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Direct inhibitory effects of Ganciclovir on ICAM-1 expression and proliferation in human coronary vascular cells (SI/MPL-ratio: >1)

PI

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Treatment of the human cytomegalovirus (HCMV) infection with ganciclovir has beneficial indirect effects on the complex interactions of HCMV with restenosis, atherosclerosis, and transplant vascular sclerosis. The current study reports on direct effects of ganciclovir on expression of ICAM-1 and cell proliferation, key events of coronary atherosclerosis/restenosis. A potential clinical relevance of the data will be evaluated with the help of SI/MPL-ratio's.

Material/Methods: Definition of the SI/MPL-ratio: relation between significant inhibitory effects *in vitro/ ex vivo* and the maximal plasma level after systemic administration in vivo (ganciclovir: 9 µg/ml). Part I of the study investigated in cytoflow studies the effect of ganciclovir (0.05–5000 µg/mL) on TNF-a induced expression of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells derived from umbilical veins (HUVEC), human coronary endothelial cells (HCAEC), and human coronary smooth muscle cells (HCMSMC). Part II of the study analysed the effect of ganciclovir (0.05–5000 µg/mL) on cell proliferation (HUVEC, HCAEC, and HCMSMC). In part III cytotoxic effects of ganciclovir (0.05–5000 µg/mL) were studied (HUVEC, HCAEC, and HCMSMC).

Results: Ganciclovir caused slight but significant inhibitory effects on expression of ICAM-1 in HUVEC, HCAEC, and HCMSMC. In all three cell types studied strong dose depending significant antiproliferative effects of ganciclovir were detected. Partially, the antiproliferative effects of ganciclovir were caused by cytotoxic effects.

Conclusions: SI/MPL-ratio's >1 in HCAEC and HCMSMC indicate that the inhibitory effects of gancliclovir on ICAM-1-expression and cell proliferation may only be expected in vivo following local high dose administration e.g. in drug eluting stents (DES).

key words: **Ganciclovir • adhesion molecule ICAM-1 • cell proliferation • Coronary Restenosis • drug eluting stents**

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BACKGROUND

The initiation of atherosclerosis results from a complex interaction of circulating factors and various cell types in the vessel wall, including endothelial cells (EC), lymphocytes, monocytes, and smooth muscle cells (SMC). Interactions of monocytes with cells of the vascular wall allows these cells to move from the bloodstream into the intima where they differentiate into macrophages. This process is mediated by a variety of adhesion molecules on EC and SMC, including the intercellular adhesion molecule-1 (ICAM-1) [1]. Excessive reactive cell proliferation of smooth muscle cells in the vessel wall has been recognized as key component following the initial inflammatory response [2].

The importance of HCMV as a pathogen has increased over the last decades, with the escalation in the number of immunosuppressed patients. Moreover infection with HCMV is at least linked with atherosclerosis, transplant vascular sclerosis, and coronary restenosis [3]. On the other hand conflicting data have been reported concerning the clinical relevance of HCMV-infection: both a clear correlation [4,5] and no correlation at all have been reported in medium sized clinical studies [6,7]. For the time being it is consensus that experimental and clinical data are not strong enough to support an antiviral therapy to prevent the vascular reactions following arterial injury.

Recently a direct antiproliferative effect of the antiviral agent ganciclovir was reported by Battiwalla et al. [8] in human T-lymphocytes *in vitro*. Ganciclovir is a nucleoside analog of guanosine targeted at the viral DNA polymerase [9]. However, this effect is only partially specific for the viral polymerase because ganciclovir also inhibits DNA synthesis in hematopoietic progenitors [10].

The current study hypothesized that ganciclovir might as well inhibit ICAM-1 expression and cell proliferation in human coronary vascular cells. A possible clinical relevance of the data has been evaluated by calculation of a SI/MPL-ratio [11,12], characterizing the relation between the concentration of an agent with a siginificant inhibitory effect *in vitro* (SI) and the maximal systemic plasma level *in vivo* (MPL).

