Combined Blockade of the Chemokine Receptors CCR1 and CCR5 Attenuates Chronic Rejection

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Background—Chemokine-chemokine receptor interaction and the subsequent recruitment of T-lymphocytes to the graft are early events in the development of chronic rejection of transplanted hearts or cardiac allograft vasculopathy (CAV). In this study, we sought to determine whether blockade of chemokine receptors CCR1 and CCR5 with Met-RANTES affects the development of CAV in a murine model.

Methods and Results—B6.CH-2 bm12 strain donor hearts were transplanted heterotopically into wild-type C57BL/6 mice (myosin heavy chain II mismatch). Recipients were treated daily with either Met-RANTES or vehicle starting on postoperative day 4 and were euthanized on postoperative days 24 and 56. We found that Met-RANTES significantly reduced intimal thickening in this model of chronic rejection and that Met-RANTES markedly decreased the infiltration of CD4 and CD8 T lymphocytes and MOMA-2+ monocytes/macrophages into transplanted hearts. Met-RANTES also suppressed the ex vivo and in vitro proliferative responses of recipient splenocytes to donor antigens. Finally, Met-RANTES treatment was associated with a marked reduction in RANTES/CCL5 and monocyte chemoattractant protein-1 gene transcript levels in the donor hearts.

Conclusions—Antagonism of the chemokine receptors CCR1 and CCR5 with Met-RANTES attenuates CAV development in vivo by reducing mononuclear cell recruitment to the transplanted heart, proliferative responses to donor antigens, and intragraft RANTES/CCL5 and monocyte chemoattractant protein-1 gene transcript levels. These findings suggest that chemokine receptors CCR1 and CCR5 play significant roles in the development of chronic rejection and may serve as potential therapeutic targets. (Circulation. 2004;109:932-937.)

Key Words: transplantation ■ lymphocytes ■ rejection ■ chemokine

Cardiac allograft vasculopathy (CAV), the primary manifestation of chronic rejection in transplanted hearts, is the leading cause of late death in heart transplant recipients. The pathogenesis of CAV is incompletely understood. Human and experimental studies indicate that activated T lymphocytes and macrophages accumulate in perivascular areas of the donor heart and play an important role in CAV development. However, the mechanism of mononuclear cell recruitment to the donor heart in CAV is not fully defined.

Chemokines are small cytokines that mediate cell chemotaxis and activation. Emerging evidence suggests that specific chemokines may play significant roles in mononuclear cell recruitment during chronic rejection. During chronic rejection of renal allografts, the chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) was persistently expressed. In murine models of CAV, production of regulated-on-activation, normal T cells expressed and secreted (RANTES/CCL5) by multiple graft-infiltrating cell types paralleled mononuclear cell recruitment and preceded the development of intimal thickening. Finally, in explanted human hearts with CAV, the chemokine RANTES/CCL5 colocalized with graft-infiltrating cells and arteries with intimal thickening.

The activity of chemokine-chemokine receptor interaction may be reduced in vivo by neutralization of the ligand or blockade of chemokine receptors. In this study, we sought to determine the effect of RANTES/CCL5 receptors CCR1 and CCR5 blockade in CAV development. Met-RANTES, an amino-terminal modified derivative of RANTES/CCL5, antagonizes the RANTES/CCL5 receptors CCR1 and CCR5 and attenuates tissue damage associated with acute kidney rejection and chronic colitis in vivo. Therefore, using an established murine model of CAV in which we have previously identified high levels of RANTES/CCL5 gene expression and protein production, we tested the effects of Met-
RANTES on intimal thickening and mononuclear cell recruitment in vivo, the proliferative responses of primed and naive splenocytes to donor antigens in vitro, and the donor heart chemokine profile.

**Methods**

**Animals**

Adult female B6C.H-2<sup>m12</sup> and wild-type (WT) C57BL/6 mice (7 to 10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Me). The B6C.H-2<sup>m12</sup> and C57BL/6 strains differ at the I-A locus of MHC II but are identical at MHC I and minor MHC loci. All animals received humane care in compliance with University of California Los Angeles guidelines and the Principles of Laboratory Animal Care published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Intraabdominal heterotopic cardiac transplantation was performed using a modification of the method outlined by Corry et al<sup>13</sup> and previously used by our group.<sup>8</sup>

Donor hearts were assessed every other day by abdominal palpation. Absence of palpable contractions was considered graft rejection.

