Cytomegalovirus Infection–Enhanced Allograft Arteriosclerosis Is Prevented by DHPG Prophylaxis in the Rat

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Background  Major risk factors for accelerated allograft arteriosclerosis include humoral and cellular immune response, hyperlipidemia, and viral infections. We demonstrated earlier that rat cytomegalovirus (RCMV) infection doubles smooth muscle cell proliferation and intimal thickening of rat aortic allografts. In this study, the effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) on RCMV-enhanced rat allograft arteriosclerosis are investigated.

Methods and Results  Aortic allografts from the DA to the WF rat strain were used. The recipients were inoculated with 10³ plaque-forming units of RCMV 1 day after transplantation. Two groups of RCMV-infected rats were treated with DHPG with an initial dose of 20 mg/kg IP and a maintenance dose of 10 mg/kg IP twice a day for a period of 14 days. In the DHPG prophylaxis group (n=22), the drug administration started 1 day before infection, and in the DHPG treatment group (n=17), 7 days after infection. One group of infected rats was left untreated (n=21). The grafts were removed 7 and 14 days and 1, 3, and 6 months after transplantation. In the DHPG prophylaxis group, no virus could be recovered by plaque assays. In the treatment group, 50% of rats were virus-positive at 1 month and 40% at 3 months. DHPG prophylaxis prevented the infiltration of inflammatory cells and their proliferation in the adventitia of RCMV-infected recipients (P<.01), with a 60% reduction in the interleukin-2 receptor expression (P<.05) and a 30% decrease in major histocompatibility complex class II expression (P=NS). DHPG prophylaxis did not significantly alter the levels of insulin-like growth factor-1, epidermal growth factor, platelet-derived growth factor-BB, transforming growth factor-β1, acidic fibroblast growth factor, and basic fibroblast growth factor messages in the allograft vascular wall. Early media necrosis was reduced. Arteriosclerotic alterations and proliferation of smooth muscle cells were both reduced 50% to 70% by DHPG prophylaxis (P<.05 at 3 months). The responses in the DHPG treatment group were quite similar but less impressive and statistically nonsignificant.

Conclusions  We consider it likely that DHPG inhibits arteriosclerotic alterations primarily by reducing the infectious virus and thereby the inflammatory response in the allograft vascular wall; another possibility is a direct antiproliferative effect on smooth muscle cell replication. A dose-dependent inhibitory effect of DHPG on smooth muscle cell replication was recorded in an in vitro study. (Circulation. 1994;90:1969-1978.)

Key Words  • transplantation • ganciclovir • smooth muscle cells • growth substances • arteriosclerosis

Major risk factors for accelerated allograft arteriosclerosis, ie, chronic rejection, in heart transplant recipients include humoral and cellular immune responses, hyperlipidemia, and viral infections. We demonstrated with our rat aortic allograft model that rat cytomegalovirus (RCMV) infection doubles allograft arteriosclerosis.

The drug of choice for the treatment of CMV infection is 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG; ganciclovir). DHPG is an acyclic analogue of the natural 2'-deoxyguanosine. The mechanism of action of this drug depends on the formation of a 5'-triphosphate derivative that is a selective inhibitor of the viral DNA polymerase. In herpes virus–infected cells, viral thymidine kinase catalyzes the production of the DHPG monophosphate derivative. However, human CMV does not encode the viral thymidine kinase. Enzymes responsible for the phosphorylation have not been identified. It has recently been shown that human CMV UL 97 open reading frame encodes a protein that controls the phosphorylation of DHPG in human CMV–infected cells. The predicted product of this gene shares regions of homology with protein kinases, guanylyl cyclase, and bacterial phosphotransferases.

In this article, we demonstrate that DHPG prophylaxis completely abolishes the enhancing effect of CMV infection on allograft arteriosclerosis. DHPG treatment initiated at the time of established infection has only a partial and nonsignificant effect on the development of this disorder. Our results postulate two possible mechanisms for the DHPG effect: a direct inhibition of the virus followed by significant reduction in the adventitial inflammatory response of the infected graft and possibly...
a direct antiproliferative effect on the secretory phenotype of vascular smooth muscle cells.

Methods

Experimental Protocol

Recipient of aortic allografts were infected intraperitoneally with RCMV 1 day after transplantation and were given DHPG either from day 0 (DHPG prophylaxis group) or day 7 (DHPG treatment group) for a period of 14 days or were left untreated and used as controls. The grafts were removed at 7 and 14 days and at 1, 3, and 6 months, and there were (5,5,4,3,4) rats in the RCMV-infected untreated group, (5,4,3,6,4) rats in the DHPG prophylaxis group, and (0,4,5,4,5) rats in the DHPG treatment group, respectively. The grafts were processed for morphometry, autoradiography, immunohistochemistry, and reverse transcriptase polymerase chain reaction (RT-PCR).

