compared to stronger bands in the control cells for the corresponding receptors after infection.

The PDGFR-α and -β mRNA and cell surface expression decreased in a dose-dependent manner; additionally we could show that the effect was mediated through HCMV-IE or -E gene products and that viral replication was needed to cause the decreased expression and that no soluble factors in the viral inoculums was responsible for the down regulation of PDGFR mRNA expression. PDGFR-β was also found to be associated with Rab4 (early endosome), Rab5 (late endosome), Lamp1 (early lysosome) and Lamp2 (late lysosome), which are proteins that are associated with secretory and endocytic pathways as well as with intracellular pathways important in protein degradation.

We found our data very consistent and solid, but still, Reinhardts et al.,'s finding puzzled us. We finally decided to include experiments with HCASMCs, the same kind of cell type used by Reinhardt et al., and we could still note a clear down regulation of PDGFR-β mRNA levels.

The experimental setup differs in many aspects between Reinhardt el al. and our study, which may explain the difference in the outcome of the study. First, in our study we used both monoclonal and polyclonal antibodies targeting PDGFR-α and -β. Secondly, we

analyzed mRNA levels of PDGFR-α and -β which clearly demonstrated a decreased expression of PDGFR-α and -β mRNA levels. Thirdly, we analyzed cells with confocal microscopy, which showed that PDGFR-β associated with proteins involved in protein degradation pathways.

The study by Soroceanu et al., discussed above, supports our finding that the cell surface expression PDGFRs decreases after HCMV-infection. The interaction with PDGFR-α upon viral attachment and entry is not completely understood but it clearly plays a significant role for a successful viral entry and viral replication. According to our study, PDGFR-α and -β is internalized after infection and later degraded while no new PDGF-α and -β receptors are expressed.

Congenital HCMV-infection is the most common infectious factor causing developmental damage to the central nervous system (CNS) with following complications such as mental retardation, hearing loss or visual impairment (Alford et al., 1990). Our observation that PDGFR-α and -β expression decreases after HCMV-infection of SMCs might be an important finding explaining defects associated with congenital HCMV-infection.

Our initial assumption, that HCMV would affect vascular disease through increased PDGFR expression, changed. Instead we realized that our finding could be an explanation for the developmental disorders often seen in congenitally HCMV-infected fetuses, as PDGFRs not only controls cellular signaling events important in vascular diseases, but also play key roles in fetal development.

4. Paper II

In the second study, we were interested to explore HCMV's possible effect on the expression of molecules involved in LT-biosynthesis. LTs are powerful mediators of inflammation and its biosynthesis has previously been considered to be restricted to myeloid cells, with some exceptions. This study intended to analyze the mRNA and protein expression of $cPLA_2$, 5-LO, FLAP, LTA_4H, LTC_4S and any possible synthesized LTs in vascular SMCs challenged by HCMV. If HCMV-infection could trigger the synthesis of bioactive 5-LO in nonmyeloid cells, thereby enabling these cells to synthesize LTs, it would offer an explanation to HCMV-induced pathogenesis in various inflammatory diseases.

4.1 A short introduction to eicosanoids

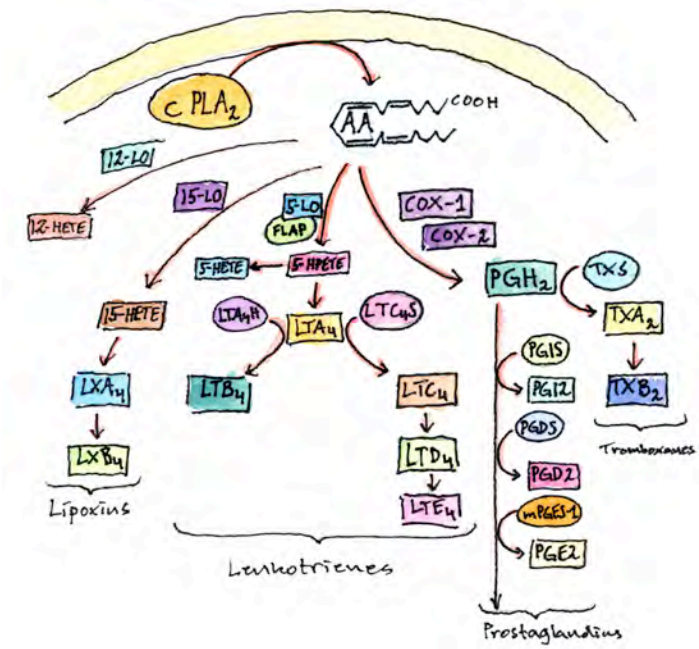

Figure 7. Simplified illustration of molecules involved in the synthesis of eicosanoids.