MATERIAL AND METHODS

Cell Culture

Endothelial cells from human umbilical veins (HUVEC) were isolated after vaginal delivery by enzymatic disaggregation with collagenase/dispase as described previously [XX 10]. Endothelial cells from human coronary arteries (HCAEC) were purchased at Lonza (Vervier, B). Cells were cultured in Endothelium Growth Medium (Lonza) and identified by the typical "cobble stone" growth pattern and positive reaction against von Willebrand factor (Dakopatts, Hamburg, D). Smooth muscle cells from the human coronary media (HCMSMC) were purchased at Lonza. HCMSMC were grown in Smooth Muscle Cell Growth Medium (Lonza). For identification of HCMSMC antibodies against smooth muscle α-actin (Sigma, Taufkirchen, D) were used.

Ganciclovir

Ganciclovir: Cymeven®, Hoffmann-La Roche AG, Grenzach-Wyhlen, D, 0.05–5000 µg/mL, dilution: aqua ad injectionem; MPL: 9 µg/mL [13].

Flow cytometry

For flow cytometry analysis of the expression of ICAM-1 in HCAEC, HUVEC, and HCMSMC cells were trypsinized and seeded into 6-well dishes (5×10^4 cells). Ganciclovir (0.05, 0.5, 5, 50, 500, 5000 µg/mL) was added to the cultures for a period of 18 h. During the last 6h of Ganciclovir incubation, the expression of adhesion molecules was stimulated by adding of TNF-α (20 ng/mL).

After Ganciclovir/TNF-α treatment, cells were washed twice with phosphate-buffered saline (pH 7.2) and trypsinized. Cells were resuspended in 100 µL of a FITC-conjugated monoclonal antibody directed against ICAM-1 (clone 84H10, Dianova Immunotech; final concentration 10 µg/mL) and incubated for 20min at 4°C. A total of 1×10^4 cells (100% gated) were analyzed immediately with a flowcytometer (BD FACSCalibur, Becton Dickinson, Heidelberg, D).

The effects of Ganciclovir (0.05–5000 µg/mL) on vitality of HCAEC, HUVEC, and HCMSMC was analyzed with propidium iodide using a flowcytometer.

Proliferation studies

HUVEC, HCAEC, and HCMSMC in passages 3–5 were seeded in a density of $3–5\times10^3$ cells × cm^{-2} in 6-well dishes. 24h after seeding the corresponding culture medium was renewed and the number of adherent cells was analyzed in a cell counter (CASY TTC®, Innovatis AG, Reutlingen, D). Subsequently Ganciclovir was added in concentrations of 0.05, 0.5, 5, 50, 500, 5000 µg/mL for another five days. Culture medium and ganciclovir were renewed at day three after seeding. Cell number after incubation with ganciclovir was calculated as relative cell number in comparison to untreated controls with the concerning solvent. Taking into account that not all cells could be successfully cultured, cell numbers of untreated controls were calculated as:

Total cell number at day 6 - cell number attached at day 1 after seeding = 100%.

Vitality of cells

In a luminescent cell viability assay (CellTiter-Glo™, Promega, Mannheim, D) the effects of ganciclovir in concentrations of 0.05, 0.5, 5, 50, 500, 5000 µg/mL (HUVEC, HCAEC, and HCMSMC) were analyzed for a period of five days in 96 well dishes (Nunc, Roskilde, DK). Luminescence of luciferase reaction as a marker of cell viability was measured in a CentroLB960 (Berthold, Technologies, Bad Wildbad, D).

SI/MPL-ratio

The SI/MPL-ratio was calculated in order to describe the relation between a significant inhibitory effect *in vitro* and the maximal plasma level after systemic administration *in vivo* [11,12].