Rats

Inbred DA (AG-B4, RT1b) and WF (AG-B2, RT1b) rat strains were used as donors and recipients, respectively. The animals were purchased from the Laboratory Animal Center, University of Helsinki, Finland. They were 2 to 3 months old and weighed 200 to 300 g. The rats were fed with regular rat food (Altromin, Standard Diet, Chr. Petersen A/S) and tap water ad libitum. The animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Aortic Allografts

A 3-cm-long segment of descending thoracic aorta was removed, thoroughly perfused with phosphate-buffered saline (PBS), and used as transplant.12 End-to-end anastomosis was performed with 9-0 continuous nylon suture. The grafts were transplanted into a heterotopic position below the renal arteries and above the bifurcation, forming a loop in the recipient abdominal cavity. Total ischemic time was 45±15 minutes, during which time the graft was kept in an ice bath at +4°C for 15 minutes. The experimental animals were anesthetized with chloral hydrate 240 mg/kg IP and were given buprenorphine 0.25 mg/kg SC (Temgesic; Reckitt & Colman) for postoperative pain relief.

Rat Cytomegalovirus

RCMV infection was established by inoculating 10⁶ plaque-forming units IP of the Maastricht strain RCMV24,25 1 day after transplantation. RCMV was passaged by infecting fibroblasts from 17-day-old DA rat embryos. The cells were cultured in flasks containing modified Eagle’s medium (MEM) (Flow Laboratories) supplemented with 200 mmol/L L-glutamine (Northumbria Biologicals Ltd), 10 000 IU/mL penicillin/1000 µg/mL streptomycin solution (Gibco Ltd), and 2% fetal calf serum (FCS) (Sera-Lab) according to standard viral culture techniques.26 The stocks of virus were stored at −70°C until use.

Plaque Assays

The potency testing of RCMV was done by plaque assays as described.27 Confluent rat embryo fibroblast monolayers were washed with Hanks’ balanced salt solution (1×) (Gibco), infected with appropriate virus dilution and incubated at +37°C in a 5% CO₂ incubator for 60 minutes. After incubation, the infected cells were overlaid with 1 mL basal medium (BME) (Eagle) (Gibco) supplemented with glutamine, penicillin, streptomycin (as above), and 5% FCS (Sera-Lab) at a final concentration of 0.25% Agarose (Sigma Chemical Co). After 7 days, the cells were fixed in 10% formalin overnight; then the solid base layer was removed, and the cell monolayers were stained with 1% aqueous crystal violet (Merck). The plaques were counted by light microscopy.

Quantification of infectious RCMV from the biopsies of salivary glands, liver, and spleen was done by the plaque assay.27 Briefly, biopsies were taken aseptically in MEM with 2% FCS (supplemented as above) and stored at −70°C. The organs were homogenized in a tissue grinder and suspended in MEM with 2% FCS (as above), and 10-fold dilutions of 10% homogenates (wt/vol) were inoculated on confluent rat embryo fibroblast monolayers. After an incubation of 7 days, the number of plaques was monitored microscopically.

ELISA

ELISA was carried out on polystyrene chloride plates coated with cellular antigens obtained from RCMV-infected rat fibroblasts. Mock antigen was used for negative controls. The RCMV antigen was prepared by the glycine-buffered extraction method.28 The polystyrene ELISA plates were coated with RCMV antigen 10 µg/mL in carbonate buffer, pH 9.6, at +4°C overnight before incubation. After the plates were washed with the buffer, serum samples at appropriate dilutions were added into the wells and incubated at +37°C for 60 minutes. After the wash with buffer, peroxidase-conjugated goat anti-rat IgG (H+L) (Cappel Laboratories) was added at a dilution of 1:2000 in buffer with 0.5% bovine serum albumin. After washing, OPD substrate citrate phosphate buffer (orthophenylenediamine; Sigma P-3888) was added and incubated for 15 minutes. To stop the color reaction, 2 mol/L H₂SO₄ was added, and the intensity was determined by Multiscan ELISA reader at 492 nm. The values are expressed as the difference of extinction between positive and negative antigen (∆ΔE). ∆E<200 is assumed to be negative.

DHPG

DHPG (ganciclovir; Cymevene 500 mg; Syntex) was dissolved in 100 mL of 0.9% NaCl at a final concentration of 5 mg/mL. DHPG was administered intraperitoneally at an initial dose of 20 mg/kg and a maintenance dose of 10 mg/kg twice a day for 14 days.

Histology

The grafts were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and examined histologically after sectioning and staining with Mayer’s hematoxylin-eosin. Cross sections were prepared for evaluation of circular changes in the graft. No difference was observed whether or not in situ fixation was performed. Nontransplanted DA rat thoracic aortas were used as normal controls.

Morphometry

Histological changes were quantified according to standard morphometric principles29 and are expressed as the mean number of points falling over a given anatomic area using a straight, cross-sectional line and a 0.02-mm grid and are given as point score units (PSU). The number of cell nuclei in the adventitia, media, and intima and intimal thickness were evaluated. Final scores were given as mean±SEM.