Eicosanoids is the common term used for unsaturated fatty acid derivates with a carbon-backbone built up by 20 carbons. The eicosanoids include products from the lipoxygenase biosynthesis

pathway, leukotrienes (LT) and lipoxins (LX), and from the cyclooxygenase biosynthesis pathway, prostaglandins (PG) and tromboxanes (TX). Eicosanoids are derived from arachidonic acids (AA) released from phospholipids in cellular membranes or other 20-carbon unsaturated fatty acids (Lee et al., 1985; Lee et al., 1984; Needleman et al., 1979).

4.2 Leukotrienes

Leukotrienes are potent proinflammatory and immune-modulating mediators divided into two classes: the chemoattractant LTB_4 and the spasmogenic cyc-LTs: LTC_4, LTD_4 and LTE_4. LTs play major roles in local and systemic inflammatory conditions, including cardiovascular disease (CVD), arthritis, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), fibrosis and certain types of cancers, (reviewed in Peters-Golden and Henderson, 2007).

The AA is released from cellular membranes through the catalysis by cytosolic phospholipase A_2 ($cPLA_2$), which when activated translocates the AA to the nuclear membrane (Clark et al., 1990; Glover et al., 1995). At the nuclear membrane, the AA is converted by 5-lipoxygenase (5-LO), with the help of 5-LO activating protein (FLAP), into 5-hydroperoxy eicosatetraenoic acid (5-HPETE) which is further converted to leukotriene A_4 (LTA_4) (Dixon et al., 1990; Panossian et al., 1982). LTA_4 can further be metabolized to leukotriene B_4 (LTB_4) by LTA_4 hydrolase (LTA_4H), or to cysteinyl-leukotrienes (cys-LTs): leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4) or leukotriene E_4 (LTE_4) by LTC_4 synthase (LTC_4S), γ-glutamyl transpeptidase and dipeptidase, respectively (Samuelsson et al., 1987).

Atherosclerotic lesions express 5-LO, FLAP, LTA_4H, LTC_4S and the receptors for LTB_4: BLT_1, BLT_2, and the receptors for CysLTs: $CysLT_1$, $CysLT_2$. LTB_4 is most likely playing a significant role in CVD including arteriosclerosis, myocardial infarction and stroke (Funk, 2005; Mehrabian et al., 2002). It has been reported that 5-LO correlates with the severity of the atherosclerotic lesion (Spanbroek et al., 2003), and instability of the atherosclerotic plaque (Qiu et al., 2006).

Previous studies investigating CMVs impact on the expression of cyclooxygenase (COX) -2 showed that COX-2 expression is required for successful viral replication, and that aspirin and indomethacin (known COX-inhibitors) attenuates HCMV infectivity in vascular smooth muscle cells (Speir et al., 1998). Speir et al., also showed that specific viral IE proteins could transactivate the COX-2 promoter. Furthermore, its been demonstrated that prostaglandin E_2 (PGE_2), the end product formed along the PG-synthesis pathway/COX-2 pathway, can restore CMVs ability to replicate in human fibroblasts treated with COX-inhibitors (Zhu et al., 2002). There is also evidence for that HCMV carries $cPLA_2$, as a host derived protein in its envelope, and that $cPLA_2$ is required for infectivity (Allal et al., 2004).

Recent studies show that LTB_4, one of the end products formed along the LT-biosynthesis pathway/5-LO-pathway, protects latently infected mice from CMV reactivation after allogenic transplantation (Gosselin et al., 2005), and that LTB_4 protects mice infected with a lethal dose of CMV and that the protective role of LTB_4 is mediated through the release of the antimicrobial peptide LL37 after interaction of the LTB_4 receptor: BLT-1 (Gaudreault and Gosselin, 2007). Interestingly, COX-2 expression has been found in different forms of cancer such as medulloblastoma and glioblastoma (Baryawno et al., 2008; Onguru et al., 2008) and 5-LO expression has been found in several types of cancer such as prostate adenocarcinoma, esophageal cancer and high-grade astrocytomas (Gupta et al., 2001; Hoque et al., 2005; Nathoo et al., 2006).

