Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages

Fina Lovren,1,2 Yi Pan,1 Adrian Quan,1,2 Paul E. Szmitko,2,4 Krishna K. Singh,1,2 Praphulla C. Shukla,1,2 Milan Gupta,1,3,6,7,8 Lawrence Chan,9 Mohammed Al-Omran,10,11 Hwee Teoh,1,2,3,8 and Subodh Verma1,3,5,8,11

Division of 1Cardiac Surgery, 2Translational Traineeship Program in Atherosclerosis, 3Cardiometabolic Risk Initiative, Keenan Research Centre in the Li Ka Shing Knowledge Institute of St. Michael’s Hospital, Toronto, Ontario; Departments of 4Medicine and 7Surgery, University of Toronto, Toronto, Ontario; 5Division of Cardiology, William Osler Health Centre, Brampton, Ontario; 7Department of Medicine, McMaster University, Hamilton, Ontario; 8Canadian Cardiovascular Research Network, Brampton, Ontario, Canada; 9Departments of Molecular and Cellular Biology and Medicine, Baylor College of Medicine, Houston, Texas; 10Division of Vascular Surgery, College of Medicine and King Khalid University Hospital, 11King Saud University-Li Ka Shing Collaborative Research Program, King Saud University, Riyadh, Kingdom of Saudi Arabia

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Lovren F, Pan Y, Quan A, Szmitko PE, Singh KK, Shukla PC, Gupta M, Chan L, Al-Omran M, Teoh H, Verma S. Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages. Am J Physiol Heart Circ Physiol 299: H656–H663, 2010. First published July 9, 2010; doi:10.1152/ajpheart.00115.2010.—Altered macrophage kinetics is a pivotal mechanism of visceral obesity-induced inflammation and cardiometabolic risk. Because monocytes can differentiate into either proatherogenic M1 macrophages or anti-inflammatory M2 macrophages, approaches that limit M1 while promoting M2 differentiation represent a unique therapeutic strategy. We hypothesized that adiponectin may prime human monocytes toward the M2 phenotype. Adiponectin promoted the alternative activation of human monocytes into anti-inflammatory M2 macrophages as opposed to the classically activated M1 phenotype. Adiponectin-treated cells displayed increased M2 markers, including the mannose receptor (MR) and alternative macrophage activation-associated CC chemokine-1. Incubation of M1 macrophages with adiponectin-treated M2-derived culture supernatant resulted in a pronounced inhibition of tumor necrosis factor-α and monocyte chemotactic protein-1 secretion. Activation of human monocytes into M2 macrophages by adiponectin was mediated, in addition to AMP-activated protein kinase and peroxisome proliferator-activated receptor (PPAR)-γ, via PPAR-α. Furthermore, macrophages isolated from adiponectin knockout mice demonstrated diminished levels of M2 markers such as MR, which were restored with adiponectin treatment. We report a novel immunoregulatory mechanism through which adiponectin primes human monocyte differentiation into anti-inflammatory M2 macrophages. Conditions associated with low adiponectin levels, such as visceral obesity and insulin resistance, may promote atherosclerosis, in part through aberrant macrophage kinetics.

adiponectin; obesity; macrophage activation

atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of abnormal lipid metabolism, endothelial dysfunction, and inflammation (35). A crucial step in this inflammatory process is the infiltration of monocytes into the subendothelial space of large arteries and their differentiation into tissue macrophages, whose activation and function are influenced by the cytokines within the inflammatory milieu of the atherosclerotic lesion.

Proinflammatory cytokines such as interferon (IFN)-γ and interleukin (IL)-1β, and inducers of tumor necrosis factor (TNF)-α such as lipopolysaccharide (LPS), promote macrophage differentiation to a “classical” activation pattern (M1) (11). M1 macrophages are associated with inflammation and tissue destruction, produce proinflammatory cytokines such as TNF-α, IL-6, and IL-12, and increase the production of reactive oxygen species sustaining the process of atherogenesis (12). In contrast, “alternative” activation program (M2) macrophages, which are induced in response to IL-4 and IL-13, dampen the inflammatory process by producing anti-inflammatory factors such as IL-10 and transforming growth factor-β, generating IL-1 receptor antagonist, scavenging apoptotic cell debris, and promoting angiogenesis and tissue repair (11, 31). A reduction in anti-inflammatory M2 macrophages and an increase in genes associated with proinflammatory M1 macrophages occur in obese states (20).

