Clinical Research

Hemoglobin Scavenger Receptor CD163 Mediates Interleukin-10 Release and Heme Oxygenase-1 Synthesis

Antiinflammatory Monocyte-Macrophage Responses In Vitro, in Resolving Skin Blisters In Vivo, and After Cardiopulmonary Bypass Surgery


Abstract—The recently described hemoglobin scavenger receptor CD163 mediates the endocytosis of hemoglobin:haptoglobin (Hb:Hp) complexes and thereby counters Hb-induced oxidative tissue damage after hemolysis. Although CD163 has been indirectly associated with antiinflammatory and atheroprotective activity, no ligand-receptor-effector pathway has yet been described for this receptor. To understand the significance of CD163 and more clearly define downstream pathways linked to inflammatory resolution, we studied the expression and function of CD163 in human monocytes/macrophages using both in vitro and in vivo models. Differentiation of human blood monocytes into macrophages either by in vitro culture or in resolving cantharidin-induced skin blisters led to an equivalent increase (>15×) in CD163 expression. Elevated CD163 levels were also noted on circulating monocytes in cardiac surgical patients during the resolution phase of the systemic inflammatory response to cardiopulmonary bypass surgery. In each case, binding of Hb:Hp to CD163-bearing cells elicited potent interleukin-10 secretion, and this was inhibited by the anti-CD163 antibody RM3/1. Release of interleukin-10, in turn, induced heme oxygenase-1 stress protein synthesis via an autocrine mechanism. Such induction of heme oxygenase-1 was observed in vivo 24 to 48 hours after the onset of cardiopulmonary bypass surgery. These results identify novel antiinflammatory and cytoprotective effector pathways in human monocytes/macrophages related to Hb scavenging and metabolism, which may have relevance in atheroprotection, wound healing, and patient recovery postoperatively. (Circ Res. 2004;94:119-126.)

Key Words: hemoglobin scavenger receptor ■ interleukin-10 ■ heme oxygenase-1 ■ antiinflammation

CD163 is a group B cysteine–rich scavenger receptor expressed exclusively by cells of the monocyte-macrophage lineage.1 This 130-kDa transmembrane glycoprotein was first identified in 19872 but has only recently been characterized as a scavenger receptor for hemoglobin, mediating endocytosis of hemoglobin:haptoglobin (Hb:Hp) complexes.3 During intravascular hemolysis, free Hb binds to the plasma protein Hp, and Hb:Hp complexes are formed. Free Hb, if not eliminated from the circulation, not only impairs renal function but may also cause tissue damage by catalyzing the production of iron-derived hydroxyl radicals by the Fenton reaction.4,5 Such Hb-induced oxidative stress is thought to modify low-density lipoproteins and may be relevant in the pathogenesis of atherosclerosis.6

After endocytosis of Hb:Hp, the heme subunit of Hb is degraded by the rate-limiting heme oxygenase (HO) enzymes. Two main isoforms of HO have been characterized, with HO-2 being constitutively present under physiological conditions and HO-1 being inducible.7 The breakdown of heme yields biliverdin, free iron, and the carbon monoxide (CO) molecule, which has antiinflammatory and cytoprotective effects.8,9 In macrophages, CO reduces proinflammatory and increases antiinflammatory cytokine secretion in response to lipopolysaccharide via a mitogen-activated protein kinase pathway.10 Interestingly, HO-1 expression is upregulated in macrophages during the resolution phase of inflammation11,12 and also in murine macrophages stimulated by interleukin (IL)-10.13 HO-1–mediated antiinflammatory effects may therefore be closely linked with antiinflammatory pathways stimulated by IL-10, such as the suppression of immune and inflammatory responses in macrophages via diminished antigen-presenting capacity and cytokine synthesis.14–16 Indeed, deficiency of HO-1 in both human and gene-targeted mice leads to a marked rise in circulating heme and subsequent oxidative vascular and tissue injury, anemia, and chronic inflammation.17,18

Previous studies have indirectly linked CD163 to antiinflammatory phenomena. Early immunohistological work showed that CD163 expression by macrophages coincided with the late phase of experimental gingivitis.2 CD163 has
also been shown to be induced experimentally on a subpopulation of macrophages polarized in response to antiinflammatory factors, such as IL-4, IL-10, and corticosteroids.\textsuperscript{19–22} Other reports suggest that upregulation of CD163 is associated with the release of unidentified antiinflammatory factors\textsuperscript{23} and that soluble CD163 once shed from the membrane can inhibit T-lymphocyte proliferation.\textsuperscript{24} However, no anti-inflammatory ligand-receptor-effector pathway has yet been described for CD163.