4.3 Results and discussion paper II

No investigators had explored HCMVs influence on the 5-LO pathway, why we became interested to do so. In addition, LTs had become a hot topic in the atherosclerotic research field as well as in other diseases characterized by inflammation. We also knew that inflammation is an important factor in the reactivation of HCMV, which made it even more attractive to explore this field in connection to HCMV-infection.

We sought to explore the possibility that HCMV-infection could induce the LT-pathway in vascular smooth muscle cells. By real-time

PCR we analyzed the mRNA expression of $cPLA_2$, 5-LO, FLAP, LTA_4H and LTC_4S.

Interestingly, we found that the mRNA expression of 5-LO was induced after HCMV-infection of HPASMC in a time- and dose-dependent manner, a cell type that normally doesn't express 5-LO. We also noted an increased expression of $cPLA_2$, while LTA_4H was stably expressed throughout the whole experiment (at 1, 3 and 7 days). In contrast, FLAP and LTC_4S had a decreased expression of the corresponding mRNA. Further experiments with UV-irradiated, non-replicative, viral inoculums, showed that viral replication was needed to induce the expression of 5-LO mRNA and that the effect was mediated by a late viral gene-product since treatment of cells with Foscavir, which inhibits viral late gene expression, largely abolished the 5-LO mRNA induction. Even though FLAP mRNA levels were decreased after HCMV-infection, it was sufficient amounts available to render SMCs capable of producing LTB_4, but not Cys-LTs, after stimulation with calcium ionophore.

To determine if this was also the case *in vivo*, we analyzed tissue sections from UC-patients with active HCMV-infection present in the bowel. In all seven patients examined we could detect cells that co-expressed 5-LO, IEA and SMC-α actin. We also examined adrenal gland tissue sections from one AIDS patient and found co-expression of 5-LO, IEA and SMC-α actin.

Taken together, we found a novel mechanism for possible LTB_4 synthesis in the human vascular wall, probably mediated by HCMV-induced $cPLA_2$, LTA4H and 5-LO expression. Our finding provides new insights into LT-biosynthesis and biological function of LTs. The finding also provides a mechanism by which HCMV may act as an etiological agent rather than a bystander in the pathogenesis in inflammatory diseases.

5. Paper III

The third study focused on HCMV's influence on the expression of different matrix metalloproteinases (MMP) and their inhibitors; tissue inhibitor of MMP (TIMP) in primary human macrophages. We aimed to analyze the mRNA expression of MMP-2, -3, -7, -9, -12, -13, -14 and TIMP-1, -2, -3 and -4 in HCMV-infected or non-infected human primary macrophages. Depending on which MMPs and TIMPs are affected by HCMV, and whether they are increased or decreased, this study would provide important information about HCMVs role in HCMV-mediated pathogenesis implicated in cancer, fibrosis and/or arteriosclerosis.

5.1 An introduction to matrix metalloproteinases

Matrix metalloproteinase's (MMPs) are closely related proteinase's belonging to a family of at least 25 proteases that can degrade all macromolecules in the connective tissue matrix, and thereby regulate the composition of the extracellular matrix.

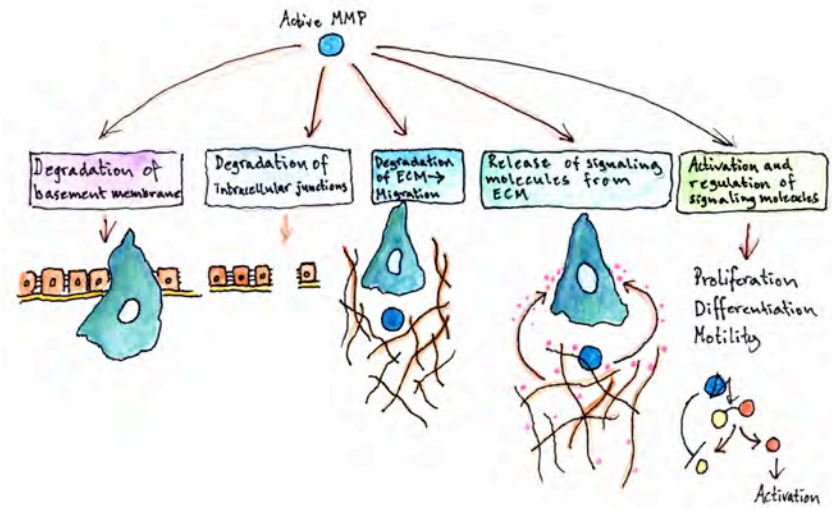

Figure 8. Illustration of MMP-exerted biological functions.