Obesity directly contributes to the pathogenesis of insulin resistance, type 2 diabetes, atherosclerosis, and cardiovascular diseases (9). Obesity-associated inflammation is characterized by an increased abundance of macrophages in the adipose tissue, which are postulated to be major sources of several molecular mediators such as TNF-α, IL-6, and C-reactive protein (CRP), which directly contribute to the proinflammatory milieu mediating vascular injury (14, 18, 19, 23). However, the adipose tissue also secretes adiponectin, an adipokine believed to confer protection against inflammation and obesity-linked insulin resistance (34). Circulating levels of adiponectin are decreased in patients with obesity, type 2 diabetes, or coronary artery disease and are inversely correlated to circulating levels of CRP and IL-6 (7, 8, 13). Similarly, adiponectin levels in human adipose tissue are also inversely affected by TNF-α and IL-6 (3). In cultured endothelial cells, adiponectin treatment inhibits the expression of adhesion molecules, whereas, in macrophages, adiponectin treatment appears to decrease TNF-α production, limits the transformation to foam cells, and stimulates the secretion of the anti-inflammatory cytokine IL-10 (16, 26, 27). Thus adiponectin appears to have anti-inflammatory and anti-atherogenic properties.

In the present study, we hypothesized that adiponectin may exert novel effects to prime human monocytes toward an anti-inflammatory alternative M2 phenotype. To this aim, we
evaluated the effects of adiponectin on human monocyte differentiation, in addition to studying macrophage phenotypes in adiponectin-deficient mice.

**MATERIALS AND METHODS**

**Materials.** Recombinant adiponectin, IL-4, and enzyme-linked immunosorbent assay (ELISA) kits were from R & D. Antibodies for flow cytometry (all from BD) included APC mouse anti-human CD206 (clone 19.2), APC mouse IgG1 isotype control, phycoerythrin (PE) mouse anti-human CD163 (clone GHI/61), and PE mouse IgG1 isotype control. Antibodies for Western blots were from Abcam (peroxisome proliferator-activated receptor (PPAR) α), Cell Signaling (AMP-activated protein kinase (AMPK) α, phospho (p-)AMPKα, IkBα, pIkBα (Ser32/36), NF-κB p65, pNF-κB p65, and PPARγ), Millipore (actin), and Santa Cruz (CD206). All other reagents were from Sigma.

**Isolation of human peripheral blood monocytes.** Approval was received from the Research Ethics Board of St. Michael’s. Participation was voluntary, and all subjects were healthy and provided informed consent. Peripheral blood mononuclear cells were isolated with Ficoll-Paque pre-filled Leucosep tubes (Greiner Bio-One). Cells were suspended in RPMI 1640 (supplemented with 10% human serum, 40 μg/ml gentamicin, and 2 mM glutamine), seeded at a density of 5 × 10^6 cells/well in six-well plates, and incubated for 2 h at 37°C with 5% CO₂. Nonadherent cells were discarded, and adherent monocytes maintained in RPMI 1640 for 7 days.

**Monocyte differentiation.** Monocyte differentiation into resting macrophages (RM) occurred after 7 days in culture. M2 macrophages were obtained by incubating freshly isolated monocytes with IL-4 (15 ng/ml) or its vehicle, was induced with acetylated low density lipoprotein (50 μg/ml).

**Real-time PCR.** First-strand cDNA was prepared with the FastLane Cell cDNA Kit (Qiagen). Primers were designed with ProbeFinder (Roche), and the sequences are provided in Supplemental Table 1 (Supplemental material for this article may be found on the American Journal of Physiology: Heart and Circulatory Physiology website.). Amplifications were performed on the Applied Biosystems StepOne Plus Real-Time PCR system. mRNA expression was analyzed using the method of Pfaffl (28), and all values were normalized against the corresponding glyceraldehyde-3-phosphate dehydrogenase levels.

**Western blots.** Proteins from whole cell lysates of macrophages were separated on 4–12% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Membranes were probed with primary antibodies and then incubated with the appropriate horseradish peroxidase-associated secondary antibodies before signals were visualized by enhanced chemiluminescence (Amersham Bioscience).

