Neointimal smooth muscle cell phenotype is important in its susceptibility to cytomegalovirus (CMV) infection: a study in rat

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Abstract

Objective: Recently, we have found that rat CMV (RCMV) infected smooth muscle cells (SMCs) in rat carotid arteries when administered 14 days after balloon injury. In the present study we investigated 1 the long term effects of CMV infection on neointimal cross-sectional area, and 2 whether the phenotype of the intimal SMCs influences their susceptibility to active CMV infection.

Methods: In the first part of the study, rats received RCMV intravenously, two weeks after balloon catheterisation of the left carotid artery and were sacrificed twenty weeks after catheterisation. Continuous BrdU infusion was performed by subcutaneously implanted osmotic pumps during the last two weeks of life. In the second part RCMV was administered eight weeks after catheterisation and rats were sacrificed two weeks later. Immunohistochemistry was used to detect viral antigens, to determine BrdU incorporation as well as the contents of α-actin, desmin and vimentin in the carotid arteries. Intima and media cross-sectional areas were determined using computerized morphometry.

Results and conclusions: RCMV infection did not induce any differences in intima or media cross-sectional areas of the injured carotid artery, nor in the extent of SMC proliferation as shown by BrdU incorporation, 20 weeks after balloon catheterisation. Eight weeks after balloon catheterisation, RCMV no longer infected neointimal SMCs. This non-responsiveness to RCMV was associated with 're-differentiation' of the eight weeks old neointima, compared with two weeks after catheterization, as shown by the contents of α-actin, desmin and vimentin. Our data suggest that intimal SMC phenotype determines its susceptibility to active RCMV infection in vivo. Since de-differentiation of neointimal SMCs is associated with enhanced proliferation of these cells it is stated that de-differentiation or proliferation is prerequisite for infection.

1. Introduction

Cytomegalovirus (CMV) is one of the herpes viruses and a well known cause of severe disease in immunocompromised patients, especially transplant recipients and patients with the acquired immunodeficiency syndrome (AIDS). Although the virus is also a common cause of infection in immunocompetent hosts, these infections in general remain asymptomatic.

CMV has been detected repeatedly in arterial tissue of humans suffering from severe atherosclerosis [1–3]. However, the exact role of the virus in atherogenesis is still questioned. A causal relation has been indicated by the findings of accelerated transplant atherosclerosis in human heart transplant recipients with CMV infection [4,5] and in rat aortic and cardiac allografts after administration of a rat specific CMV (RCMV) [6–8]. Since arterial smooth muscle cells (SMCs) play a key role in the development of an atheromatous plaque and of the neointima after vascular injury [9–11], we used a balloon angioplasty model in the rat to study the susceptibility of arterial SMCs to CMV infection and the effect of the virus on neointima development. In this model we have previously shown that administration of RCMV 14 days after balloon catheterisation of the carotid artery induces an abundant active infection of SMCs in the neointima [12], albeit without influencing the neointimal cross-sectional area. This observation is in contrast with our findings in a rat aorta transplantation model where RCMV administration does increase SMC prolifera-
tion and neointimal thickness [6,7]. Moreover, in humans it has been described that CMV was associated with restenosis after coronary angioplasty [13,14]. The apparent discrepancy between our earlier findings in the rat balloon injury model and these latter data may be explained by the possibility that we only studied the neointima two weeks after RCMV administration, which may have been too short to detect any virus-induced effect on intimal thickness. Therefore, in the present study we examined long-term effects of RCMV infection on neointimal area.

Also in our previous experiments, RCMV did infect intimal but not medial SMCs. Thus, we hypothesized that it is well possible that distinctive phenotypic features of the intimal SMC may influence its susceptibility to RCMV infection. Since the phenotype of SMCs in the neointima that develops after balloon injury has been shown to change over time [15,16], we investigated the susceptibility of the neointima to RCMV infection eight weeks after balloon catheterisation, at which time SMC phenotype had changed from a synthetic (de-differentiated) to a more contractile (re-differentiated) phenotype [15,16].