Since MMPs also regulate connective tissue remodeling, thereby determining the expansion and stability of the atherosclerotic plaque and the ability of smooth muscle cells to proliferate, MMPs have been implicated in atherogenesis and acute coronary syndrome. Macrophages are proposed to be the main source of MMP activity in atherosclerotic plaques (Brauer, 2006; Libby et al., 2002; Newby,

2005) and it has been shown that differentiation of monocytes into macrophages markedly increases their proteolytic capacity (Whatling et al., 2004).

One of the characteristics of MMPs is the catalytic zinc-binding site, another feature of the MMPs is the cystein-switch, most MMPs are secreted in a latent pro-form and activation requires cleavage of the pro-peptide that blocks the active site. The regulation of MMPs is controlled in multiple steps. Promoter regulation may govern a steady state expression, induced expression or repressed expression. Further, intracellular storages may control the rate by which the MMPs are secreted, while extracellular matrix molecules may bind MMPs, which then are immobilized. In addition, TIMPs have the ability to inactivate MMPs, and thus play a role in the regulation of MMPs.

MMPs have also been described to take part in other types of disease such as cancer, rheumatoid arthritis, and fibrosis. The expression of MMPs and their inhibitors: tissue inhibitor of metalloproteinase's (TIMPs), is in general found to be increased in various diseases (Brauer, 2006; Curran and Murray, 1999; Fiedorczyk et al., 2006; Galis and Khatri, 2002).

HCMV-infection downregulates MMP activity in endothelial cells and cytotrophoblasts, impairing endothelial cell migration and placental cytotrophoblast invasiveness (Yamamoto-Tabata et al., 2004). In contrast, HCMV-infection upregulates MMP-2 protein levels and activity in human coronary artery smooth muscle cells (Reinhardt et al., 2006).

5.2 Results and discussion paper III

In the third paper we sought to elucidate whether HCMV-infection could affect the expression of MMPs and their inhibitors. MMPs have been suggested to play important roles in the pathogenesis of various inflammatory diseases such as vascular disease, cancer and fibrosis. Earlier reports showed that HCMV-infected microvascular endothelial cells and cytotrophoblasts had decreased MMP-9 expression (Yamamoto-Tabata et al., 2004) and that HCMV-infected SMCs had an increased expression of MMP-2 (Reinhardt et al.,

2006). Macrophages are major producers of MMPs in the human atherosclerotic plaque.

Studies in apoE/MMP-9 double knockout mice show that MMP-9 might have a protective role in plaque development. If the MMP-9 gene is knocked out in apoE mice, the mice get larger plaques, more plaque ruptures, fewer SMCs and an increased number of macrophages in the plaque (Johnson et al., 2005).

We wanted to analyze if HCMV-infection could affect MMPs and their inhibitors in another cell-type, relevant in inflammation, and we decided to work with primary human macrophages. In this study we analyzed the mRNA expression of MMP-2, -3, -7, -9, -12, -13 and -14 as well as the mRNA expression of TIMP-1, -2, -3 and -4, in HCMV-infected or non-infected human primary macrophages.

We found that MMP-9, -12, -14 and TIMP-2 and -3 were significantly decreased in infected macrophages as compared to control cells. In contrast, we found that TIMP-1 mRNA expression was increased after infection by HCMV. Since the most profound effects were seen on MMP-9 and TIMP-1 mRNA expression, and MMP-9 has been described to be one key molecule in vascular disease, we focused our further studies on MMP-9 and its major inhibitor TIMP-1. More detailed analysis showed that MMP-9 mRNA levels started to decrease early after infection, in fact, a significant decrease could be observed as early as 2 hours after infection. In contrast, TIMP-1 mRNA levels were found to be increased 72 hours after infection. We could also demonstrate that the effect occurred in a dose-dependent manner. Wetern blot and ELISA experiments revealed that the total cellular content of MMP-9 protein decreased after infection, while in contrast, TIMP-1 total cellular protein content increased. Further, in a gelatin zymography assay, we could show that the MMP-9 activity decreased. In another set of experiments we were able to show that the decrease in MMP-9 mRNA expression was mediated through an HCMV-IE or –E gene product, while TIMP-1 mRNA expression was affected by a late viral gene product.