Autoradiography

All rats received 300 µCi of (methyl-¹H)thymidine (H-TdR; Amersham International plc) IV 3 hours before they were killed. Histology was processed from paraffin cross sections, and emulsion film autoradiography (Ilford L.4; Ilford) was performed. The numbers of labeled nuclei were counted in the adventitia, media, and intima of aortas.
Immunohistochemistry

A piece of transplant was taken in OCT compound (Tissue-Tek, Miles Inc), snap-frozen, and stored at −70°C. Cross sections 4 μm thick were stained with a three-layer indirect immunoperoxidase technique30 using the following monoclonal antibodies with appropriate dilutions: interleukin (IL)-2 (CD25), a monoclonal antibody to rat IL-2 receptor (a generous gift from Dr J. Kupiec-Weglinski, Harvard Medical School, Boston, Mass), and OX6 (Sera Lab), a mouse IgG1 monoclonal antibody to rat major histocompatibility complex (MHC) class II common determinant. A peroxidase-conjugated rabbit anti-mouse Ig antibody (Dako-immunoglobulins A/S) and a peroxidase-conjugated goat anti-rabbit Ig antibody (Caltag Laboratories) were used as second and third antibodies, and the reaction was revealed by chromogen 3-amino-9-ethylcarbazole containing hydrogen peroxidase. Mayer’s hemalum was used for counterstaining. Positive staining for IL-2R antibody was quantified as the number of positive adventitial inflammatory cells per aortic cross section, and positive reaction for MHC class II antibody was scored from 0 to 5 (0, no positive staining; 1, very weak; 2, weak; 3, moderate; 4, intense; and 5, very intense).

Total RNA Isolation

Immediately after graft removal, a piece of graft was stored in liquid nitrogen. Total RNA was extracted with guanidine-isothiocyanate preparation.31 Briefly, frozen tissues were mixed with 3 mL of 4 mol/L guanidine-isothiocyanate buffer and homogenized with Ultra-Turrax (Janke & Kunkel). Two milliliters of 5.7 mol/L CsCl buffer was put into polyallomer ultracentrifuge tubes (Beckman Instruments), and the guanidine-lysed samples were added onto the top. The samples were centrifuged at 32,000 rpm for SW 50.1 at +20°C for 21 hours. After centrifugation, the supernatant was removed, and RNA was resuspended with TES buffer (10 mmol/L TRIS, pH 7.4; 5 mmol/L EDTA, pH 7.4; 1% sodium dodecyl sulfate) in an ice bath for 2 to 3 hours. The resuspension was put into microfuge tubes, and the same volume of phenol was added. The samples were mixed and spun down to separate phases. The upper phase was removed to a new tube, and the phenol purification was repeated twice. After that, phenol-chloroform (1:1) and, in the last step, only chloroform was added to samples to yield purified RNA. Aqueous RNA was precipitated with 3 mol/L sodium acetate (pH 5.2) and 2.5× frozen ethanol. RNA concentrations were measured spectrophotometrically.

RT-PCR

RNA (1 μg) was reverse transcribed to cDNA by a commercial Perkin Elmer Cetus protocol. One microgram from the RT reaction was taken for PCR, which was performed in PCR buffer (500 mmol/L TRIS, pH 8.8; 15 mmol/L MgCl2; 150 mmol/L [NH4]SO4; 0.1% gelatin; 1% Triton-X-100) using 0.2 mmol/L of each nucleotide, 1 μmol/L of each primer, and 2.5 U Taq DNA polymerase. The samples were amplified for 25 to 28 cycles each, denaturated at +94°C for 1 minute, annealed at +57°C for 1 minute, and extended at +72°C for 2 minutes. As a control, PCR was also made with GAPDH primers. The samples were electrophoresed in agarose gel and Southern blotted. Radiolabeled oligo probes were used to detect the PCR products. The intensity of hybridization signals was measured by densitometry (ELECTROPHORESIS Data Center, Helsinki Laboratories), and the results are expressed as a ratio of growth factor mRNA to GAPDH mRNA ±SEM.

The primers and probes were prepared at the Molecular/Cancer Biology Laboratory, Department of Pathology, University of Helsinki, Finland. The rat cDNA sequence of transforming growth factor (TGF)-β1 was obtained from the EMBL (accession number X52498 for TGF-β1), and all other sequences were obtained from the GenBank (accession numbers are M15480 for insulin-like growth factor [IGF]-1, M63585 for epidermal growth factor [EGF], Z14117 for platelet-derived growth factor [PDGF]-BB, M22427 for basic fibroblast growth factor [bFGF], and M17710 for GAPDH). No cDNA sequence is available for rat acidic FG (aFGF), and the primer and probe sequences were prepared with sequences of mouse (M30641), bovine (X13221), and human (S90026) aFGF.