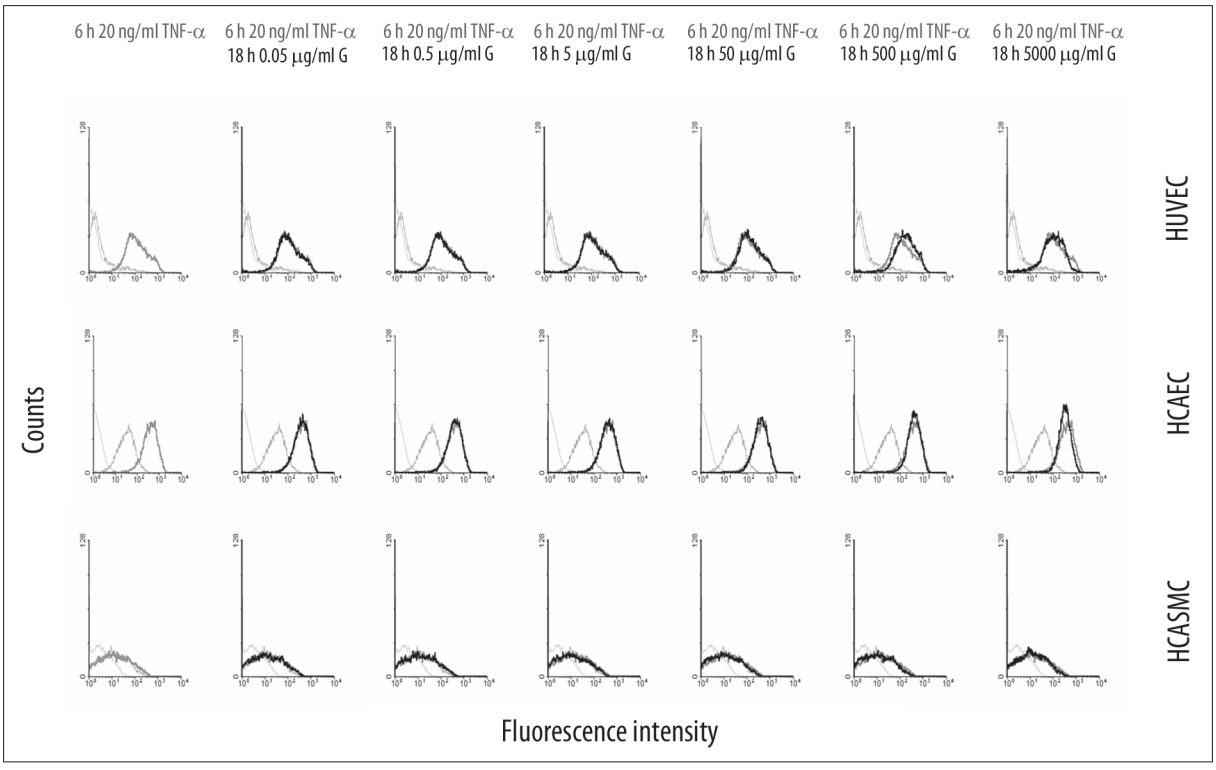

Figure 1. The effect of ganciclovir in concentrations of 0.05 μg/mL, 0.5 μg/mL, 5 μg/ml, 50 μg/mL, 500 μg/mL, and 5000 μg/mL on expression of the intercellular adhesion model-1 (ICAM-1) is demonstrated. Ganciclovir caused slight significant inhibitory effects on expression of ICAM-1 in HUVEC, HCAEC and HCMSMC.

Statistical analysis

Data of cytoflow and proliferation studies are presented as mean ±S.D. Statistical significance of differences between controls and drug-treated cells was determined by paired Student's t-test. Statistical significance was accepted for $P<0.05$.

Results

Identification of cells

Monocultures of HUVEC and HCAEC were identified by positive reaction with antibodies directed against von Willebrand factor and by the typical "cobblestone" growth pattern in culture. Monocultures of HCMSMC exhibited the "hill and valley" growth pattern and reacted positively with antibodies against smooth muscle α-actin.