The exact mechanisms responsible for the observed altered expression pattern of MMP-9 and TIMP-1 mRNA and protein

expression is yet to be determined. However, HCMV-infection triggers IFN-γ production that mediates the activation of CIITA, which has been suggested to suppress MMP-9 expression by competitive binding of the CREB-binding protein (CBP) (Nozell et al., 2004), a protein with known histone acetyltransferase (HAT) -activity. In addition to the IFN-γ/CIITA –mediated competitive binding of CBP, the HCMV IE86 protein has been reported to interact with CBP (Hsu et al., 2004). Competitive binding of CBP by IE86 may thus be another possible explanation of HCMV-mediated suppression of MMP-9 mRNA expression. Another possible explanation or contributing factor might be the elevated IL-10 and cmvIL-10 levels following HCMV-infection (Yamamoto-Tabata et al., 2004). The HCMV-encoded IL-10 homolouge, cmvIL-10, has been shown to be secreted by HCMV-infected cells, furthermore, cmvIL-10 has also been shown to be functional, increasing the human IL-10 production, impairing the migration of cytotrophoblasts, by affecting the MMP-9 expression (Yamamoto-Tabata et al., 2004). IL-10 is known to decrease MMP-9 production and increase TIMP-1 production (Lacraz et al., 1995), therefore the HCMV-induced IL-10 and cmvIL-10 might be responsible for the altered MMP-9/TIMP-1 balance observed in our study.

Further studies are needed to elucidate if cmvIL-10 may affect the MMP-9/TIMP-1 balance in primary human macrophages or if the IE86 competitive binding of CBP might be the actual cause.

In summary, our findings supports the notion that HCMV-infection contributes to vascular disease and that the altered MMP-9/TIMP-1 balance mediated by HCMV, offers a molecular mechanism that link this virus to the development of vascular disease or possibly other inflammatory diseases.

6. Paper IV

Study number four aimed to investigate HCMV's effect on telomerase activity in human diploid fibroblasts (HDF) and malignant glioma (MG) cell lines. HCMV has been implicated in oncogenesis, but the HCMV-mediated mechanisms are not well defined. Recent studies have shown that HCMV protein and DNA are present in more than 90% of glioblastoma multiforme tumor specimens. Cellular immortalization/transformation requires telomerase activation and approximately 90% of cancers exhibit active telomerase. In this study, we wanted to explore if HCMV-infection could trigger telomerase activation in HDFs, which normally don't have telomerase activity, and in MG cell lines derived from primary glioma tumor specimens. If HCMV is able to trigger telomerase activity we wanted to find out how this is mediated by HCMV.

6.1 A short introduction to telomerase

The human enzyme telomere terminal transferase (telomerase) is a ribonucleoprotein with the capacity to synthesize TTAGGG repeats (Morin, 1989). Telomerase was first discovered in *Tetrahymena thermophilia* (Greider and Blackburn, 1985) and some years later it was established that telomerase itself contained the RNA temlplate CAACCCCAA that encodes for the TTAGGG DNA-repeats that builds up telomeres, and that this ribonucleoprotein in fact was a reverse transcriptase (RT), an RNA dependent DNA polymerase (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990). In humans, telomerase has two vital components: human telomerase mRNA component (hTR or hTERC) that is the RNA template needed for telomeric DNA synthesis; and the catalytic component human telomerase reverse transcriptase (hTERT) with its RT activity that adds the repeats onto the chromosomal ends. hTR is ubiquitously expressed in almost all human cells, while hTERT expression is tightly repressed. Therefore, induction of hTERT expression is required for telomerase activation (Cong et al., 2002).

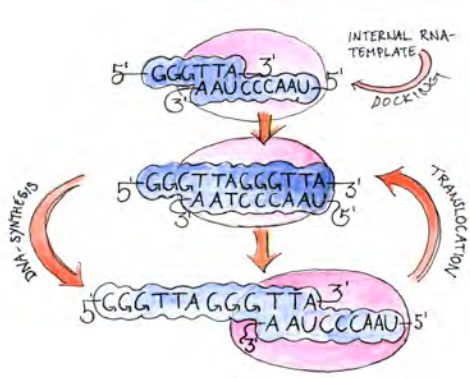

Figure 9. Illustration of telomer elongation by the telomerase complex.