Smooth Muscle Cell Culture

Smooth muscle cells were isolated from 9- to 11-day-old DA rat thoracic aortas.32 The aortas were opened longitudinally, and the intimal layer was gently scraped off; the media and the adventitia were carefully separated and sliced into small pieces. The different layers were digested with 0.1% collagenase and 0.02% DNAse in PBS at +37°C for 1 to 2 hours. Isolated smooth muscle cells from the media were centrifuged, suspended in culture medium (as above), seeded in plastic flasks, and incubated at +37°C. Passages 2 through 4 were used for investigation.

Smooth muscle cells were seeded in 96-microtiter wells (5000 cells/well) in Dulbecco’s MEM (Flow Laboratories) supplemented with 10% FCS (Sera-Lab), 2 μmol/mL glutamine, and 1000 μg/mL streptomycin/10 000 U/mL penicillin. DHPG was added at concentrations of 0, 1, 10, 100, and 1000 μg/mL. The cells were exposed daily to 1 μCi/mL of 3H-Tdr for 24 hours to quantify DNA synthesis. After 24 hours, the wells were washed three times with PBS, and the cells were detached with 1.25% trypsin + versene and mixed with Optiphase “HiSafe” 3 (LKB-Wallac). Radioactivity was measured with a Rackbeta liquid scintillation counter (LKB-Wallac). To assess the viability of cultured cells and to exclude any toxic effect of the drug on smooth muscle cells in vitro, the medium was removed from additional cultures on day 4 and replaced with conditioned medium from smooth muscle cell bulk cultures. Furthermore, the cells were monitored daily by light microscopy for their viability.

Statistical Analyses

Data are expressed as mean±SEM or, in the case of nontransplanted DA rat thoracic aortas, as mean±SD (normal controls). The total variation between the groups was analyzed by the nonparametric Kruskal-Wallis one-way analysis by ranks (Statview 52.2*, BrainPower Inc). The rank sums obtained with the Kruskal-Wallis test were used for the Dunn test at the significance level of 5% (Medstat; Astra Group AB) to find out which of the group(s) differed significantly from the others.33 Finally, linear regression analysis34 was applied to evaluate the significances of histological changes between DHPG-treated and RCMV-infected allografts within time intervals. In regression analysis, the statistical differences between regression coefficients (slopes) of linear plots have been calculated. Values of P < .05 were regarded as statistically significant.

Results

Effect of DHPG on RCMV Infection

Aortic allograft recipients were infected with 10⁶ plaque-forming units of RCMV 1 day after transplantation. The rats were divided into three different categories according to the DPHG therapy: (1) no therapy, (2) DHPG prophylaxis beginning 1 day before inoculation of the virus, and (3) DHPG treatment beginning at the time when infection was established.

In nontreated RCMV-infected rats (Table 1), infectious virus could usually be recovered from the biopsies
of liver/spleen 7 days after transplantation but not thereafter (acute infection). In contrast, plaque assays from the salivary glands did not yield infectious virus until 1 and 3 months after inoculation (chronic infection). Six months after transplantation, no infectious virus could be demonstrated from organ biopsies (latent infection), and the rats were assumed to be latently infected.

In the DHPG prophylaxis group, no infectious virus could be isolated by plaque assays from the organ biopsies during the whole observation time. In the DHPG treatment group, the virus could be isolated only from salivary glands in 50% (3/6) of the rats 1 month after infection and in 40% (2/5) of the rats 3 months after infection (Table 1).

Effect of DHPG on the Humoral Immune Response to RCMV Infection

The RCMV-IgG antibody level was measured by ELISA 3 and 6 months after transplantation. High-level antibody formation to RCMV was observed in non-treated RCMV-infected allografts, as shown in Table 2. In the DHPG prophylaxis group, antibody formation was below the threshold level. In the DHPG treatment group, low humoral immune response to RCMV was detected in 67% and 75% of the rats at 3 and 6 months, respectively.

Effect of DHPG on RCMV-Enhanced Allograft Arteriosclerosis

Aortic allografts were transplanted from the DA to the WF rat strain and harvested 7 and 14 days and 1, 3, and 6 months after transplantation. Histological changes were quantified by morphometry and are given as PSU±SEM.

Adventitia

There were only a few cells, mainly fibroblasts, in the adventitia of nontransplanted DA aortas (0.5±0.5 PSU, ±SD). In RCMV-infected allografts, an intense adventitial inflammatory response to 11.6±1.5 PSU occurred 7 days after transplantation. The inflammatory response was increased to 15.6±1.5 PSU 1 month after transplantation, after which it declined.13 The early inflammatory response in RCMV-infected allografts was inhibited by DHPG prophylaxis to the level of 6.1±0.2 PSU (P<.01) at 7 days (Fig 1a). DHPG treatment had no influence on the early inflammatory response (Fig 2a). The peak inflammatory response of both DHPG groups occurred at 1 month but was reduced by 30% compared with RCMV-infected allografts (P<.05 for DHPG prophylaxis). Thereafter, the number of adventitial cell nuclei in the DHPG groups showed the same decline as in the control group.