Effects of Ganciclovir on ICAM-1 expression

The effects of ganciclovir (0.05, 0.5, 5, 50, 500, 5000 μg/mL) on TNF-α induced expression of ICAM-1 are demonstrated in Figure 1. A small significant inhibition of ICAM-1 expression was detected in HUVEC ($p<0.05$, SI/MPL-ratio: 0.005), HCAEC ($p<0.01$, SI/MPL-ratio: 555) and HCMSMC ($p<0.05$, SI/MPL-ratio: 5.55).

In HUVEC, treatment with TNF-α increased the mean fluorescence levels (%) of ICAM-1 expression 23.26-fold from 4.3% to 100%. Incubation of HUVEC with ganciclovir in concentrations of 0.05 μg/mL, 0.5 μg/mL, 5 μg/mL caused a small significant inhibition of ICAM-1 expression by 10.1% ($p\leq0.01$), 6.3% ($p<0.05$), and 5.9% ($p\leq0.01$), corresponding to SI/MPL-ratio's of 0.005, 0.05, and 0.5. Ganciclovir in a concentration of 50 μg/mL and 500 μg/mL caused a minimal inhibitory effect by 1.2% (n.s.), respectively a small stimulatory effect of 8% (n.s.). An inhibition of ICAM-1 expression by 25.5% was detected after incubation of HUVEC with ganciclovir in a concentration of 5000 μg/mL ($p\leq0.01$; SI/MPL-ratio: 555).

In HCAEC, treatment with TNF-α increased the mean fluorescence levels (%) of ICAM-1 expression 6.06-fold from 16.5% to 100.00%. After incubation of HCAEC with ganciclovir in concentrations of 0.05 mg/mL, 0.5 μg/mL, 5 μg/mL, 50 μg/mL, and 500 μg/mL no significant effects were detected, ICAM-1 expression was 97.3±4.3%, 98.3±3.6%, 104.4±1.9%, 101.6±2.2%, and 101.3±3.3%. Ganciclovir in the maximal concentration of 5000 μg/mL resulted in an inhibition of ICAM-1 expression by 25.4% ($p=0.01$, SI/MPL-ratio: 555).

In HCMSMC, treatment with TNF-α increased the mean fluorescence levels (%) of ICAM-1 expression 1.74-fold from 57.6% to 100.00%. Incubation of HCMSMC with ganciclovir in concentrations of 0.05 mg/mL, 0.5 μg/mL, 5 μg/mL, 50 μg/mL, and 500 μg/mL, and 5000 μg/mL caused small inhibitory effects of ICAM-1 expression by 8.5%, 6.9%, 14.2%, 12.5%, 14.2%, and 15% reaching statistical significance at the concentration of 50 μg/mL ($p<0.05$, SIMPL-ratio: 5.55) and 5000 μg/mL ($p<0.05$, SI/MPL-ratio: 555).

Effects of Ganciclovir on cell proliferation

The effects of ganciclovir (0.05 μg/mL, 0.5 μg/mL, 5 μg/mL, 50 μg/mL, 500 μg/mL, 5000 μg/mL) on proliferation of