Telomeric repeats at chromosomal ends are needed in order to maintain stability of the chromosomes, e.g. to avoid chromosomal end-to-end fusion, protect against recombination and avoidance of being recognized as damaged DNA. With each cell division in normal somatic cells the telomeres shortens, eventually leading to senescence or apoptosis. Most normal somatic cells have an undetectable telomerase activity due to the tight repression of its catalytic component: hTERT; exceptions are normal somatic cells that undergo clonal expansion such as T- and B-cells, stem cells and cells in tissue with high proliferative capacity, (reviewed in Cong et al., 2002). In contrast, hTERT expression and telomerase activity are observed in up-to 90% of human cancers (Kim et al., 1994; Shay and Bacchetti, 1997). The activation of telomerase through hTERT expression is a critical step in the transformation of normal cells into cancer cells with telomere dysfunction (Shay and Wright, 2005). These cancer cells thereby acquire the capacity to divide indefinitely, escaping senescence. In fewer cases, there is an alternative DNA recombination pathway referred to as: the alternative lengthening of telomeres (ALT) (Bryan et al., 1997), that makes it possible for the cell to escape its normal destiny, and instead transform into a cancer cell. Indeed, depletion of hTERT or telomerase activity impairs the proliferation and survival of cancer cells mediated through telomere dysfunction (Hahn et al., 1999).

Except its fundamental role in the maintenance of telomere length to protect the chromosomes from end-to-end fusion and instability,

and apart from its significance in cellular immortalization and transformation, telomerase has been suggested to take part in other important biological processes. Telomerase has been suggested to protect against apoptosis and to promote cellular proliferation independently of its telomere lengthening function, (reviewed in Cong and Shay, 2008). Telomerase might thereby also play a role in the proliferation and survival of cancer cells.

The transcription of hTERT is governed through multiple transcription factors including Sp1 and the c-Myc/Max network. Five Sp1 binding sites and two c-Myc binding E-boxes are located at the proximal promoter region of the hTERT gene (Kyo et al., 2000; Takakura et al., 1999). In contrast, histone deacetylases (HDACs) has been reported to take part in transcriptional repression of hTERT, and additionally, Sp1 is dependent on the inhibition of HDACs to mediate hTERT promoter activation and hTERT expression (Cong and Bacchetti, 2000; Hou et al., 2002). A switch from c-Myc/Max to Mad1/Max E-box binding at the hTERT proximal promoter has been shown to have a negative regulatory effect on hTERT expression in differentiating HL60 cells (Xu et al., 2001). The transcription factor Mad1 has the possibility, through available E-boxes at the proximal hTERT promoter, to recruit HDACs to the hTERT promoter, and thereby repress the transcription through deacetylation-mediated condensed chromatin structure. Interestingly, it has also been shown that TSA induces hTERT/telomerase, and moreover, that TSA facilitates HCMV replication.

Human tumor viruses specifically target hTERT/telomerase to transform cells, (reviewed in Bellon and Nicot, 2008). Epstein-Barr virus, Kaposi sarcoma–associated herpesvirus, human papillomavirus, hepatitis-B and -C virus, and human T-cell leukemia virus-1 all induce hTERT transcription and activate telomerase through various mechanisms, (reviewed in Bellon and Nicot, 2008).

6.2 Results and discussion paper IV

In this paper we aimed to explore the possibility that HCMV-infection could trigger the expression of hTERT and the activation of telomerase. Telomerase activation is critical for cellular immortalization and malignant transformation (Shay and Wright, 2005). As discussed above, telomerase activity is tightly regulated through the expression or repression of the catalytic component, hTERT (Shay and Bacchetti, 1997). Human tumor viruses have been shown to target hTERT and thereby induce telomerase activity (Bellon and Nicot, 2008) but so far no one had looked at the possibility that HCMV could target hTERT expression with following telomerase activation.

HCMV was already during the 1970s suspected to contribute to cancer development. It was shown by Geder et al., that HCMV-infection could transform human cells (Geder et al., 1976; Geder et al., 1977), unfortunately they failed to detect any HCMV-DNA or proteins in the transformed cells after long time passage, and later studies failed to transform cells. In later studies HCMV was shown to trigger the expression of the proto-oncogenes c-fos, c-jun and c-myc (Boldogh et al., 1990). Furthermore, HCMV has been demonstrated to cause specific DNA-breaks in chromosome 1, more specifically at 1q42 and 1q21 (Fortunato et al., 2000a). Interestingly, there is an association between 1q42 aberrations and glioblastoma (Li et al., 1995) and an association with 1q21 and breast carcinoma (Bieche et al., 1995). HCMV has been detected in several cancers such as colon cancer (Harkins et al., 2002), malignant glioma (Cobbs et al., 2002), prostatic carcinoma (Samanta et al., 2003), cervix cancer (Pacsa et al., 1975) and EBV-negative Hodgkin lymphoma (Huang et al., 2002).

