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PRE-CLINICAL RESEARCH

A New Monocyte Chemotactic Protein-1/Chemokine CC Motif Ligand-2 Competitor Limiting Neointima Formation and Myocardial Ischemia/Reperfusion Injury in Mice

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Objectives A nonagonist monocyte chemotactic protein-1 (MCP-1/CCL2) mutant (PA508) with increased affinity for glycosaminoglycans and thus competing with CCL2 was evaluated as a candidate for preventing neointima formation or myocardial ischemia/reperfusion injury.

Background Myocardial infarction (MI) remains a major cause of death worldwide despite improved interventional and therapeutic options. Therefore, the discovery of drugs that limit restenosis after intervention and post-MI damage remains an important challenge.

Methods The function of PA508 was assessed in functional assays in vitro and in mouse models of wire-induced neointima formation and experimental MI.

Results PA508 was functionally inactive in CC chemokine receptor 2 (CCR2) binding and calcium influx but inhibited monocyte chemotaxis or transendothelial migration toward CCL2, suggesting that it interferes with CCL2 presentation. In wild-type but not CCR2-deficient mice, PA508 reduced inflammatory leukocyte recruitment without affecting differential leukocyte counts, CCL2 levels, organ function, or morphology, indicating that it specifically attenuates the CCL2-CCR2 axis. Compared with vehicle, daily intraperitoneal injection of PA508 significantly ($p < 0.05$, $n = 5$) reduced neointimal plaque area and mononuclear cell infiltration in carotid arteries of hyperlipidemic apolipoprotein E-deficient mice while increasing smooth muscle cell content. In C57Bl/6J mice that underwent myocardial ischemia/reperfusion, treatment with PA508 significantly reduced infarction size, monocyte infiltration, and collagen and myofibroblast content in the infarction area and preserved heart function compared with vehicle ($p < 0.05$, $n = 4$ to 8).

Conclusions Here we demonstrate that administration of a rationally designed CCL2 competitor reduced inflammatory monocyte recruitment, limited neointimal hyperplasia, and attenuated myocardial ischemia/reperfusion injury in mice and could therefore be envisioned as a combined therapeutic approach for restenosis and MI. (J Am Coll Cardiol 2010;56:1847–57) © 2010 by the American College of Cardiology Foundation

The major cause of death in Western countries, atherosclerosis and subsequent myocardial infarction (MI), triggers a complex inflammatory reaction accompanied by cytokine and chemokine release, which remains incompletely understood and not sufficiently targeted by current therapeutic strategies (1–5). Chemokines are considered major arbitrators that modulate

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Abbreviations and Acronyms

CCL = chemokine CC motif ligand

CCR = chemokine CC motif receptor

GAG = glycosaminoglycan

I/R = ischemia/reperfusion

LV = left ventricular

MCP = monocyte chemotactic protein

MI = myocardial infarction

this inflammatory response during the development of atherosclerosis (5,6), but are also involved in myocardial healing and scar formation after MI (1,7,8). Chemokines have highly conserved and well-defined structures tailored to their function as inflammatory mediators. In particular, most chemokines can form multimeric structures, and specific amino acid motifs confer high affinity for glycosaminoglycans (GAGs), allowing effective presentation on the surface of vascular cells. Chemokines are functionally intolerant to modifications of the N terminus, a property that has been exploited for regulated on activation, T-cell expressed, and secreted (RANTES/CCL5) to create the antagonist Met-CCL5 (9). Mutations that affect oligomerization and/or GAG interaction of chemokines have also generated chemokine variants with antagonistic properties (10,11).