**Experimental Groups**

B6C.H-2<sup>m12</sup> strain donor hearts (allografts) were transplanted into WT C57BL/6 recipient mice. Allograft recipients received either Met-RANTES (20 μg IP daily in 0.5 mL of PBS) or vehicle (0.5 mL of PBS IP daily) beginning on postoperative day 4. The donor hearts were harvested either on day 24 after transplant (n=6 in each group) or on day 56 after transplant (n=4 in each group). The Met-RANTES was synthesized by A.E.I.P. as previously described,<sup>10</sup> and the Met-RANTES dose used was selected based on its efficacy in prior rodent models of disease.<sup>11,12</sup> No immunosuppression was given. C57BL/6 strain donor hearts were also transplanted in B6C.H-2 bm12 strain donor (B6C.H-2bm12) recipients and harvested on day 24 (n=6) or day 56 (n=4) after transplant (isografts).

**Morphometric Analysis**

Elastica von Gieson stains were performed on transverse sections obtained from basal half of allografts and isografts. Vessel size was measured with computer-based software (Optimas); only vessels exceeding 80 μm in diameter were included.<sup>14</sup> For vessels and Met-RANTES-treated groups, 6±2 vessels (on posttransplant day 24) and 7±2 vessels (on posttransplant day 56) per animal were analyzed. Luminal (L) and intimal+luminal (I+L) areas were traced, and areas were quantitated with the Optimas program. Intimal thickening (% stenosis) was calculated according to the formula intimal thickening=I/I+L and expressed as a percentage.

**Graft-Infiltrating Cell Isolation and FACS Analysis**

Apical halves of hearts, harvested on day 24 after transplant, were digested in Collagenase D (2 mg/mL; Worthington Biochemical). Isolated cells were counted after lysis of erythrocytes. Cells were subsequently incubated with fluorochrome-conjugated antibodies specific for cell-surface antigens (rat anti-mouse CD4, CD8, and MOMA-2 Abs, Pharmingen and Serotec USA). FACS analysis of labeled cells was carried out with a single-laser argon flow cytometer (Becton Dickinson).

**Histology, Grading, and Immunohistochemistry**

Frozen sections (4 μm) of transplanted mouse hearts were either stained with H&E or immunostained for CD4, CD8, MOMA-2, α-actin, and vascular cellular adhesion molecule (VCAM)-1 with standard avidin-biotin technique (Vectastain ABC, Vector Laboratories). Mononuclear cell infiltration in H&E stains was graded in blinded fashion as absent (0), rare (1), mild (2), moderate (3), or diffuse (4), as described previously.<sup>7</sup> Sections immunostained for CD4, CD8, MOMA-2, and VCAM-1 were also graded as 0 to 4, as previously described.<sup>7</sup>

**Ribonuclease Protection Assay**

Total cellular RNA was isolated from apical ventricular tissue using the Trizol method (Life Technologies). RNA quality was confirmed with gel electrophoresis. Chemokine gene expression was determined using an RNase protection assay (Pharmingen) as previously described.<sup>7</sup>

**Mixed Lymphocyte Reaction**

Spleens from control and Met-RANTES-treated mice were removed at the time of euthanasia. After lysis of erythrocytes, 8×10<sup>6</sup> splenocytes were incubated with an equivalent number of irradiated donor (B6C.H-2<sup>m12</sup>) splenocytes in triplicates. Control wells were set up using syngeneic stimulator cells. Proliferation was measured after each reaction well was pulsed with 0.5 μCi [³H]thymidine (Amersham) for 24 hours. The cells were then harvested and counted on a β scintillation counter. Naïve responder splenocytes from untransplanted C57BL/6 mice were similarly set up in MLRs in the presence of Met-RANTES (0.1, 1.0, and 10 μg/mL), albumin (10 μg/mL; Sigma), and no protein. Proliferation was measured as described above.

**Statistics**

Data are presented as mean±SEM. ANOVA was used to compare mean cell number, proliferative responses, and intimal thickening between different groups. A value of P<0.05 was considered significant.

**Results**

**Characterization of Intimal Lesions in This Model of CAV**

Our prior studies with this model have demonstrated that the intimal lesions consist of moderate numbers of T-lymphocytes, monocytes/macrophages, and few smooth muscle cells on day 24 after transplantation<sup>15</sup> (Figure 1, column I). On day 56 after transplantation, the intimal lesions consisted of fewer mononuclear cells (Figure 2, column 1). The pathologic characteristics of the intimal lesions in Met-RANTES-treated recipients were similar (Figure 1, column 2; Figure 2, column 2). The donor hearts in Met-RANTES-treated recipients survived to 56 days, whereas 1 donor heart in vehicle-treated recipient was lost to rejection on day 26 (P=NS).