A recently described skin-blistering model used to study in vivo leukocyte extravasation and cytokine production during tissue inflammation in humans involves the topical application of the vesicant cantharidin to healthy skin.\textsuperscript{25} Although initially described for the study of the acute phase of inflammation, the technique also allows functional studies to be performed on tissue macrophages isolated from resolving blisters.

Cardiac surgery using cardiopulmonary bypass (CPB) provokes a systemic inflammatory response.\textsuperscript{26,27} This is mainly triggered by contact activation of blood components on the artificial surfaces of the extracorporeal bypass circuit. Although often remaining subclinical and resolving promptly after CPB, in its most extreme form this inflammatory response is associated with multiple-organ failure and high patient mortality. Hemolysis is a well-recognized consequence of CPB, being triggered by shear stresses generated within the bypass circuit.\textsuperscript{28,29} Reports show that plasma-free Hb rises as high as 50 mg/dL by the end of CPB in association with a fall in Hp. The significance of the CD163 receptor in immunomodulation and clearance of Hb:Hp complexes after CPB, thereby promoting resolution of the inflammatory response and patient recovery, is unknown.

In the present study, we have examined whether Hb:Hp binding to CD163 is linked to antiinflammatory effector pathways. We demonstrate that Hb:Hp binding to CD163 triggers IL-10 and HO-1 induction in human macrophages in vitro and in tissue macrophages ex vivo. Functional CD163 is also expressed on circulating monocytes in vivo after coronary artery bypass graft (CABG) surgery with CPB. This in vivo CD163 expression is associated with HO-1 induction postoperatively. These studies therefore identify novel downstream effector pathways induced by Hb:Hp binding to CD163, which may coordinate hemoglobin catabolism with inflammatory resolution after injury.

**Materials and Methods**

**Reagents and Antibodies**

Human Hb (A\textsubscript{a}), human Hp (phenotype 1-1), and fluorescein isothiocyanate (FITC; isomer 1) were purchased from Sigma-Aldrich (Poole, UK). Cantharidin (Cantharon) was purchased from Dormer Laboratories Inc (Rexdale, Ontario, Canada). Anti-human CD163 monoclonal antibody clones RM3/1, Ki-m8, and SC5-FAT were purchased from Bachem (Merseyside, UK), clone GHI/61 from BD Pharmingen (Oxford, UK), and clone Ber-MAC3 from Dako (Copenhagen, Denmark). Mouse IgG\textsubscript{1} control antibody and FITC-conjugated goat anti-mouse IgG secondary antibody were purchased from Sigma-Aldrich. Directly conjugated FITC:anti-CD163 antibody, RPE-conjugated anti-L-selectin antibody, and relevant control antibodies were purchased from BD Pharmingen, ECD-conjugated anti-CD14 antibody and relevant control antibody were purchased from Beckman Coulter (High Wycombe, UK). Neutralizing anti-human IL-10 polyclonal antibody and control goat IgG antibody were purchased from R&D Systems (Abingdon, UK). Polyclonal anti-HO-1 antibody was purchased from Stressgen Biotechnologies Corp (Victoria, Canada), and anti-HO-2 antibody from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). Anti-CD64 antibody 10.1 was generously provided by Dr Nancy Hogg (Cancer Research UK, London, UK).