Even though many years have past since the first reports about HCMV's oncogenic properties were reported, no clear mechanistic explanation to HCMV's role in cancer has been shown.

In this study we initially studied the mRNA expression of hTERT in HCMV-infected and non-infected fibroblasts and MG-cells. Interestingly we found that HCMV-infection triggered hTERT expression in these cells. UV-irradiated viral inoculums (replication deficient virus) could not mediate the effect, indicating that no

soluble factors in the viral inoculums mediated the induced mRNA expression of hTERT; rather that viral replication is needed to achieve this induction. We could also detect hTERT protein expression in fibroblasts by immunofluorescence techniques and the telomerase activity was confirmed using a telomerase PCR ELISA kit and a TRAPEZE kit. Further, using wt hTERT luciferase reporter construct as well as hTERT luciferase reporter constructs with mutations in Sp1 or c-Myc motifs, we could determine that HCMV-induced hTERT mRNA expression was partially Sp1-dependent.

Thereafter we used IE72 or IE86 expression plasmids together with the hTERT luciferase reporter construct, and found that the IE72 protein activated hTERT expression, while IE86 had no effect on hTERT expression. Immunoprecipitation experiments showed that HCMV-IE proteins interacted with HDAC-1 and -2. Western blot experiments show an increase of Sp1 protein in the infected cells and ChIP assay experiments revealed that Sp1 and IE proteins are present at the hTERT proximal promoter region, while HDACs are sequestered from the promoter region. In the ChIP assay we also detected H3 acetylation, which is known to open up the chromatin structure and facilitate the transcription of hTERT.

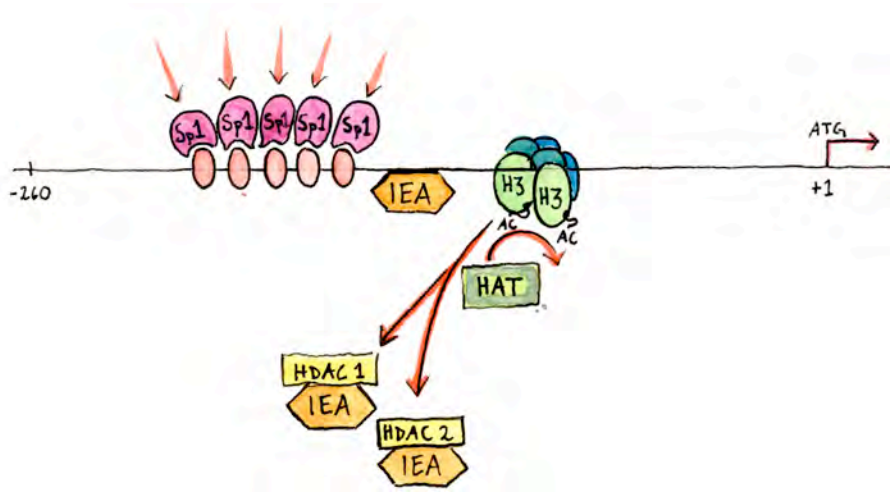

Figure 10. Illustration of the hTERT promoter region and what happens after HCMV-infection.

Finally, and most important, we could detect co-expression of hTERT and IE proteins in cells present in glioma specimens derived from patients with malignant glioblastoma. Interestingly, we could see that tumors with “low grade” HCMV-infection had a low levels of hTERT protein. In contrast, we found that tumors with “high grade” HCMV-infection had higher levels of hTERT protein present in the tumor. These observations suggest a tight link between the levels of HCMV and hTERT protein expression in tumors. Thus hTERT expression is not associated to tumor cells in this case, but rather associated to HCMV.

In summary, our finding provides a novel mechanism that may explain HCMV-induced oncogenesis, a mechanism that may be critical in the understanding of the relationship between HCMV and cancer.

7. Summary

My thesis work has focused on understanding molecular mechanisms of HCMV-pathogenesis in different diseases.

In paper one we found that the mRNA and protein expression of the receptors for PDGF-α and -β were decreased after HCMV-infection in human smooth muscle cells. Furthermore, we could show that PDGFR-β associates with molecules coupled to endosomes and lysosomes, which would suggest that the receptor is degraded after infection. This finding provides a possible mechanism to explain HCMV-induced birth defects often seen in children that have previously experienced a congenital HCMV-infection.