**Met-RANTES Attenuates CAV Development**

Morphometric analysis of donor hearts from vehicle-treated mice revealed significant CAV (52±5%) at day 24 after transplant. In contrast, donor hearts from Met-RANTES-treated mice developed markedly less intimal thickening (9±4%; P<0.05 compared with vehicle-treated mice) (Figure 1A). Isografts had no intimal thickening (0±0%, P<0.05 versus Met-RANTES).

The inhibitory effect of Met-RANTES on CAV development persisted on day 56 after transplantation (Figure 2A). Hearts transplanted into Met-RANTES-treated mice had significantly less intimal thickening (13±7%) than hearts transplanted in the vehicle-treated mice (55±7%, P<0.05).

**Met-RANTES Decreases Mononuclear Cell Recruitment During CAV Development**

To determine whether Met-RANTES attenuated CAV by decreasing mononuclear cell recruitment, we examined sections of transplanted hearts by histology and immunohistology and quantitated graft-infiltrating mononuclear cells by...
flow cytometry. Mononuclear cell infiltration grade by H&E staining at 24 days after transplantation was significantly lower in mice treated with Met-RANTES (0.5 ± 0.1) than in mice treated with vehicle (3.0 ± 0.5, P < 0.05). On day 56 after transplantation, the grade of graft-infiltrating mononuclear cells in the Met-RANTES–treated group remained significantly less than the grade in the vehicle-treated group (0.3 ± 0.3 versus 2.7 ± 0.7, P < 0.05). In addition, immunohistochemical stains confirmed that Met-RANTES significantly decreased the recruitment of CD4+ and CD8+ T lymphocytes and MOMA-2+ macrophages on day 24 after transplantation (Figures 1B through 1D) and on day 56 after transplantation (Figures 2B through 2D). Analysis of graft-infiltrating cells by FACS on day 24 revealed a minimum of 9-fold decrease in the number of CD4+ T lymphocytes, CD8+ T lymphocytes, and MOMA-2+ monocytes/macrophages with Met-RANTES treatment (Figures 3A and 3B). The graft-infiltrating cells on day 56 in the Met-RANTES–treated group were too few to be isolated and quantitated (data not shown). Met-RANTES did not affect the numbers of peripheral lymphoid CD4+ T lymphocytes, CD8+ T lymphocytes, or MOMA-2+ macrophages on days 24 and 56 after transplantation (data not shown).

**Met-RANTES Inhibits Proliferative Responses Ex Vivo and In Vitro**

To additionally delineate the mechanism of Met-RANTES–induced CAV inhibition, we sought to determine the effect of Met-RANTES on proliferative responses to donor antigens ex vivo and in vitro. Splenocytes from vehicle-treated recipients exhibited a robust proliferative response (22,811 ± 2665 cpm). In contrast, splenocytes from Met-RANTES–treated C57BL/6 recipients were markedly hypoproliferative in response to donor splenocytes (9041 ± 982 cpm, P < 0.01 versus vehicle; Figure 4). Splenocytes from both vehicle-treated and Met-RANTES–treated recipients had normal mitogenic responses in response to concanavalin A (data not shown).

Met-RANTES also inhibited the proliferation of naive C57BL/6 splenocytes to donor bm12 antigens in a dose-dependent fashion. Adding 0.1 µg/mL Met-RANTES to the mixed lymphocyte reaction only slightly reduced proliferation (9983 ± 2740 cpm versus control MLR 14,975 ± 2910; P = NS versus controls). In contrast, addition of 10 µg/mL of Met-RANTES decreased proliferation by >66% (4925 ± 1050 cpm; P < 0.05 versus controls). Proliferation was not altered by the addition of albumin to the MLR (data not shown).

**Met-RANTES Does Not Affect Endothelial Cell VCAM-1 Expression**

Mouse endothelial and smooth muscle cells do not express VCAM-1 at rest. However, VCAM-1 expression is upregulated on both microvascular endothelial cells and smooth muscle cells in alloimmune responses, suggestive of vessel wall cell activation and capacity to recruit αβ integrin–positive cells. 16,17 We therefore sought to determine whether Met-RANTES affects vessel wall activation and VCAM-1 expression. The expression of VCAM-1 on microvascular endothelial and smooth muscle cells was similar in the donor hearts harvested from Met-RANTES versus vehicle-treated recipients on day 24 after transplantation (2.7 ± 3.8 versus 3.8 ± 0.4, P = NS) (Figure 1F) and on day 56 after transplantation (2.7 ± 0.6 versus 3.7 ± 0.4, P = NS).