**Monocyte-Macrophage Isolation and In Vitro Culture**

Human monocytes were isolated from venous blood using a Dextran/Percol sedimentation technique as previously described.\textsuperscript{30} Isolated monocytes were cultured on 24-well plates at 5×10\textsuperscript{5} cells/well in IMEM supplemented with l-glutamine (Invitrogen Ltd), 10% autologous serum, and 100 U/mL of penicillin and streptomycin (Invitrogen Ltd). Cells were incubated for up to 7 days, and growth medium was replenished on days 1 and 3 of culture.

**Hb:Hp Treatment of Monocyte-Macrophage Cultures**

Hb:Hp complexes were generated by dissolving equimolar amounts of Hb and Hp in growth medium, as previously described.\textsuperscript{3} Hb, Hp, or Hb:Hp complexes were added at final concentrations of 1 mg/mL, unless otherwise stated, to monocyte-macrophage cultures before incubation for 24 hours and collection of supernatants or cell lysates for IL-10 and HO-1 analysis, respectively. Supernatants and cell lysates were stored in aliquots at −70°C before analysis. In some experiments, anti-CD163 mAbs were added at a final concentration of 20 μg/mL.

**Cantharidin-Induced Skin Blisters**

To investigate in vivo macrophage CD163 expression and IL-10 production at sites of tissue inflammation in healthy, human volunteers, the vesicant Cantharidin was used topically to induce skin blister formation and leukocyte extravasation as previously described.\textsuperscript{22} Blister fluid was collected at 16 and 40 hours, corresponding to the proinflammatory and resolving phases of blister formation, respectively. Blister cells were analyzed by 3-color flow cytometry for CD163 and L-selectin expression on CD14\textsuperscript{+} cells (see below) and by ex vivo culture in the presence of Hb:Hp (1 mg/mL), with or without blocking RM3/1 antibody (20 μg/mL), for 24 hours in Costar 96-well half-area microplates (Corning Inc). IL-10 levels in culture supernatants were corrected for variable monocyte-macrophage content in 16- and 40-hour blisters by expressing IL-10 production in terms of picograms per milliliter generated per 10\textsuperscript{5} CD14\textsuperscript{+} cells.

**Flow Cytometric Analysis**

In vitro cultured human monocytes/macrophages were prepared for flow cytometric analysis by detaching adherent monolayers with ice-cold PBS supplemented with 2.5 mmol/L EDTA for 15 minutes, followed by scraping and washing into IMEM growth medium,\textsuperscript{30} and CD163 and L-selectin expression in whole blood was determined using the immunolysed whole-blood lysing technique (Beckman Coulter) as previously described.\textsuperscript{31} The monocyte population in whole blood was identified by characteristic forward- and side-scatter properties and in cantharidin blister exudate fluid by gating with anti-CD14 antibody in the fluorescent (FL)-3 channel. CD163 and L-selectin expression were measured in the FL-1 and FL-2 channels, respectively. For ligand-binding studies, Hb:Hp complexes were initially FITC conjugated, as previously described,\textsuperscript{32} before incubating with in vitro differentiated macrophages in 24-well plates at 100 μg/mL for 2 hours in the presence and absence of blocking RM3/1 antibody (20 μg/mL) or control anti-CD64 antibody (20 μg/mL). Flow cytometric analysis was carried out using an EPICS XL-3 cytometer (Beckman Coulter).
CABG
Sixteen patients (11 male, 5 female; age, 47 to 76 years) referred for elective CABG surgery to the Hammersmith Hospital, London, were included in the study. Emergency cases, combined procedures, or redo operations were excluded, and none of the patients had signs of infection preoperatively. All had un complicated postoperative recoveries. Regional ethics committee approval for the study protocol was gained, and informed consent was obtained from each subject. All patients underwent a routine surgical procedure using standardized anesthetic and CPB techniques. Mean CPB time was 74.8±16.7 minutes.

Ex Vivo Culture of Monocytes From CABG Patients
Blood was collected preoperatively by antecubital fossa venesection and at 24 hours after onset of CPB from the central venous catheter. Blood monocytes were isolated and cultured ex vivo in 24-well plates at 5×10⁶ cells per well using the same procedures as described above. Supernatants and cell lysates were collected and stored at −70°C before IL-10 and HO-1 analysis.