In paper two we showed that HCMV-infected human smooth muscle cells induce the mRNA and protein expression of 5-LO. Furthermore, we demonstrate that HCMV-infected SMCs are capable of synthesizing LTB_4, which is normally produced by inflammatory cells. Finally we could show that SMCs from patients with IBD co-expressed 5-LO, IEA and SMC-α actin *in vivo.* This finding offers an important link between diseases characterized by inflammation and HCMV. Through this mechanism HCMV may burst and sustain the inflammation, and thereby also worsening an inflammatory disease.

In paper three we showed that HCMV-infection alters the balance between MMP-9 and TIMP-1 in human macrophages. We found that the mRNA and protein expression of MMP-9 decreased. We could also demonstrate that the MMP-9 activity was decreased. In contrast we found that TIMP-1 mRNA and protein expression was induced. HCMV has been associated with vascular disease, and this finding offers one possible mechanism how this virus could affect plaque stability through altering the MMP-9/TIMP-1 balance.

In paper four we showed that HCMV-infection induces the mRNA and protein expression of hTERT followed by activation of the telomerase complex in human fibroblasts and malignant glioma cell lines. Furthermore, we could detect co-expression of IE and hTERT proteins *in vivo*, in tissue biopsies from patients with malignant glioblastoma. This finding offers a novel mechanism used by HCMV

that may be critical in the understanding of HCMV's possible oncogenic properties.

In summary, my thesis includes four molecular mechanisms that may be important in the understanding how HCMV through its ability to control cellular functions, may participate in the pathogenesis of different diseases.

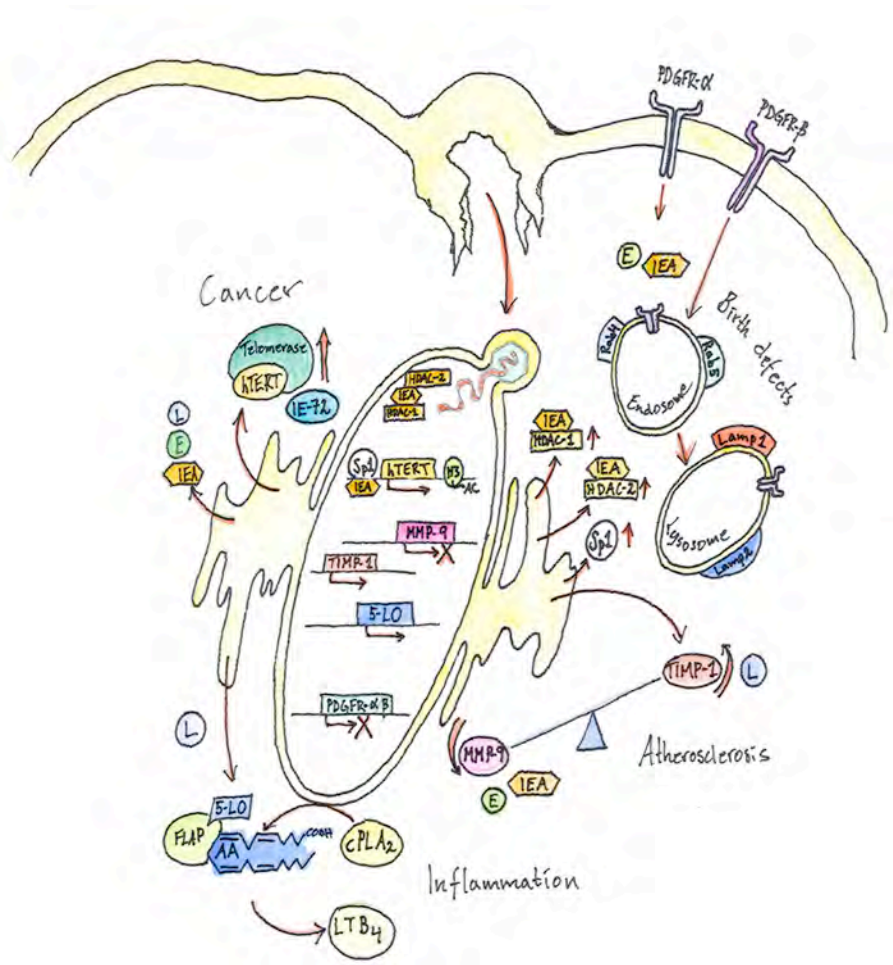

Figure 11. This illustration summarizes my findings that are presented in paper I-IV.

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