**Met-RANTES Alters the Donor Heart Chemokine Profile**

To determine the impact of Met-RANTES treatment on chemokine profile of the grafts, we analyzed the donor hearts for CCR1 and CCR5 ligand gene transcript levels at both 24 and 56 days after transplantation. The donor hearts in the vehicle-treated recipients displayed a significant rise in RANTES/CCL5 and MCP-1 gene transcript levels on days 24 and 56 after transplantation compared with isografts (Figure 5). We could not detect macrophage inflammatory protein-1α (MIP-1α)/CCL3 or MIP-1β/CCL4 gene transcripts at the examined time points. These results are consistent with our
prior studies examining the time course of chemokine expression at both gene transcript and protein levels in this model. Interestingly, the RANTES/CCL5 and MCP-1 gene transcript levels were markedly lower in the donor hearts of Met-RANTES–treated recipients compared with the control recipients. This finding was consistent at both days 24 and 56 after transplantation (Figure 5). Similar to the control group, the donor hearts in the Met-RANTES–treated recipients did not express MIP-1α/CCL3 or MIP-1β/CCL4.

Discussion
In this model of CAV, combined CCR1 and CCR5 blockade by Met-RANTES significantly reduced intimal thickening. The mechanism of Met-RANTES–induced CAV inhibition included reduction of CD4+ and CD8+ T lymphocytes and MOMA-2+ monocyte/macrophage recruitment; suppression of donor-specific recipient splenocyte proliferation; and reduction of intragraft RANTES/CCL5 and MCP-1 chemokine levels.

The modification of RANTES by addition of a single methionine moiety yields Met-RANTES, which has antagonistic effects on chemokine receptors CCR1 and CCR5. Met-RANTES has been shown to be effective in controlling several chronic diseases. In a murine model of collagen-induced arthritis, Met-RANTES–treated mice developed less severe disease than control animals. Donor hearts in CCR-1–deficient mice treated with cyclosporine or anti-CD4 antibody were protected against the development of chronic rejection. The findings of the present study also suggest that direct blockade of CCR1 and CCR5 receptors by Met-RANTES can significantly reduce the severity of intimal thickening, a manifestation of chronic rejection in heart transplantation.

Figure 2. Representative sections of donor hearts from vehicle-treated and Met-RANTES–treated recipients harvested on day 56 after transplantation. A, Met-RANTES resulted in significant reduction in the severity of intimal thickening compared with control group. Similar to 24-day groups, Met-RANTES treatment was also associated with attenuation of CD4 lymphocyte (B), CD8 lymphocyte (C), and MOMA2+ cell infiltration (D); VCAM-1 expression was not different between Met-RANTES and vehicle-treated groups (not shown). Panels are representative sections from each group; there were 4 animals per group (magnification ×100).

Figure 3. Met-RANTES treatment resulted in significant reduction in the number of graft-infiltrating CD4 and CD8 lymphocytes (A) and MOMA2+ monocyte/macrophages (B) by FACS at 24 days after transplantation. Data are from 6 animals in each group.

Figure 4. Met-RANTES significantly reduced the ex vivo and in vitro alloreactivity of recipient (C57BL/6) splenocytes to donor (B6C.H-2bm12) antigens in the mixed lymphocyte reaction (MLR). Splenocytes isolated from Met-RANTES–treated heart transplant recipients at 24 days were 50% less proliferative to donor antigens than splenocytes from vehicle-treated controls. Splenocytes demonstrated normal mitogenic responses to concanavalin A (data not shown). Data are results of 6 experiments in each group, performed in triplicate. Syn indicates syngeneic; Allo, allogeneic.
Met-RANTES Reduces Mononuclear Cell Recruitment

Met-RANTES substantially reduced (>80%) the recruitment of CD4+ and CD8+ T lymphocytes and MOMA-2+ monocytes/macrophages to the donor heart. Because CAV in this model is a T lymphocyte–dependent process, inhibition of T lymphocyte recruitment alone may be the most important mechanism by which Met-RANTES reduces CAV. However, macrophages are also believed to play a pathogenic role in human CAV. Notably, activated macrophages express CCR1 and CCR5 and Met-RANTES reduces in vitro monocyte arrest on endothelium and in vivo monocyte recruitment in other disease models. Thus, a direct inhibition of monocyte/macrophage recruitment may independently contribute to the reduction of CAV by Met-RANTES. Notably, Met-RANTES did not completely prevent T lymphocyte/macrophage recruitment or CAV development, suggesting that other chemokine/chemokine receptor interactions may be involved. In addition, one cannot presently rule out the possibility that CAV might eventually develop at longer-term follow-up (beyond 56 days), particularly if the Met-RANTES is not continuously administered.