Enzyme-Linked Immunosorbent Assays
IL-10 concentrations in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) technique (Duosets; R&D Systems) according to the manufacturer’s recommendations. All samples were measured in duplicate, and results are expressed as mean IL-10 concentrations (pg/mL)±SEM from n=3 experiments. Intracellular HO-1 concentrations in cell lysates were quantified by HO-1–specific ELISA (Stressgen Biotechnologies Corp.). Results were normalized with respect to lysate protein content using the Bio-Rad D, protein assay (Bio-Rad).

HO-1 and HO-2 Western Blot Analysis
Monocytes/macrophages were lysed in buffer containing 1% Triton X-100, 25 mmol/L sodium deoxycholate, 150 mmol/L NaCl, 50 mmol/L Tris, pH 7.4, 4 mmol/L EDTA, 200 μmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, and 5% protease inhibitor cocktail (Sigma Aldrich). Lysates were subjected to SDS-PAGE on 12.5% gels, and separated proteins were transferred to Immobilon-P membranes (Millipore Corporation). Equal loading of lanes was confirmed by estimation of lysate protein content using the Bio-Rad D, protein assay (Bio-Rad). Blots were developed with an enhanced chemiluminescence substrate (Amer sham Pharmacia Biotech UK Ltd).

Statistical Analysis
Statistical comparisons between groups were made using an unpaired Student’s t test (GraphPad Prism Software, Inc).

Results

HB:Hp Complex Induction of IL-10 via the CD163 Receptor in In Vitro Differentiated Macrophages
Human monocytes were differentiated for up to 7 days in culture to allow macrophage maturation. CD163 expression increased progressively with time in culture (Figure 1A), consistent with acquisition of a characteristic “fried egg” morphology and expression of a range of macrophage (but not dendritic cell) markers. To investigate whether HB:Hp elicited an antiinflammatory response from matured macrophages, day 7 cells were cultured an additional 24 hours in the presence of HB:Hp, Hb, or Hp alone (1 mg/mL), followed by determination of IL-10 levels in culture supernatants. These experiments revealed that HB:Hp, but not Hb or Hp alone, induced high levels of IL-10 secretion (4885.1±1077.7 pg/mL; mean±SEM; Figure 1B). Induction of IL-10 was dependent on the concentration of HB:Hp complex, with significantly elevated levels being detectable at 30 μg/mL (244.8±56.4 pg/mL; mean±SEM; Figure 1C). Accumulation of IL-10 was gradual, with detectable levels first seen at 6 hours after addition of complexes, rising to a maximum at 48 hours (Figure 1D).

To demonstrate that IL-10 induction was mediated via the CD163 receptor, a panel of anti-CD163 antibodies with no previously assigned functional properties was screened for blocking activity. This identified antibody clone RM3/1 as a potent inhibitory antibody, thus confirming that HB:Hp-induced secretion of IL-10 in macrophages was mediated via CD163 (Figure 2A). Clone Ki-M8 was a nonblocking antibody, and other clones (5C6-FAT, GHI/61, and Bermac-3) exhibited partial blocking activity. Ki-M8 also possessed agonistic properties and induced significant IL-10 secretion in the absence of the HB:Hp ligand (Figure 2B). The RM3/1-blocking antibody was next used to confirm ligand binding of HB:Hp complexes to CD163 (Figure 2C). This experiment confirmed that binding of FITC-conjugated HB:Hp complexes to in vitro differentiated macrophages was blocked by RM3/1 but not by control anti-CD64 antibody.
To investigate whether IL-10 induction attributable to Hb:Hp uptake was linked to other pathways of heme catabolism, intracellular HO-1 protein synthesis was investigated in the presence and absence of blocking antibodies against IL-10. These experiments demonstrated strong induction of HO-1 in macrophages exposed to Hb:Hp in vitro, which was blocked by antibodies against CD163 or IL-10 (Figure 3). Constitutive levels of HO-2 remained unchanged in the presence or absence of Hb:Hp ligand complexes. Weak induction of HO-1 was also observed in the presence of Hb alone, as previously reported.33,34 Also, considering the capacity of IL-10 to induce CD163 expression,19,20 these experiments therefore identify IL-10 as a link between accelerated Hb:Hp scavenging and HO-1 production in macrophages.