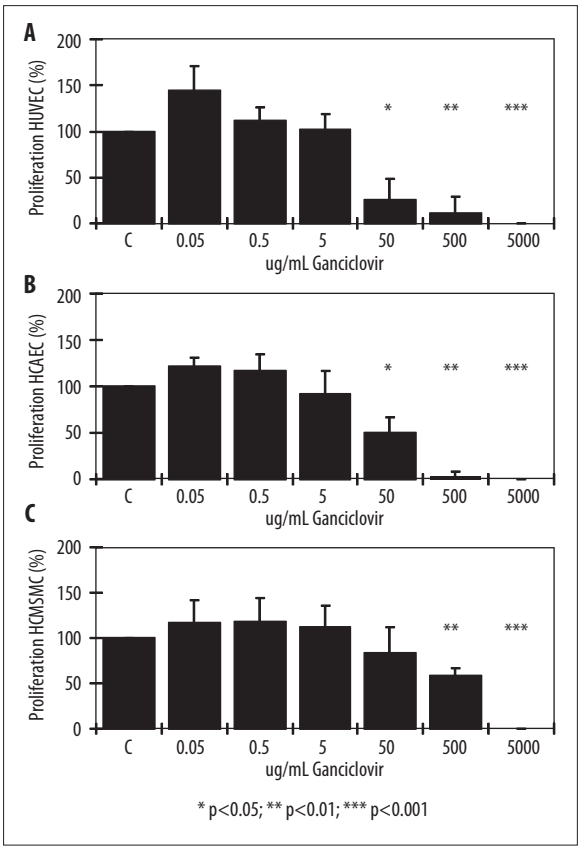

Figure 2. The effects of ganciclovir (0.05 µg/mL, 0.5 µg/mL, 5 µg/ml, 50 µg/mL, 500 µg/mL, and 5000 µg/mL) on proliferation of HUVEC (**A**), HCAEC (**B**), and HCMSMC (**C**) are demonstrated. After incubation with ganciclovir dose dependend significant antiproliferative effects were detected (C – control, p<0.05 – *; p<0.01 – **; p<0.001 – ***; paired Student's t-test).

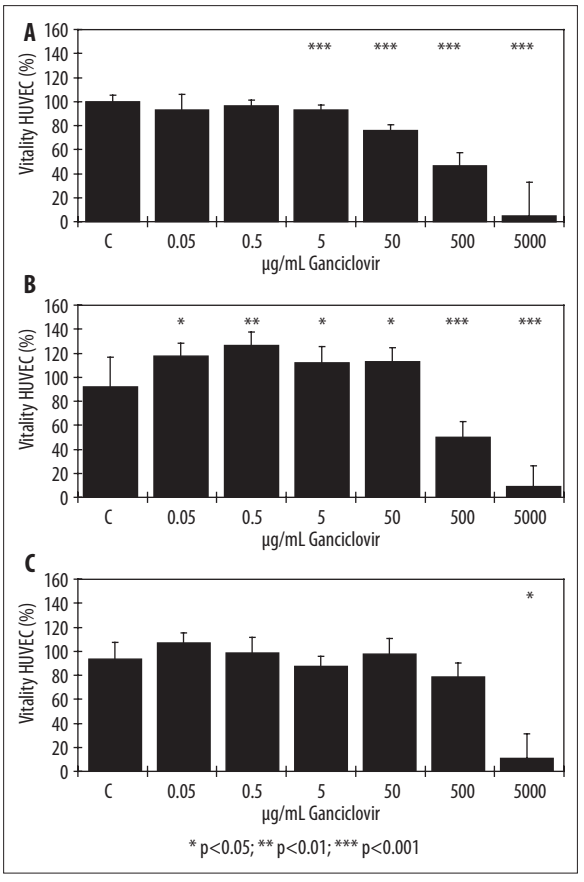

Figure 3. Cell vitality of HUVEC (**A**), HCAEC (**B**), and HCMSMC (**C**) was analyzed after incubation with ganciclovir in concentrations of 0.05 µg/mL, 0.5 µg/mL, 5 µg/ml, 50 µg/mL, 500 µg/mL, and 5000 µg/mL. Cell vitality of HUVEC, HCAEC, and HCMSMC was significantly decreased after incubation with ganciclovir. (C – control, p<0.05 – *; p<0.01 – **; p<0.001 – ***; paired Student's t-test).

HUVEC, HCAEC, and HCMSMC are demonstrated in Figure 2. After incubation with ganciclovir dose dependent significant antiproliferative effects were detected. Second, strong dose dependent significant antiproliferative effects were detected after incubation of HUVEC (p<0.05, SIMPL-ratio: 5.55), HCAEC (p<0.05, SIMPL-ratio: 5.55), and HCMSMC (p<0.01, SIMPL-ratio: 55.5).