Our study describes the interaction of RCMV with neointimal SMCs in vivo in a bidirectional way. On the one hand, the long term effect of RCMV on neointimal formation was studied and on the other hand a possible influence of the phenotype of the neointimal SMC on its susceptibility to RCMV infection was investigated.

2. Methods

2.1. Animals and virus

15 Weeks old Wistar–Kyoto rats were used that had been bred under specific pathogen-free conditions at the Department of Experimental Animal Service at our University. The experiments were performed according to local institutional guidelines. Animals were given standard rat chow and tap water at libitum.

RCMV consisted of a pool of homogenized salivary glands of acutely infected laboratory rats [17]. The rats were intravenously infected with \(10^5\) plaque-forming units (PFU) of RCMV, after being immunosuppressed by a total body roentgen irradiation of 5 Gy. This protocol was used since, analogue to human CMV infection, a decreased immunocompetence is a prerequisite for symptomatic RCMV infection [18], including infection of the rat aorta and carotid artery and microvascular vessel wall ([12], own experimental results, submitted for publication). Control animals were mock infected with a salivary gland homogenate derived from non-infected rats.

2.2. Experimental design

In all rats, balloon injury of the left common carotid artery was performed as described before [19]. The right carotid artery was left untraumatized and served as an internal control.

2.2.1. Experiment 1: Effect of RCMV infection on neointima thickness

Rats were randomly divided into two groups (A and B, \(n = 9–10/\)group). All animals received total body roentgen irradiation thirteen days after balloon injury, followed by RCMV (group A) or mock (group B) infection, 1 and 3 days later (Fig. 1). The rats were killed 18 weeks after RCMV or mock infection. 4 Weeks after infection the animals were anesthetized with ether and biopsies were taken from the salivary glands to ascertain active RCMV infection. Two weeks before the animals were killed, an osmotic mini-pump (Alzet model 2002, Alza Corp., Palo Alto, CA, USA) previously filled with 5'-bromo-2'-deoxyuridine (BrdU, 20 mg/ml 0.9% NaCl 0.5 \(\mu\)l/h) was inserted subcutaneously between the shoulder blades to measure SMC DNA synthesis.

2.2.2. Experiment 2: Susceptibility of neointimal SMC to RCMV infection

Two groups of rats (C and D, \(n = 9–10/\)group) were formed at random.

All rats received total body roentgen irradiation 8 weeks after balloon injury and were RCMV (group C) or mock (group D) infected 1 and 3 days later (Fig. 1). The rats were killed 2 weeks after RCMV or mock infection. Salivary glands were obtained post mortem to confirm the presence of replicative virus.

All rats (experiments 1 and 2) were killed by aortic bleeding about one hour after they had received 0.5% Evans blue in 0.9% NaCl intravenously to stain non-endothelialized vascular tissue. In situ perfusion was performed with 0.9% NaCl containing 100 mg/l sodium nitroprus-
side (Merck). Several circular segments were taken from both Evans blue retaining and non-retaining areas of the injured left common carotid artery. Control segments were taken from the non-injured right common carotid arteries. All tissues were fixed in 3.7% formaldehyde in phosphate buffered saline (pH 7.4), routinely processed and paraffin-embedded. For haematoxylin-eosin, Lawson elastin and immunocytochemical staining procedures 4 μm thick cross-sections were cut.

2.3. Immunohistochemistry

2.3.1. Viral antigens

Injured carotid arteries as well as salivary glands were screened for the presence of RCMV antigens. Immunostaining procedures were performed as described previously [12], using mouse monoclonal antibodies against nuclear and cytoplasmic RCMV early antigens [20]. Subsequent incubations were performed with peroxidase conjugated rabbit antimouse IgG (Dako) and diaminobenzidine substrate.

To rule out nonspecific antigen binding, parallel sections were stained with an anti-human CMV monoclonal antibody, which does not react with RCMV, nor with control mouse ascites fluid (Sigma Immuno Chemicals). Spleen sections from an acutely infected rat served as positive controls.