Table 2. Effect of DHPG on Humoral Immune Response to RCMV Infection

<table>
<thead>
<tr>
<th>Time After Transplant</th>
<th>RCMV</th>
<th>DHPG Prophylaxis</th>
<th>DHPG Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mo</td>
<td>1700±0</td>
<td>70±50</td>
<td>270±60</td>
</tr>
<tr>
<td>6 mo</td>
<td>1560±190</td>
<td>50±40*</td>
<td>440±60</td>
</tr>
</tbody>
</table>

RCMV indicates rat cytomegalovirus. Anti-RCMV IgG antibody formation was assessed by ELISA, which was carried out to cellular antigens of RCMV-infected rat fibroblasts coated on polystyrene chloride plates. Mock antigen was used for negative controls. The intensity of color reaction was determined at 492 nm, and the values are expressed as the difference of extinction (ΔE) between positive and negative antigen ±SEM. ΔE ≤200 is assumed to be negative. *P<.01 by the Kruskal-Wallis and the Dunn tests.
Fig 1. Semilogarithmic plots showing the effect of DHPG prophylaxis on rat cytomegalovirus (RCMV)-enhanced aortic allograft arteriosclerosis. The rats were infected intraperitoneally (IP) with 10⁵ plaque-forming units of RCMV 1 day after transplantation. The infected rats either received DHPG prophylaxis IP from day 0 with an initial dose of 20 mg/kg and a maintenance dose of 10 mg/kg twice a day for 14 days (solid line with squares) or were left nontreated and used as controls (dotted line with circles). The grafts were removed on days 7 and 14 and at 1, 3, and 6 months. The numbers of rats in the DHPG prophylaxis group were 5, 4, 3, 6, and 4 and in the nontreated group, 5, 4, 4, and 3, respectively. The responses are quantified by point score units (PSU), ie, the number of cell nuclei in the adventitia, media, and intima, and as the thickness of intima, and the results are given as mean ± SEM. Framed area indicates nontreated DA rat thoracic aortas, mean ± SD. *P < .05, **P < .025, and ***P < .01. The total variation between the groups was analyzed by the nonparametric Kruskal-Wallis test, which yielded the rank sum used for the calculation of significances by the Dunn test. In addition, linear regression analysis revealed a significant reduction in the intimal thickness by DHPG prophylaxis up to 3 months (P < .05; slope 0.5 in the DHPG prophylaxis group vs slope 1.7 in DA-WF + RCMV rats).

Media

The number of cell nuclei in the media of nontransplanted DA aortas was 3.1 ± 0.1 PSU (± SD). In RCMV-infected allografts, media necrosis with loss of media nuclei developed after 1 month, declining to 0.6 ± 0.2 PSU 3 months after transplantation. In the DHPG prophylaxis (Fig 1b) and treatment (Fig 1b) groups, the number of media cells remained approximately within the normal variation up to 1 month compared with RCMV-infected allografts (P < .05 for the DHPG-prophylaxis group). In the prophylaxis and treatment groups, media necrosis was first observed at 3 months, reaching 0.5 ± 0.3 PSU in the prophylaxis and 0.3 ± 0.1 PSU in the treatment group at 6 months after transplantation.

Intima

The most important effects of DHPG were observed in the intima and are depicted in Figs 1c, 1d, 2c, and 2d.

Fig 2. Semilogarithmic plots showing the effect of DHPG treatment on rat cytomegalovirus (RCMV)-enhanced aortic allograft arteriosclerosis. The rats were infected intraperitoneally (IP) with 10⁵ plaque-forming units of RCMV on day 1. The infected rats were either given DHPG treatment IP from day 7 with an initial dose of 20 mg/kg and a maintenance dose of 10 mg/kg twice a day for 14 days (solid line with triangles) or left nontreated and used as controls (dotted line with circles). The rats were killed on day 14 and at 1, 3, and 6 months. The number of rats in the DHPG prophylaxis group were 0, 4, 4, 5, and 4 and in the nontreated group 5, 5, 4, 4, and 3, respectively. For symbols see Fig 1.

Intimal cells. The luminal surface of nontransplanted DA aortic wall is normally lined by an endothelial cell monolayer, ie, on average 0.9 ± 0.2 PSU (± SD) nuclei only. In RCMV-infected allografts, the number of intimal nuclei increased to 1.6 ± 0.1 PSU 7 days after transplantation. It reached a peak of 3.6 ± 0.7 PSU at 1 month, remained unchanged at 3 months, and declined.

Fig 3. Autoradiograms for proliferating cells in the three layers of allograft vascular wall after DHPG prophylaxis. The rats received ³H-TdR intravenously 3 hours before graft removal, and the autoradiograms were quantified as the number of thymidine-incorporating nuclei in cross sections of different layers of aortas. For symbols see Fig 1.
slightly at 6 months after transplantation. DHPG prophylaxis reduced the number of intimal cell nuclei to 1.1±0.1 PSU on day 7 (P<.025), to 2.1±0.5 PSU at 1 month (P=NS), and to 2.1±0.2 PSU at 3 months (P<.05). Thereafter, the number of intimal cell nuclei declined slightly, as did in nontreated RCMV-infected allografts, and settled at that level (P=NS). DHPG treatment reduced the number of intimal nuclei to 1.7±0.2 PSU at 1 month (P=NS) and to 2.6±0.1 PSU at 3 months (P=NS). Thereafter, the number of intimal nuclei reached the level of nontreated RCMV-infected allografts.