**IL-10 Induction by Hb:Hp Complexes in Macrophages Isolated From Skin Blisters In Vivo**

To extend the physiological relevance of these in vitro observations to tissue macrophages in humans, we investigated CD163 expression and antiinflammatory activity by monocyte-macrophages isolated from cantharidin-induced skin blisters. CD163 expression was measured on CD14+ gated leukocytes recovered from blister fluid at 16 and 40 hours after initiation of the blister response to cantharidin during the acute and resolving phases, respectively. Fewer than 5% of CD14+ cells stained positively for CD163 in whole blood (Figure 4A) or in 16-hour blister fluid (Figure 4B). However, most CD14+ cells were CD163+ in 40-hour blister fluid (Figure 4C). Loss of L-selectin expression on monocyte extravasation provided a marker for blood-blister transmigration, as shown in Figures 4B and 4C.35 To additionally examine the functional role of CD163 in skin inflammation, blister exudate cells were cultured ex vivo in the presence and absence of Hb:Hp or inhibitory RM3/1 antibody. These experiments showed that Hb:Hp stimulated IL-10 from 40-hour blister macrophages (but not 16-hour blister cells) and that this was blocked by anti-CD163 mAb.
The abolition of IL-10 secretion by RM3/1 strongly suggests that CD163 is the sole scavenging receptor on macrophages for Hb:Hp complexes in this model.

**IL-10 and HO-1 Induction in Circulating Monocytes From Patients After CABG Surgery on CPB**

To examine the role of CD163 in the systemic inflammatory and hemolytic responses after CPB surgery, blood samples were collected from 16 patients undergoing elective CABG operations using CPB. We found that CD163 was significantly upregulated on circulating monocytes at 24 hours after the onset of bypass (Figures 5A and 5B). To examine whether elevated expression of CD163 at 24 hours was linked to antiinflammatory IL-10 and HO-1 effector pathways, monocytes were isolated from three CABG patients and cultured ex vivo for 24 hours in the presence and absence of Hb:Hp or RM3/1 antibody. No detectable IL-10 was induced from monocytes collected preoperatively, whereas exposure to Hb:Hp complexes significantly stimulated CD163-dependent IL-10 secretion from monocytes collected 24 hours postoperatively (2928.45±782.18 pg/mL; Figure 5C). Immunoblotting furthermore demonstrated that intracellular HO-1 protein was strongly induced by Hb:Hp in 24-hour monocytes but not in preoperative monocytes and that this was ablated by antibody RM3/1 (Figure 5D). To extend the in vivo relevance of our findings, we examined the presence of HO-1 protein in freshly isolated monocytes from patients undergoing CABG surgery and found a significant induction at 24 and 48 hours after CPB onset (Figure 5E).

**Discussion**

Previous studies have indirectly implicated the CD163 receptor with the resolving phase of inflammation. 2,19–22 In this...
study we demonstrate for the first time that Hb:Hp ligand binding to CD163 on human monocyte-macrophages isolated in vitro and in vivo elicits a direct antiinflammatory effect via the secretion of IL-10. Neither Hb nor Hp alone significantly induced IL-10. This is consistent with previous work showing that Hb:Hp recognition and clearance by CD163 depends on a prior conformational change to Hp triggered by the binding of free Hb.3 Because IL-10 is known to upregulate CD163,19,20 it is likely that IL-10 synthesis will amplify Hb:Hp scavenging via a positive feedback loop on receptor expression. The signaling mechanisms involved in the release of IL-10 are the subject of ongoing investigations, but the slow kinetics of secretion observed suggest that they will be distinct from CD163-associated calcium flux and inositol trisphosphate events previously described.36

Our studies have also described for the first time an inhibitory anti-CD163 antibody, RM3/1. This antibody is a potent blocker of Hb:Hp complex binding and IL-10 secretion. Previously, only antibody clone EDHU-1 had been assigned functional properties, inasmuch as it was shown to transmit activating signals on cross-linking of CD163.36 Coincidentally, we also identify clone Ki-m8 as an agonistic antibody.