Met-RANTES Reduces Antigen-Specific Splenocyte Proliferation

Prior studies have demonstrated that the CCR1 and CCR5 ligands RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, and MCP-1 can each stimulate lymphocyte proliferation in vitro. We found that splenocytes isolated from Met-RANTES–treated mice had less than half the proliferative response to donor antigens than did splenocytes isolated from vehicle-treated controls. Furthermore, Met-RANTES produced a dose-dependent reduction of recipient splenocyte proliferation to donor antigens in vitro. Thus, the mechanism of Met-RANTES–dependent CAV attenuation may involve not only decreased cellular recruitment to the transplanted heart but also a diminished proliferative response to donor antigens. These results are consistent with those of Gao et al, who found that CCR1-deficient mice had a 20% to 25% reduction in splenocyte proliferation versus wild type mice.

Met-RANTES Does Not Affect Vessel Wall VCAM-1 Expression

Shear-resistant adhesion of monocytes to endothelial cells has been shown to be mediated, at least in part, by interaction of immobilized RANTES/CCL5 on the surface of activated endothelial cells with its receptors and by interaction of monocytes α, and β, integrins with intracellular adhesion molecule-1 or VCAM-1. Met-RANTES has been shown to be quite effective in blocking RANTES/CCL5-mediated shear-resistant adhesion, with minimal effect on integrin-mediated firm adhesion. The findings of our study also suggest that Met-RANTES treatment does not affect endothelial cell activation and VCAM-1 expression. Hence, the beneficial effect of Met-RANTES in diminishing mononuclear cell infiltration is primarily mediated via interference of RANTES/CCL5-CCR1 and CCR5 interaction and not attributable to control of endothelial cell activation.
Met-RANTES Attenuates Intragraft RANTES/CCL5 and MCP-1 Chemokine Levels

In addition to its chemotactic properties, RANTES/CCL5 is also known to stimulate the production of proinflammatory cytokines such as interleukin-2, interleukin-1β, and tumor necrosis factor-α.23,24 These inflammatory cytokines then in turn stimulate production of more RANTES/CCL5.25 Blockade of RANTES/CCL5 chemokine receptor interaction may control this cycle and reduce RANTES level. The findings of this study indeed demonstrate that RANTES/CCL5 chemokine receptor blockade with Met-RANTES was associated with a marked reduction in RANTES/CCL5 and MCP-1 gene transcript levels in the donor hearts. Prior studies have demonstrated a correlation between the upregulation of these 2 chemokines, mononuclear cell recruitment, and development of intimal lesions in this model.7,8 Therefore, in addition to its effect on mononuclear cell chemotaxis and T-lymphocyte proliferation, Met-RANTES also decreases the production of chemokines (RANTES/CCL5 and MCP-1) that participate in the development of CAV.

Which C-C Chemokine Is Antagonized by Met-RANTES in This Model?

The chemokine receptors CCR1 and CCR5 bind multiple CC chemokines in vitro, including RANTES/CCL5, MIP-1α/CCL3, and the family of MCPs. CCR5 also binds to MIP-1β/CCL4.26 Analyses of CCR1 and CCR5 ligands in this model of CAV showed that RANTES/CCL5 and MCP-1 gene transcripts, but not MIP-1α/CCL3 and MIP-1β/CCL4 mRNAs, are upregulated. Because the primary ligand for CCR1 in the mouse is MIP-1α/CCL3, not RANTES/CCL5,27 we suggest that Met-RANTES may reduce CAV in this model by interrupting the interactions of RANTES/CCL5 with CCR5 and MCPs with CCR1 and CCR5.

In conclusion, the present study provides evidence that antagonism of the CCR1 and CCR5 receptors attenuates chronic rejection. In this model, the mechanisms of Met-RANTES-induced CAV inhibition include a reduction in mononuclear cell recruitment, a decreased proliferative response, and a reduction in RANTES/CCL5 and MCP-1 gene transcript levels. Chemokine receptor blockade may be of therapeutic benefit in controlling the development of chronic rejection in transplanted hearts.

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References

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