2.3.2. BrdU incorporation

Incorporation of the thymidine analogue BrdU was visualized by staining with an anti-BrdU monoclonal antibody as previously described [12]. Subsequent incubation steps were performed with biotinylated rabbit anti-mouse IgG (Amersham) and with an avidin/biotin-peroxidase complex (Vectastain-ABC kit, Vector Lab Inc.) and diaminobenzidine.

2.3.3. α-Actin, desmin and vimentin

Staining of the SMC differentiation markers α SMC actin, desmin and vimentin, was performed with monoclonal antibodies.

For α-actin and desmin staining incubation with respectively anti-α SMC IgG (Dako) and anti-desmin II (Organon Technika, Boxtel, The Netherlands) was followed by subsequent incubations with biotin labelled sheep anti-mouse IgG (Amersham), alkaline phosphatase conjugated biotin-streptavidin complex (Amersham) and alkaline phosphatase substrate (kit, Vector Lab. Inc).

For vimentin staining, incubation with the monoclonal anti-vimentin IgG (Boehringer) was followed by incubations with peroxidase conjugated rabbit anti-mouse IgG and diaminobenzidine substrate. To check for possible effects of roentgen irradiation and/or infection, staining of α-actin, desmin and vimentin was also performed on carotid sections of rats that had not been irradiated or infected, at 2, 8 and 20 weeks after balloon catheterisation (n = 5/group).

2.4. Cross-sectional areas

Neointimal and medial cross-sectional areas of carotid arteries were measured using a computer-assisted morphometry system (Quantimet 570, Leica) on Lawson stained cross-sections as described before [12].

2.5. Statistics

For comparisons of carotid intimal and medial cross-sectional areas from rats of experimental and control groups (group A vs. B and group C vs. D) within both experiments, a one-way ANOVA was used. One-way ANOVA was also used to compare left and right medial cross-sectional areas within groups. A two-way ANOVA was used to compare corresponding cross-sectional areas from both experiments (group A/B vs. group C/D) and to compare weights at different moments within both experiments. All data are expressed as mean ± SD.

3. Results

3.1. Experiment 1

At the start of the experiments mean body weights for experimental (group A) and control (group B) groups were 354 g and 358 g, respectively. Also, at 18 weeks after RCMV infection no weight differences were observed between the two groups (Table 1).

3.1.1. Viral antigens

Immunohistochemistry, using specific anti-RCMV monoclonal antibodies, showed the presence of early viral antigens in the salivary glands of rats from group A (Fig. 2), which proved that early active RCMV infection had taken place in these animals.

Table 1

<table>
<thead>
<tr>
<th>Rat body weights at time of different experimental events</th>
<th>Body weight (g) at time of</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>balloon injury</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>group A</td>
<td>353 ± 23</td>
</tr>
<tr>
<td>group B</td>
<td>358 ± 20</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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<tr>
<td>group C</td>
<td>323 ± 20</td>
</tr>
<tr>
<td>group D</td>
<td>328 ± 16</td>
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</tbody>
</table>

\(^{a}\) P < 0.05 as compared with \(Δ\)weight in group D.

\[^{b}\]ΔWeight = weight at death minus weight at RCMV or mock infection. Expressed is the mean ± SD of \(Δ\)weights for individual rats.

Values are expressed as mean ± SD.
Table 2
Medial and neointimal cross-sectional areas of RCMV infected carotid arteries at different times after balloon catheterisation

<table>
<thead>
<tr>
<th>Cross-sectional area (mm²)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>group A</td>
<td>group B</td>
</tr>
<tr>
<td>neointima</td>
<td>0.13±0.03</td>
<td>0.15±0.04</td>
</tr>
<tr>
<td>media</td>
<td>0.13±0.01</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>lumen</td>
<td>0.13±0.04</td>
<td>0.13±0.06</td>
</tr>
</tbody>
</table>

| Right carotid artery       |              |              |
| media                      | 0.11±0.01    | 0.10±0.01    | 0.13±0.02    | 0.12±0.02    |

Expressed are the neointimal cross-sectional areas without an endothelial layer.
No neointima was present in the non-injured right carotid artery.
Values are expressed as mean±SD.