**Flow cytometry.** RMs and M2 macrophages were collected by gentle scrapping, washed in PBS, and suspended in 1% BSA-supplemented PBS (BSA-PBS) with the appropriate antibody or isotype control (1:100). After 1 h on ice, cells were washed two times before suspension in BSA-PBS. Antibody-tagged cells were enumerated on a FACSCalibur (BD) system, and the results were evaluated with the FACSDiva and FlowJo software (BD). Nonspecific binding was avoided by incubating macrophages with PBS/10% human serum for 20 min.

**Inflammatory cytokine and chemokine measurements.** M2 macrophages were cultured in the presence or absence of adiponectin (10 μg/ml) for 7 days before being maintained in fresh medium for the following 24 h. The medium (20%) was subsequently introduced to 7-day-old RMs for 24 h before the cells were activated with LPS (100 ng/ml) for 4 h. The resultant M1 macrophages were cultured for a further 24 h in fresh medium before TNF-α, monocyte chemotactic protein-1 (MCP-1), and CCL-3 levels in the supernatants were measured by ELISA.

**Animal studies.** All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the St. Michael’s Hospital Animal Care Committee. Macrophages were isolated from the peritoneal cavity of male adiponectin knockout mice (Adipoq^-/-) and their wild-type littermates (Adipoq^+/+) as
previously described (10, 21). Briefly, peritoneal cavities were lavaged with cold PBS, and the cells were washed two times before suspension in RPMI 1640. Macrophages, pooled from five mice, were seeded at a density of $2 \times 10^5$ cells/ml and treated with or without recombinant rat IL-4 for 3 days in the presence of adiponectin or its vehicle.

Statistical analysis. Results are presented as means $\pm$ SE. Differences between two groups were compared with the Student’s t-test. Intergroup comparison of means was performed by ANOVA followed by the Student’s t-test. Significance was set at $P < 0.05$.

RESULTS

Adiponectin promotes the alternative activation of human monocytes into M2 macrophages. To evaluate the effect of adiponectin on M2 differentiation, primary human monocytes were differentiated into alternative macrophages with IL-4 alone (M2) or in the presence of adiponectin (M2-Adipo). Signaling through the IL-4 receptor, as well as the IL-13 receptor, leads to activation of the mannose receptor (MR, CD206) and alternative macrophage activation-associated CC chemokine-1 (AMAC-1) and downregulation of CD163 expression (10, 15, 33). To characterize the macrophage activation phenotype, we evaluated the mRNA expressions of these markers in M2 and M2-Adipo cells. Both MR and AMAC-1 expressions were strongly induced by IL-4 in M2 macrophages, and their levels were further amplified by adiponectin cotreatment (Fig. 1, A and B). In contrast, expression of the macrophage scavenger receptor CD163 was significantly reduced after IL-4 stimulation and was further suppressed with concomitant adiponectin treatment (Fig. 1C). Macrophage CD163 expression appears to be elevated in type 2 diabetes and obesity, and our findings are consistent with a previous report that suggested adiponectin reduces CD163 expression in monocytes (32).

Adiponectin-activated M2 macrophages demonstrate increased MR protein expression. Flow cytometry revealed higher MR protein levels in M2 macrophages compared with RMs. Moreover, MR expression was enhanced in M2-Adipo macrophages. Representative results and the summarized data are shown in Fig. 2A. In contrast, significantly decreased CD163 expression was observed in M2 macrophages with CD163 levels further diminished in M2-Adipo macrophages (Fig. 2B). The observed changes in mRNA expressions and protein levels of M2 markers in M2-Adipo macrophages suggest that adiponectin promotes alternative M2 differentiation.