The present communication identifies IL-10 as a new link between HO-1 synthesis in macrophages and Hb:Hp binding via CD163. This link may enable macrophages to coordinate heme scavenging and breakdown with antiinflammatory activity. Thus, not only can cells effectively scavenge and metabolize Hb:Hp complexes from their environment via CD163 and HO-1, they can also exert antiinflammatory and cytoprotective effects via the release of IL-10 and the heme metabolite CO.

The human skin blister data confirm and extend the in vitro monocyte-derived macrophage data in two ways. First, CD163 expression is upregulated on transmigrated monocytes on differentiation into macrophages, and this process occurs more quickly in vivo (relative fluorescent intensity of 32.7 at 40 hours) than in vitro (relative fluorescent intensity of 36.5 at 7 days). Second, CD163+ cells from resolving blisters secrete IL-10 when challenged with Hb:Hp. The appearance of functional CD163 receptors in resolving blisters therefore suggests that antiinflammatory scavenging of Hb:Hp may play a key role in wound healing after trauma or surgery where extravascular hemolysis occurs in wound hematomas.

Interestingly, intimal Hb:Hp scavenging may attenuate the inflammatory mechanisms underlying atherosclerosis. CD163 is expressed on macrophages in atherosclerotic plaques,37 and free Hb, particularly when glycosylated in diabetes, promotes atherogenesis by oxidizing low-density lipoprotein.
lipoproteins. Recent epidemiological studies have shown that susceptibility to cardiovascular disease in diabetes is markedly influenced by Hp phenotype and clearance rate of Hb:Hp complexes by CD163. We suggest that the upregulation of CD163 in human monocytes is a response to the stabilization and removal of pro-oxidant Hb from the vessel wall by Hp and CD163 but also on the subsequent induction of antiinflammatory pathways through IL-10 and HO-1.

Our study reveals for the first time an increase in CD163 expression on circulating human monocytes 24 hours after the onset of CPB surgery, a time when resolution of the inflammatory response to bypass is apparent. Interestingly, this upregulation of CD163 may be linked to a prior surge in plasma IL-10 that we observed at 90 minutes after the onset of CPB (data not shown) and consistent with previous studies. Also, we demonstrate a significant induction of CD163 and HO-1 on circulating monocytes after CPB surgery. Based on both the time course of this response and our in vitro findings, it is possible that this occurs secondary to CD163 upregulation and Hb:Hp complex scavenging. Therefore, expression of CD163 and HO-1 on circulating monocytes after CPB may play a dual role in patient recovery after surgery, first, through a direct effect by the endocytic clearance and catabolism of potentially nephrotoxic, proinflammatory free Hb from the circulation, in association with Hp, and, second, through an indirect antiinflammatory effect promoting resolution of the systemic inflammatory response and wound healing. It is possible that clinical outcome after CPB surgery in the future could be improved by manipulation of the inflammatory response before surgery. The principle of such an intervention has already been proven with the demonstration that transplant-associated arteriosclerosis can be ameliorated by prior exposure to CO. An approach relevant to CPB might be to prime patients with CABG to express CD163-bearing cells before surgery by administering polarizing cytokines, such as IL-4 or IL-10, or corticosteroids, thus promoting heme catabolism and antiinflammatory wound healing.

In conclusion, we have identified novel antiinflammatory and cytoprotective effector pathways downstream of the hemoglobin scavenger receptor CD163. These pathways may enable CD163+ monocytes/macrophages to directly ingest and metabolize Hb:Hp complexes from their environment via CD163 and HO-1, respectively, while exerting antiinflammatory and atheroprotective effects via the release of IL-10 and the heme metabolite CO. Hb scavenging may thus act as a molecular switch to promote inflammatory resolution and wound healing, with therapeutic implications for wound healing and the prevention or treatment of atherosclerosis.

Acknowledgments
This study was supported by the British Heart Foundation.

References
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_Circ Res._ 2004;94:119-126; originally published online December 1, 2003; doi: 10.1161/01.RES.0000109414.78907.F9

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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