18 Weeks after infection none of the carotid arteries from group A rats contained viral antigens. This was the case in both the endothelialized and non-endothelialized parts. Salivary glands and carotid arteries from rats of control group B were always negative.

3.1.2. BrdU labelling
SMC nuclei in the intima and media of injured carotid arteries from group A rats only very sporadically stained positive with anti-BrdU. Although labelling fractions have not been quantitated, there were no apparent differences between group A and B in the amount of BrdU labelling, which was less than 0.1% over 14 days.

3.1.3. Cross-sectional areas
Neointimal cross-sectional areas of left injured carotid arteries from infected (group A) and non-infected rats (group B) were comparable (Table 2) as were medial cross-sectional areas. Similarly, there were no significant differences between medial cross-sectional areas of non-injured right carotid arteries from animals of group A and B.

3.2. Experiment 2
At the start of the experiments the mean body weights were 323 g (group C) and 328 g (group D). Two weeks after infection, however, the weights differed significantly between the two groups. Rats from group C lost weight during these two weeks (−18 g ± 8), while all rats from group D gained weight in the same period (17 g ± 8, p < 0.05, Table 1).

3.2.1. Viral antigens
The salivary glands of group C rats contained viral antigens as shown by immunohistochemistry, confirming RCMV infection in these animals.
In the carotid arteries that were obtained from the rats of group C, RCMV antigens could never be detected in the media, nor in the intima, which practically excludes active RCMV infection. The absence or presence of endothelial cells had no effect on the RCMV staining.
Viral antigens were never present in the mock infected rats from group D (data not shown).

3.2.2. Cross-sectional areas
RCMV administration in the rats of group C did not affect the carotid neointimal cross-sectional area of injured left carotid arteries as compared with group D. Neither were there significant differences between the lumina on comparison of the two groups. Medial cross-sectional areas of balloon injured carotid arteries did not differ between the two groups and were also comparable with the medial cross-sectional areas of the non-injured carotid arteries (Table 2). Finally, as seen in Table 2, intimal and medial cross-sectional areas did not differ significantly from corresponding the cross-sectional areas in experiment 1.

Table 3
Immunohistochemical staining of α-actin, desmin and vimentin of rat carotid arteries at different times after balloon catheterisation

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 5)</th>
<th>Experiment 1 (group A and B = 20 weeks)</th>
<th>Experiment 2 (group C and D = 10 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 weeks</td>
<td>8 weeks</td>
<td>20 weeks</td>
</tr>
<tr>
<td>Desmin</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Actin</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

Staining extent was assessed semi-quantitatively; no — or very rarely — staining was scored as −; light staining and small staining foci were scored as +; moderate staining as + +; intensive staining as + + +.
Fig. 3. Immunohistochemical staining of desmin in carotid arteries at different times after balloon injury (100×). (a) Desmin staining of carotid artery, 2 weeks after injury; (b) desmin staining of carotid artery, 10 weeks after injury; (c) desmin staining of carotid artery, 20 weeks after injury.

3.3. SMC phenotype; α SMC actin, desmin and vimentin

The phenotypic change was best illustrated with the desmin immunoreactivity. Two weeks after injury neointimal SMCs were only very sporadically positive for desmin. The amount of desmin-positive SMCs gradually increased over time; higher levels were found at 8 weeks, while 20 weeks old neointimas contained the highest levels of SMCs being positive for desmin (Table 3 and Fig. 3).

It proved to be more difficult to differentiate between the amount of α-actin and vimentin containing SMCs at different times after balloon injury, using these immunostaining techniques. However, the intensity of α SMC actin staining per SMC in a 2 weeks old neointima was less than in a neointima at 8 and 20 weeks after balloon injury (Table 3). No differences were observed between α-actin staining of 8 and 10 weeks (experiment 1) on the one hand and 20 weeks old neointimas (including experiment 2) on the other hand.

The intensity of vimentin staining was the highest in a 2 weeks old neointima. Vimentin staining of 8–10 and 20 weeks old neointimas were comparable (Table 3).