**Intimal thickness.** In nontransplanted DA aortas, the intima consists of a single layer of cells (0.1±0.1 PSU±SD), only. In RCMV-infected allografts, intimal thickness increased rapidly to 5.2±0.9 PSU 3 months after transplantation. The enhanced increase in intimal thickness continued in infected allografts, although more slowly, reaching 6.7±1.6 PSU at 6 months. DHPG prophylaxis significantly reduced intimal thickness to 0.3±0.1 PSU on day 7 (P<.025), to 0.8±0.1 PSU at 1 month (P<.05), and to 1.7±0.4 PSU at 3 months (P<.05). At 6 months, intimal thickness in the DHPG prophylaxis group was half (2.4±1.0 PSU) of that observed in RCMV-infected allografts, but the difference was not statistically significant. In addition, linear regression analysis revealed that DHPG prophylaxis significantly reduced intimal thickness from the time of transplantation to 3 months after transplantation (P<.05; slope 1.7 in the nontreated group versus slope 0.5 in the prophylaxis group). On the other hand, DHPG treatment significantly reduced intimal thickening only at 1 month but not thereafter; to 0.8±0.2 PSU at 1 month (P<.05), to 2.7±0.2 PSU at 3 months (P=NS), and to 3.4±1.0 PSU at 6 months (P=NS).

**Effect of DHPG on RCMV-Enhanced Cell Proliferation in the Allograft**

All rats were given 3H-TdR intravenously 3 hours before graft removal. Vascular wall cell proliferation was quantified as the number of labeled nuclei per aortic cross section ±SEM. In normal aortas, no nuclear labeling is detected after 3H-TdR pulse.

**Adventitia**

In RCMV-infected allografts, the peak in cell proliferation occurred 7 days after transplantation, reaching 227±21 labeled nuclei per aortic cross section. The proliferative response declined to 115±24 labeled nuclei at 1 month, after which only low-level proliferative response was observed. In the DHPG prophylaxis group compared with RCMV-infected nontreated allografts (Fig 3a), the early proliferative peak of inflammatory cells on day 7 was reduced to 91±20 labeled nuclei (P<.05), remained at the same level up to 1 month, and declined to the level observed in RCMV-infected allografts at 3 months. The proliferative response in the DHPG treatment group was less affected (Fig 3a).

**Media**

In RCMV-infected allografts, a gradual proliferative response occurred, increasing to 14±4 labeled nuclei at 7 days, 22±2 labeled nuclei at 14 days, and 19±7 labeled nuclei at 1 month and declining thereafter. In the DHPG prophylaxis group (Fig 3b), the early proliferative response of media cells was inhibited to half of that observed in RCMV-infected allografts, reaching 6±3 labeled nuclei at 7 days (P<.05) and 11±2 labeled nuclei at 14 days (P<.05). The same degree of inhibition was also observed in the DHPG treatment group, with...
number of proliferating intimal cells at 7 days (P<.01) and a less significant reduction at 3 months (P<.05). In the DHPG treatment group (Fig 4c), a significant reduction in the number of proliferating intimal cells was observed only 3 months after transplantation (P<.05).

**Effect of DHPG on IL-2 Receptor and MHC Class II Molecule Expression**

Frozen sections of aortic allografts were incubated with monoclonal antibodies to IL-2R and MHC class II to quantify the immune activation in the adventitial inflammation. In normal aortas, there are no IL-2R-expressing lymphocytes and, with the exception of few dendritic cells, no cells expressing class II either.

**IL-2R**

In RCMV-infected allografts, there were 29±9 IL-2R-expressing cells 7 days and 299±46 IL-2R-expressing cells 1 month after transplantation. There was a significant reduction (P<.05) in the number of IL-2R-positive cells in the DHPG prophylaxis group at 1 month (Fig 5a). In the DHPG treatment group, no significant reduction was observed (Fig 5b).

**MHC Class II**

In RCMV-infected allografts, a prominent class II expression with a score of 4.5±0.3 was seen 7 days after transplantation and remained at the same level at 1 month. In the DHPG prophylaxis group (Fig 5c), the expression of MHC class II molecule was slightly reduced to 3.2±0.2 at 1 month (P=NS), whereas in the DHPG treatment group (Fig 5d), MHC class II was moderately present during the whole observation time (P=NS).
Effect of DHPG on Growth Factor mRNA Expression

Growth factor mRNA expression in the allograft vascular wall was approximately similar in the DHPG prophylaxis and treatment groups, and thus, only the results of DHPG prophylaxis are given in Fig 6. DHPG prophylaxis did not significantly alter the levels of IGF-1, EGF, PDGF-BB, TGF-β1, aFGF, and bFGF messages in the allograft vascular wall compared with nontreated RCMV-infected allografts; this is shown in Fig 6.