Adiponectin-stimulated M2 macrophages exert paracrine anti-inflammatory effects on M1 macrophages. Adiponectin has a crucial role in suppressing macrophage activity, and it has been shown to cause decreased production of proinflammatory cytokines in macrophages, both in vivo and in vitro (37). To determine whether M2-Adipo macrophages can influence the inflammatory status of activated M1 macrophages, indirect coculture experiments were performed, and cytokine and chemokine release by M1 macrophages was subsequently quantified by ELISA. Incubation of M1 macrophages with M2-derived culture supernatant resulted in pronouncedly decreased secretion of the proinflammatory molecules TNF-α,
MCP-1, and CCL-3 (Fig. 3). This inhibitory effect was more evident when M1 macrophages were incubated with the supernatant derived from M2 macrophages primed in the presence of adiponectin. These findings indicate that M2 macrophages can suppress the inflammatory status of surrounding M1 macrophages and that M2 macrophage priming with adiponectin enhances the anti-inflammatory properties of M2 macrophages. Adiponectin does not switch RM, M1, or foam cells into an M2 phenotype. Previous studies have shown that macrophages are plastic cells and can be switched from one activated state to another and vice versa (2, 30). Therefore, we tested whether RM, M1 macrophages, or foam cells can be reverted to the M2 phenotype following adiponectin treatment. M2 phenotype was evaluated by determining the mRNA expressions of MR, AMAC1, and CD163. Adiponectin exposure did not appreciably alter the transcript expressions (Fig. 4) or protein levels (data not shown) of any of these M2 markers, suggesting that it does not influence the expression of M2 markers in RM, M1, or foam cells.

Adiponectin activates M2 macrophages via PPAR-α and the NF-κB-1kB signaling module. Macrophages constitutively express the adiponectin receptors AdipoR1 and AdipoR2 (6). The downstream targets of these receptors include the PPAR-α, AMPK, and p38 mitogen-activated protein kinase (38). In M2-Adipo macrophages, we found similar AMPK and PPAR-γ transcript expression and protein levels (Figs. 5A, 6A, and 6B) but observed significantly higher levels of PPAR-α transcript and protein (Figs. 5B and 6B) compared with those of both RM and M2 macrophages. We further noted that the PPAR-α antagonist GW-6471 blocked M2-Adipo macrophage differentiation by altering the expression of the M2 macrophage markers MR (Fig. 5C) and AMAC1 (Fig. 5D). As shown in Fig. 6C, we observed suppressed NF-κB and IκB phosphorylation in M2 macrophages, effects that were further accentuated in M2 macrophages that had been treated with adiponectin. Collectively, our results suggest that adiponectin-activated M2 differentiation appears to be intricately linked with the PPAR-α and PPAR-γ receptors as well as the NF-κB-1kB signaling module.

Adiponectin deficiency alters peritoneal monocyte activation favoring an inflammatory phenotype. As shown in Fig. 7A, expression of the M2-specific marker MR was significantly lower in macrophages derived from Adipoq−/− mice compared with those from Adipoq+/+ littermates. Adiponectin treatment improved MR transcript and protein expressions in IL-4-treated macrophages from Adipoq−/− mice (Fig. 7, A and C). Macrophage expression of CD163 was greater in Adipoq−/− mice relative to their Adipoq+/+ littermates (Fig. 7B). In contrast, adiponectin-treated Adipoq−/−-derived macrophages...
demonstrated similar CD163 expression to those from Adipoq+/−/− mice. Taken together, these results provide further support for a role for adiponectin in promoting differentiation of monocytes toward an anti-inflammatory M2 phenotype.

DISCUSSION

In the present study, we have demonstrated that the adipokine, adiponectin, promotes the differentiation of monocytes into the anti-inflammatory M2 macrophage phenotype. Adiponectin treatment stimulated the expression of markers of M2 activation such as MR and AMAC-1 but did not influence the transition of differentiated macrophages, such as M1 macrophages, RM, or foam cells, toward the anti-inflammatory M2 phenotype. Furthermore, adiponectin enhanced the anti-inflammatory properties of M2 macrophages on M1 macrophages as demonstrated by indirect coculture experiments. PPAR-α activation as well as the NF-κB-IκB signaling module seem to be involved in facilitating monocyte differentiation to the M2 phenotype. Finally, the observation that peritoneal monocytes in adiponectin knockout mice tended to exhibit decreased markers of M2 activation, and that adiponectin supplementation partially abrogated this effect, support our in vitro observations.