For all three antibodies there were no differences between the staining results of carotid arteries of RCMV infected and mock-infected rats. Similarly, roentgen irradiation did not influence staining intensities of any of these markers of SMC differentiation, as may be concluded from comparable staining results of carotid arteries from experimental and control rats that had not been irradiated or infected (Table 3).

4. Discussion

The data presented here show that RCMV infection of a rat carotid neointima does not induce an increase of neointimal thickness, even at 18 weeks after infection. Moreover, this study suggests that the susceptibility of neointimal SMCs to RCMV may be transient. While an active RCMV infection can be induced in neointimal SMCs 2 weeks after balloon catheterisation [14], they seem to have become insusceptible to RCMV infection 8 weeks after this injury.

The lack of a RCMV effect on neointimal cross-sectional area is in contrast with our finding of an increased neointimal thickness and SMC proliferation in a rat allograft aortic transplant model [6,7]. These stimulatory effects of RCMV were, however, not noticeable until months after transplantation, which was one of the reasons to search for long-term RCMV effects on neointimal thickness in the balloon injury model in the present study. Finding, as presented here, no virus induced differences in neointimal cross-sectional area 20 weeks after balloon catheter induced injury stresses the different nature of neointimal development in both models. In the transplantation model the allogenic aorta induces immune responses which are apparently crucial for RCMV to increase neointimal cross-sectional area and SMC proliferation, since in syngeneic aorta transplants no such increases were found [6,7]. The absence of allogenic immune responses after arterial balloon injury may explain why we did not find RCMV induced differences in neointimal thickness after arterial balloon catheterisation.

In immunocompetent humans, however, an association
has been described between the occurrence of arterial restenosis after coronary balloon angioplasty and (previous) infection with CMV [13,14]. Of course restenosis of atherosclerotic human arteries — in which a prominent proliferation response may be lacking [24,25] and remodeling may be more important [21–23] — differs from the neointima formation in rats after arterial balloon injury experiments.

At 20 weeks after balloon injury no RCMV early antigens has been found in the rat carotid neointima, indicating that active RCMV infection does not persist for such a long time. In our previous experiments we showed that soon after arterial injury massive active RCMV infection persists for at least two weeks in the neointima, while all other organs are negative for RCMV except the salivary glands [14]. In the latter, the virus is known to persist for a long time [17]. The arterial intima likewise may be a preferential site for CMV infection and it would be of great interest to find out if RCMV remains latent present in arteries.

The observation that an eight weeks old rat carotid neointima can not be infected with high concentrations of infectious RCMV indicates the transient nature of the susceptibility of the neointimal SMCs to RCMV infection, since administration of the virus two weeks after injury results in an abundant neointimal infection [12]. This finding makes it attractive to hypothesize that the phenotype of the intimal SMC may be an important regulator in its susceptibility to RCMV. Indeed, our immunohistochemical staining results show that the phenotype of neointimal carotid SMCs changes over time and indicate that neointimal SMCs change from a de-differentiated state to re-differentiation as time after injury progresses. Comparable data were published by many other authors, albeit in rats that had not been immunosuppressed [15,16]. Thus de-differentiation may be a prerequisite for neointimal SMC susceptibility to active RCMV infection. Alternatively, it may be that the proliferation rate of neointimal SMCs regulates their susceptibility to RCMV infection. It is known that the fraction of proliferating cells in the neointima reaches a maximum approximately 1 week after balloon injury and gradually diminishes over time to very low levels at the time rats were infected in our study (8 weeks) [19]. Interestingly, de-differentiation of SMC has been frequently associated with proliferation [27]. The in vivo data presented here do not allow us to determine if de-differentiation and proliferation (or both) are crucial for RCMV infection of neointimal SMC.

In conclusion, this study suggests SMC de-differentiation and/or increased SMC proliferation are prerequisites for RCMV infection. This is especially interesting since intimal SMCs with cytoskeletal features of de-differentiation and increased SMC replication have been found in arterial pathologies that have been associated with CMV infection, such as atheromatous plaques, arterial grafts and post-angioplasty arteries [25–29].

References

[20] Bruning JH, Debe WHM, Dormans PHJ, Meijer H, Bruggeman CA. The development and characterisation of monoclonal antibodies...