Effect of DHPG on Smooth Muscle Cell Proliferation in Vitro

Since DHPG therapy showed a distinct inhibitory response both in smooth muscle cell proliferation in the allograft media and intima and in the generation of arteriosclerotic changes in the allograft, the question arises whether DHPG also has a direct antiproliferative effect on smooth muscle cell replication. Therefore, we investigated the effect of DHPG on smooth muscle cell proliferation in vitro. Smooth muscle cells isolated from aortas of newborn rats were seeded in 96-well microtiter wells. DHPG was added at various concentrations (0 to 1000 μg/mL) on day 0, and 3H-TdR incorporation was quantified after 24-hour pulses once a day for a period of 6 days.

As depicted in Fig 7, DHPG inhibited smooth muscle cell replication in a dose-dependent fashion. The peak of smooth muscle cell DNA synthesis was reduced by 50% at DHPG concentrations of 1 and 10 μg/mL. The 3H-TdR incorporation into smooth muscle cell DNA was inhibited by almost 100% at higher concentrations, ie, 100 and 1000 μg/mL of DHPG. When monitored by light microscopy, the viability of the cells was good during the whole experiment. After replacement of the drug-containing medium with conditioned medium, control cells showed another proliferative response. Instead, the inhibitory effect of DHPG proved not to be reversible.

Discussion

Clinical studies have shown that CMV infection plays an important role in the generation of cardiac allograft arteriosclerosis. We demonstrated earlier that CMV infection occurring during the immediate posttransplant period enhances aortic allograft arteriosclerosis in the rat. CMV infection induced an early prominent inflammatory response and proliferation of inflammatory cells in the allograft adventitia. Furthermore, it doubled the proliferation of smooth muscle cells and the arteriosclerotic alterations in the intima. If the point of infection was postponed by 2 months, the alterations in RCMV-infected allografts were nearly nonexistent. In syngeneic grafts and in the native control aortas, no histological effect of RCMV infection was observed. These observations suggest that acute allogeneic response must coincide with acute viral infection to induce accelerated allograft arteriosclerosis.

DHPG is effective against RCMV infection both in vitro and in vivo. In our present study, DHPG prophylaxis prevented the development of CMV infection, as demonstrated by the plaque assay. In addition, no seroconversion was observed. DHPG treatment initiated during the infection abolished the infection in half of the animals and postponed it in the remaining animals. In the DHPG treatment group, the level of antibodies to RCMV was reduced to 25% compared with nontreated RCMV-infected rats. These results indicate that for an effective seroconversion, active and prolonged viral replication is necessary: apparently the input of infection per se does not render a sufficient immunological stimulus for seroconversion.

The main message in our communication is that DHPG prophylaxis completely abolished the enhancing effect of CMV infection on allograft arteriosclerosis. DHPG treatment initiated at the onset of acute infection also inhibited accelerated arteriosclerosis, but the effect was only partial and statistically nonsignificant. First, DHPG prophylaxis prevented the early prominent adventitial inflammatory response in RCMV-infected recipients and was associated with a clear reduction in the number of cells expressing IL-2 receptor. Second, the extent of early media necrosis was reduced. And third, the proliferation of smooth muscle cells and the arteriosclerotic alterations in the allograft intima were significantly reduced by >50% by the DHPG prophylaxis. Later, the proliferation of smooth muscle cells in the intima and media declined even further, remaining at zero level at 3 months. The proliferation was still moderate in the intima of RCMV-infected allografts at this time.

The association of peptide growth factors in allograft arteriosclerosis has been established. We also recently demonstrated that allograft arteriosclerosis unmodified by RCMV infection is accompanied by a prominent upregulation of IGF-1, PDGF-BB, and TGF-β1 messages in the allograft vascular wall, but EGF messages remain unchanged. In RCMV-enhanced allograft arteriosclerosis, a prominent, additional elevation of PDGF-BB and TGF-β1 messages was observed and a less prominent upregulation of aFGF and EGF mRNA expression. PDGF is a potent chemoattractant and mitogen for smooth muscle cell proliferation. Autocrine or paracrine production of IGF-1 is believed to be necessary for PDGF to exert its mitogenic effects on smooth muscle cells. Depending on the other signal molecules present, TGF-β1 is acting as a mitogen or growth inhibitor. Furthermore, studies with balloon catheter denudation suggest that bFGF may be a key mitogen for the initiation of smooth muscle cell replication, but it is not important in the continuation of intimal smooth muscle cell replication. In our study, DHPG prophylaxis did not alter the (elevated) expression levels of these mitogens, suggesting that other pathways for the regulation of smooth muscle cell replication exist.