Several studies have demonstrated that low concentrations of adiponectin, as observed in obese individuals, are independently associated with the prevalence of coronary artery disease in men and that higher concentrations of adiponectin, independent of glycemic or lipid status, confer a lower risk of myocardial infarction (17, 29). Adiponectin, on a molecular level, appears to exert an antiatherogenic effect upon the vasculature by enhancing endothelial nitric oxide synthesis, attenuating the attachment of monocytes to endothelial cells by inhibiting the proinflammatory TNF-α- and IL-8-induced synthesis of adhesion molecules (e.g., intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin), interfering with NF-κB activation, reducing foam cell formation, and suppressing both the proliferation and migration of human aortic smooth muscle cells (1, 4, 26, 27). However, several features of adiponectin indicate that it may function as a cytokine that modulates immune function. It can regulate immune responses by promoting the clearance of apoptotic cells by macrophages, it decreases macrophage TNF-α production, and it stimulates the secretion of the anti-inflammatory cytokine IL-10 (16, 36). Our findings further support a role for adiponectin in immune function modulation, since it appears to stimulate monocyte differentiation toward an anti-inflammatory phenotype. These observations are consistent with recent observations that adiponectin treatment results in an increase in the levels of M2 markers and a reduction in reactive oxygen species generation (25).

Human atherosclerotic lesions appear to contain both M1 and M2 macrophages (2). M2 macrophages are present at locations distant from the lipid core in more stable zones of the plaque and appear to exert anti-inflammatory properties...
Our observations support a potential role for adiponectin-activated M2 macrophages to exert a paracrine anti-inflammatory effect on M1 macrophages. Native monocytes, in the presence of an M2 stimulus such as IL-4, can be primed by adiponectin toward an enhanced M2 phenotype. This presents yet another molecular pathway via which adiponectin exerts its anti-inflammatory effects upon the vasculature, inhibiting plaque formation and stabilizing established atherosclerotic plaques. This reduction in lesion inflammation may result in plaque stabilization by increasing collagen content and diminishing fibrous cap thinning. Indeed, in vivo studies conducted in adiponectin knockout mice demonstrate increased neointimal thickening and vascular smooth muscle cell proliferation following vascular injury compared with wild-type controls, with the extent of neointimal proliferation being considerably attenuated after adenovirus-mediated reexpression of adiponectin (24). However, adiponectin does not influence the expression of M2 markers in RM, M1 programmed macrophages, or foam cells that are already differentiated. The underlying reason(s) behind why adiponectin on its own does not propel the activation of RM, M1, and foam cells into a more M2 phenotype remains unknown.

The mechanism through which adiponectin primes monocytes toward the M2 phenotype seems to involve PPAR-α activation and the NF-κB-IκB signaling module. PPAR-α is a nuclear receptor that regulates the expression of genes encoding proteins involved in lipid metabolism, fatty acid oxidation, and glucose homeostasis and appears to exert anti-inflammatory effects in the vascular wall by modulating atherosclerosis-associated inflammatory responses (39). PPAR-α activation has been demonstrated to reduce the production of Th1 cytokines, such as IFN-γ and IL-1β, which induce the classical activation profile M1 (5, 22). NF-κB has been implicated as a promoter of inflammation in multiple experimental models. In our hands, adiponectin

Fig. 7. mRNA levels of MR (A) and CD163 (B) in peritoneal monocytes isolated from wild-type (Adipoq+/+) mice and adiponectin knockout (Adipoq−/−) littermates as measured by real-time PCR with GAPDH acting as the housekeeping gene. C: MR protein levels in peritoneal monocytes from Adipoq−/− mice as detected by Western blotting with actin acting as the housekeeping protein. Monocytes were cultured with IL-4 alone or in the presence of adiponectin or its vehicle for 3 days; n = 5 for A and B, n = 3 for C. *P < 0.05 vs. Adipoq+/+. †P < 0.05 vs. Adipoq−/−.
treatment was associated with enhanced PPAR-α activation and reduced NF-κB-IκB phosphorylation. Thus adiponectin priming of monocytes via PPAR-α and the NF-κB-IκB signaling module may further enhance the M2 anti-inflammatory phenotype as observed in the current study.

In conclusion, our present study revealed that adiponectin primes human monocyte differentiation toward anti-inflammatory M2 macrophages via PPAR-α. Adiponectin-activated M2 macrophages suppress the secretion of pro-inflammatory molecules by M1 macrophages, an effect that may promote atherosclerotic plaque stability. Our data provide additional insight into how adiponectin deficiency may promote vascular inflammation and atherosclerosis, in part, through aberrant macrophage kinetics and provide an entirely novel biological basis for the atheroprotective effects of this adipokine.

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DISCLOSURES

The authors report no conflict of interest pertinent to the data presented in this manuscript.

REFERENCES