In HUVEC incubation with ganciclovir in the concentrations of 0.05 µg/mL, 0.5 µg/mL, and 5 µg/mL caused stimulatory effects on cell proliferation by 44.8% (n.s.), 11.3% (n.s.), and 2% (n.s.). Strong dose dependent inhibitory effects were detected following incubation of HUVEC with ganciclovir in the concentrations of 50 µg/mL, 500 µg/mL, and 5000 µg/mL, cell proliferation was reduced by 74.5% (p<0.05, SI/MPL-ratio: 5.55, 89.3% (p=0.01, SI/MPL-ratio: 55.5, and 100% (p<0.001, SI/MPL-ratio: 555).

In HCAEC (Figure 2B) incubation of HCAEC with ganciclovir in the concentrations of 0.05 µg/mL and 0.5 µg/mL caused small stimulatory effects on cell proliferation, significance was not achieved. Dose dependent inhibitory effects by 8.3% (n.s.), 49.6% (p<0.05, SI/MPL-ratio: 5.55), 95.9% (p<0.001, SI/MPL-ratio: 55.5), and 100% (p<0.001, SI/MPL-ratio: 555) were detected after incubation of HCAEC with ganciclovir in the concentrations of 5 µg/mL, 50 µg/mL, 500 µg/mL, and 5000 µg/mL.

After incubation of HCMSMC (Figure 2C) with ganciclovir in the concentrations of 0.05 µg/mL, 0.5 µg/mL, and 5 µg/mL stimulation of cell proliferation by 16.6%, 17.9%, and 11% was detected, statistical significance was not achieved. Ganciclovir in the concentrations of 50 µg/mL, 500 µg/mL, and 5000 µg/mL resulted in dose depending inhibitory effects by 16.7% (n.s.), 41.8% (p=0.01, SI/MPL-ratio: 55.5), and 100% (p<0.001, SI/MPL-ratio: 555).

Effects of Ganciclovir on cell vitality

Cell vitality of HUVEC, HCAEC, and HCMSMC was analyzed after incubation with ganciclovir in concentrations of 0.05 µg/mL,0.50 µg/mL, 5 µg/ml, 50 µg/mL, 500 µg/mL, and 5000 µg/mL. Parts of the inhibitory effects of ganciclovir were caused by cytotoxic effects (Figure 3).

In HUVEC (Figure 3A) cell vitality was slightly decreased by 6.8% (n.s.), 3.5% (n.s.), and 6.9% (p<0.001, SI/MPL-ratio: 0.55) following incubation with ganciclovir in concentrations of 0.05 µg/mL, 0.5 µg/mL, and 5 µg/mL. Strong

inhibitory effects on cell proliferation by 24.3% ($p<0.001$, SI/MPL-ratio: 5.55), 53.2% ($p<0.001$, SI/MPL-ratio: 55.5), and 95.4% ($p<0.001$, SI/MPL-ratio: 555) were detected after incubation of HUVEC with ganciclovir in the concentrations of 50 µg/mL, 500 µg/mL, and 5000 µg/mL.

In HCAEC (Figure 3B) cell vitality was both significantly stimulated and inhibited. Ganciclovir in the concentrations of 0.05 µg/mL, 0.5 µg/mL, 5 µg/mL, and 50 µg/mL caused significant stimulatory effects on cell vitality by 27.6% ($p<0.05$, SI/MPL-ratio: 0.005), 37.4% ($p<0.01$, SI/MPL-ratio: 0.05), 21.7% ($p<0.05$, SI/MPL-ratio: 0.5), and 22.7% ($p<0.05$, SI/MPL-ratio: 5). Strong inhibitions of cell vitality were detected after incubation with ganciclovir in the concentration of 500 µg/mL ($p<0.001$, SI/MPL-ratio: 55.5) and 5000 µg/mL ($p<0.001$, SI/MPL-ratio: 555).