The distinct inhibitory response by DHPG therapy both in smooth muscle cell proliferation in the allograft media and intima and in the generation of arteriosclerotic changes in the allograft suggests that the inhibitory effect of DHPG on virus infection–enhanced allograft arteriosclerosis is not linked only to an inhibition of the viral replication but that additional pathways may also...
exist: either direct inhibition of inflammatory cells may lead to indirect inhibition of smooth muscle cell proliferation or the drug itself may have a direct antiproliferative effect on smooth muscle cell replication. We know that DHPG inhibits the proliferation of noninfected cells, especially of the rapidly proliferating ones (like bone marrow cells), but with substantially higher concentrations than required to inhibit the replication of CMV. On the other hand, 100-fold higher concentrations are achieved in CMV-infected cells than in noninfected cells. A dose-dependent inhibitory effect of DHPG on smooth muscle cell replication was recorded in our study in vitro. The replication of cultured smooth muscle cells, previously shown to be of a secretary phenotype similar to smooth muscle cells in atherosclerotic plaques, was reduced by 50% at clinically relevant concentrations of 1 and 10 μg/mL of DHPG and by almost 100% at concentrations of 100 and 1000 μg/mL. The inhibitory effect of DHPG proved not to be reversible on removal of the drug and replacement of fresh conditioned medium. Under light microscopy, the smooth muscle cell viability was good, but this does not exclude the possibility that concentrations of 100 and 1000 μg/mL might be toxic. Biron and coworkers demonstrated that once DHPG triphosphate is formed in the cell, it persists for several days in both human CMV-infected and -noninfected cells. The observed effects of the drug on noninfected smooth muscle cells may be due to DHPG incorporation in the nucleoside analogue of the cellular genome, thus affecting cellular metabolism. This incorporation might be more effective in cells with a high proliferative state, eg, as a result of viral infection.

Study Limitations

There are certain limitations to our study. Our rat aortic allograft model may not be entirely analogous to the coronary wall in human cardiac allografts. However, rat aortic allografts developed arteriosclerotic alterations that are virtually indistinguishable from those observed during chronic cardiac rejection in humans. With this aortic allograft model, we gain insight into the pathophysiological mechanisms of chronic rejection in the vascular wall itself, whereas in a heart transplant model, the coronary arteries should be dissected free from the surrounding tissue to obtain growth factors and cytokines from the vascular wall of the allografts.

Although the natural history of rat CMV infection is quite similar to that seen in human CMV infection, there are some differences. The incubation time for RCMV infection is only 7 days, whereas in humans, most CMV infections occur within 60-60 days after cardiac transplantation. This implies that the optimal duration and the timing needed for DHPG prophylaxis to prevent the development of CMV-enhanced allograft arteriosclerosis in humans will be difficult to determine on the basis of our experimental data.

Unfortunately, only a limited number of growth factors and cytokines were investigated in this study. DHPG prophylaxis prevented the prominent inflammatory response and immune activation in the vascular wall of RCMV-infected allografts. No inhibitory effect was seen in growth factor messages. This finding suggests that the growth factors, previously shown to be elevated in the vascular wall during unmodified and RCMV-enhanced allograft arteriosclerosis, are not the only regulatory molecules for smooth muscle cell replication.

Our observations suggest that DHPG may be useful in clinical prophylaxis of CMV infection in heart transplant patients, not only as it decreases morbidity in acute infection, but also as it inhibits accelerated allograft arteriosclerosis. A randomized doubled-blind multicenter clinical trial should be conducted to validate these experimental observations in humans.

Conclusions

Our study demonstrates that DHPG prophylaxis entirely abolishes the accelerating effect of RCMV infection on allograft arteriosclerosis in the rat. Furthermore, early prophylaxis of RCMV infection with DHPG prevents the development of viral infection. The effect of DHPG treatment in the inhibition of virus infection and virus-enhanced arteriosclerotic process was less prominent compared with DHPG prophylaxis. These results suggest that DHPG might be useful in clinical prophylaxis of CMV infection among heart transplant recipients, not only as it decreases morbidity in acute infection but also as it inhibits accelerated allograft arteriosclerosis.

Acknowledgments

This study was supported by The Academy of Finland, The Sigrid Juselius Foundation, The University of Helsinki, The Aarne Koskelo Foundation, Finnish Medical Society Duodecim, and The Finnish Heart Association, Helsinki, Finland. We thank E. Wasenius, RN, Lab, and T. Lahtinen, RN, Lab, for excellent technical assistance.

References

8. Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE. Cytomegalovirus infection is associated with
34. Lemström KB, Aho PT, and Häyry PJ. Cytomegalovirus infection enhances mRNA expression for platelet-derived growth factor-BB and transforming growth factor-β1, in rat aortic allografts: possible mechanism for cytomegalovirus-enhanced graft arteriosclerosis. *Arterioscler Thromb.* In press.
Cytomegalovirus infection-enhanced allograft arteriosclerosis is prevented by DHPG prophylaxis in the rat.
K B Lemström, J H Bruning, C A Bruggeman, P K Koskinen, P T Aho, S Yılmaz, I T Lautenschlager and P J Häyry

doi: 10.1161/01.CIR.90.4.1969

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