The CC motif chemokine monocyte chemoattractant protein (MCP)-1/CCL2 is a potent mononuclear cell chemoattractant involved in various diseases characterized by monocyte-rich leukocyte infiltrates (12). CCL2 can directly influence wound angiogenesis by mediating endothelial cell migration (13) and can modulate fibrous tissue deposition by stimulating extracellular matrix production and differentiation of fibroblasts toward a myofibroblast-like phenotype (14,15). CCL2 is also essential in the development of restenotic changes after coronary intervention by mediating monocyte infiltration and their subsequent activation (16). Mice that lack CCR2, the receptor for CCL2, show a marked reduction in neointima formation, monocyte recruitment, and neointimal macrophage content after arterial injury (17). After ischemia/reperfusion (I/R) injury, these mice exhibit a decrease in macrophage infiltration and infarct size compared with wild-type mice (15,18). Similarly, CCL2-deficient mice display a decreased and delayed macrophage infiltration and myofibroblast accumulation associated with an improvement of left ventricular (LV) dysfunction and regional hypocontractility after MI (19,20). In line with these findings, CCL2 can induce a novel transcription factor that causes cardiac cell death and ventricular dysfunction (21). Hence, pharmacological inhibition of CCL2 might represent an attractive approach to prevent detrimental vascular remodeling and to attenuate damage after MI. Indeed, the inhibition of CCL2 by an antibody resulted in reduction of atherosclerosis and inflammation in *Apoe*$^{-/-}$ mice, inducing a stable atherosclerotic plaque phenotype (22) and thus demonstrating the feasibility of this approach. However, antibodies are large proteins difficult to be economically produced in sufficient amounts, need to be humanized, and carry the risk of undesired binding to other antigens, which may affect safety of long-term treatment.

The purpose of our study was to explore beneficial effects of a recombinant CCL2 competitor (PA508), a CCR2-activation incompetent variant of CCL2, which was engineered to have an increased affinity for GAGs, as a novel therapeutic approach for the prevention of neointimal formation and myocardial I/R injury in mice.

Methods

Generation of PA508. The amino acid mutations in human CCL2 complementary DNA were introduced by standard molecular cloning (23).

Surface plasmon resonance. Equilibrium binding of CCL2 and PA508 to heparin pentasaccharide (Sanofi-Aventis, Paris, France) was determined (24,25) and used for Scatchard plot analysis and calculation of dissociation constants (kilodaltons).

Calcium mobilization. Calcium transients in Fluo-4–labeled THP-1 cells were recorded in a microplate reader. After baseline measurement, cells were stimulated with H_2O (control), CCL2, or PA508 (50 ng/ml).

Chemotaxis and transendothelial migration. The effects of PA508 on THP-1 cell migration toward CCL2 for 2 h at 37°C were assessed using 5-μm Transwell filters seeded without or with human umbilical vein endothelial cells and analyzed by flow cytometry.

Chemokine receptor binding studies. PA508 binding studies to CCR2-transfected cells were performed using ^{125}I-CCL2 displaced by PA508 or Met-CCL2 in comparison with wtCCL2 in Hank's balanced salt solution buffer.

Pharmacokinetics of PA508. Female Balb/cOlaHsd mice (Harlan Laboratories, Horst, Belgium) were injected intraperitoneally with Met-CCL2 or PA508 20 μg per mouse. Serum concentrations of Met-CCL2 or PA508 were determined by species-specific enzyme-linked immunosorbant assay for human CCL2 (Anogen, Yes Biotech Laboratories Ltd., Mississauga, Ontario, Canada) at various time points.

PA508 treatment of mice. Mice were treated intraperitoneally with PA508 (0, 1, 5, or 10 μg in phosphate-buffered saline). All animal experiments and study protocols were approved by local authorities, complying with German animal protection laws.

Examination of PA508-treated mice. Venous blood was obtained from C57Bl/6J mice before treatment (6 mice per group) and at 1, 3, and 8 days and assayed for alanine-aminotransferase, creatinine, and CCL2 levels. Organs were harvested for histological analysis. Blood cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey).

Leukocyte recruitment in vivo. An air-pouch model (26) was performed in C57Bl/6J (8 per group) or CCR2$^{-/-}$ mice (4 per group) treated intravenously with phosphate-buffered saline, PA508 (10 μg), or heat-inactivated PA508 (HI-PA508) (10 μg, n = 4).

Wire-injury and ex vivo perfusion models. Eight-week-old *Apoe*$^{-/-}$ or *Ccr2*$^{-/-}$*Apoe*$^{-/-}$ mice underwent carotid