In HCMSMC (Figure 3C) small stimulatory (n.s.) and inhibitory effects (n.s.) on cell vitality were detected after incubation with ganciclovir in concentrations of 0.05 µg/mL, 0.5 µg/mL, 5 µg/mL, and 50 µg/mL. After incubation with ganciclovir in the concentrations of 0.05 µg/mL, 0.5 µg/mL, and 50 µg/mL cell vitality was increased by 14.2% (n.s.), 5.6% (n.s.), and 4.6% (n.s.). A small inhibitory effect by 6.6% (n.s.) was found after incubation of HCMSMC with ganciclovir in a concentration of 5 µg/mL. After incubation of HCMSMC with ganciclovir in concentrations of 500 µg/mL and 5000 µg/mL cell vitality was inhibited by 25.8% (n.s.) and 88.5% ($p<0.05$, SI/MPL-ratio: 555).

Discussion

The present *in vitro* study investigated direct effects of ganciclovir on key pattern of human coronary vascular disease. Three basic conclusions were determined. First, ganciclovir caused a small but significant inhibition of ICAM-1 expression in HUVEC ($p<0.05$, SI/MPL-ratio: 0.005), HCAEC ($p<0.01$, SI/MPL-ratio: 555) and HCMSMC ($p<0.05$, SI/MPL-ratio: 5.55). Second, strong dose depending significant antiproliferative effects were detected after incubation of HUVEC ($p<0.05$, SIMPL-ratio: 5.55), HCAEC ($p<0.05$, SIMPL-ratio: 5.55), and HCMSMC ($p<0.01$, SIMPL-ratio: 55.5). Third, partially these inhibitory effects of ganciclovir were caused by cytotoxic effects.

In vivo and *in vitro*, HCMV infects all of these cell types, and aside from immunologic clearance, viral infection modifies many of the host cellular functions that promote tissue repair [3]. Therefore, two groups have suggested already 15 years ago that an antiviral therapy may be effective in the treatment of human coronary restenosis [14,15]. Although this was an attractive hypothesis it required further clarification. *In vitro* it was demonstrated that HCMV infection resulted in recruitment of inflammatory effectors such as chemokines and cytokines. In addition, CMV modifies a number of cellular factors involved in angiogenesis and wound repair processes, including adhesion molecules, growth factors and receptors [3]. Upregulation of all these agents that initiate endothelial adhesion and those that promote wound healing provides a means for HCMV to enhance the adherence and infiltration of inflammatory cells that drive vascular disease. On the other hand conflicting data have been reported from clinical restenosis trials: both a clear correlation [4,5] and no correlation at all have been reported in medium sized clinical studies [6,7]. Recently our group has successfully infected a human arterial organ culture model with three different HCMV strains. However the data did not support the hypothesis that infection with HCMV triggered key events of restenosis as cell proliferation and neointimal hyperplasia in a period of 56 days [16].

At present time the data are not strong enough to support an antiviral therapy with e.g. ganciclovir in patients suffering from vascular injury. Ganciclovir is a nucleoside analog of guanosine that competitively inhibits the incorporation of dGTP by viral DNA polymerase [17]. However, this effect is only partially specific for the viral polymerase, because ganciclovir also inhibits DNA synthesis in hematopoietic progenitors [10]. Recently Battiwalla et al. [8] noticed that although cytopenia following ganciclovir administration *in vivo* is a well recognized complication *in vivo*, the possibility of a direct antiproliferative effect of ganciclovir has not yet been addressed. In a corresponding *in vitro* study the group were the first to demonstrate direct inhibitory effects of ganciclovir on proliferation of human T-lymphocytes [8]. These reports triggered the current investigation studying direct effects of ganciclovir on ICAM-1 expression and cell proliferation, key events of atherosclerosis, graft disease, and postangioplasty restenosis [1,2].

ICAM-1, a member of the immunoglobulin superfamily, is involved in atherosclerosis through the regulation of monocyte recruitment into atherosclerosis-prone areas [81, review]. Recently our group has demonstrated in a threedimensional model of monocyte attack that TNF-α induced upregulation of ICAM-1 increases adhesion and chemotaxis of human monocytes [18]. Expression of ICAM-1 has also been detected in human smooth muscle cells, indicating that these cells are as well capable of interacting with leukocytes [19]. The present data demonstrate in HCMSMC a slight significant inhibition after administration of ganciclovir in concentrations of 5000 µg/mL in HCAEC and 50 µg/mL and 5000 µg/mL. In HCAEC a slight inhibitory effect on ICAM-1 expression was detected after incubation with ganciclovir in a concentration of 5000 µg/mL. Both in HCMSMC and HCASEC the corresponding SI/MPL-ratio's were >1, indicating that the effects can't be expected *in vivo* following systemic administration of the agent. What remains difficult to comprehend is the inhibitory effect of ganciclovir in HUVEC after administration of ganciclovir in concentrations of 0.05 µg/mL, 0.5 µg/mL, 5 µg/mL followed by a missing effect after incubation with the increased concentration of 50 µg/mL and 500 µg/mL. Due to the fact that both in HUVEC and HCAEC expression of ICAM-1 was significantly inhibited after incubation with ganciclovir in a concentration of 5000 µg/mL this concentration seems to be accurate for a direct inhibitory effect on ICAM-1 expression in human endothelial cells. Moreover problems arise to figure out the differences between endothelial and smooth muscle cells, indicating a higher sensitivity of smooth muscle cells. Both in endothelial cells and smooth muscle cells expression of ICAM-1 is regulated via the nuclear factor-κB pathway [20]. Recently our group has demonstrated that HCAEC are more sensitive to an inhibitory antisense strategy directed against the nuclear factor-κB pathway in comparsion to HCMSMC [21]. This is in contrast to current data presented. One possible explanation might be the existence of pathways beyond the nuclear factor-κB pathway in the regulation of ICAM-1 expression [22].

Recently Battiwalla et al. [8] reported on a direct inhibitory effect of ganciclovir in a concentration of 10 µg/mL and 50µg/mL in human T-cell proliferation, no effect was detected after incubation with ganciclovir in a concentration of 2 µg/mL. These data are in accordance with the current data demonstrating significant antiproliferative effects of ganciclovir in concentrations of ≥50 µg/mL in HCAEC and ≥500 µg/mL in HCMSMC. Partially the antiproliferative effects of ganciclovir were caused by cytotoxic effects. After incubation of HCMSMC with ganciclovir in concentrations of 500 µg/mL and 5000 µg/mL cell vitality was inhibited by 25.8% (n.s.) and 88.5% ($p<0.05$, SI/MPL-ratio: 555).

Conclusions

In comparison to the strong antiproliferative effects in human vascular smooth muscle cells described earlier by our group after administration of cytostatic agents [23], steroid agents [24], and immunosuppressive agents [25] the antiproliferative effects of ganciclovir are comparable with inhibitory effects of 41.8% (500 µg/mL) and 100% (5000 µg/mL), respectively.

However, in contrast to these agents the antiproliferative effects in HCMSMC were detected with concentrations of ganciclovir 55.5 times and 555 times the maximal plasma level, resulting in SI/MPL-ratio's of 55.5 and 555. Therefore this effect can't be expected after systemic administration. Local high dose administration of ganciclovir, however, may be however possible with e.g. drug eluting stents (DES's) or other local devices [26,27]. Recently our group has suggested to calculate a SI/DES ratio [12] for appropriate dosing in DES's. Due to the fact that agents are commonly underdosed after systemic administration [11] and overdosed in DES's [12], we would like to suggest SI/MPL- and SI/DES-ratio's of 1/100 as an orientation.

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The authors of the manuscript certify that they comply with the principles of Ethical Publishing in the International Journal of Cardiology [28].

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