Hypoxia Induces Netrin-1 and Unc5b in Atherosclerotic Plaques
Mechanism for Macrophage Retention and Survival


Objective—Hypoxia is intimately linked to atherosclerosis and has become recognized as a primary impetus of inflammation. We recently demonstrated that the neuroimmune guidance cue netrin-1 (Ntn1) inhibits macrophage emigration from atherosclerotic plaques, thereby fostering chronic inflammation. However, the mechanisms governing netrin-1 expression in atherosclerosis are not well understood. In this study, we investigate the role of hypoxia in regulating expression of netrin-1 and its receptor uncoordinated-5-B receptor (Unc5b) in plaque macrophages and its functional consequences on these immune cells.

Approach and Results—We show by immunostaining that netrin-1 and Unc5b are expressed in macrophages in hypoxia-rich regions of human and mouse plaques. In vitro, Ntn1 and Unc5b mRNA are upregulated in macrophages treated with oxidized low-density lipoprotein or inducers of oxidative stress (CoCl₂, dimethyloxalylglycine, 1% O₂). These responses are abrogated by inhibiting hypoxia-inducible transcription factor (HIF)-1α, indicating a causal role for this transcription factor in regulating Ntn1 and Unc5b expression in macrophages. Indeed, using promoter-luciferase reporter genes, we show that Ntn1- and Unc5b-promoter activities are induced by oxidized low-density lipoprotein and require HIF-1α. Correspondingly, J774 macrophages overexpressing active HIF-1α show increased netrin-1 and Unc5b expression and reduced migratory capacity compared with control cells, which was restored by blocking the effects of netrin-1. Finally, we show that netrin-1 protects macrophages from apoptosis under hypoxic conditions in a HIF-1α-dependent manner.

Conclusions—These findings provide a molecular mechanism by which netrin-1 and its receptor Unc5b are expressed in atherosclerotic plaques and implicate hypoxia and HIF-1α–induced netrin-1/Unc5b in sustaining inflammation by inhibiting the emigration and promoting the survival of lesional macrophages. (Arterioscler Thromb Vasc Biol. 2013;33:1180–1188.)

Key Words: apoptosis ■ guidance ■ HIF-1 ■ macrophage ■ migration ■ Ntn1

Atherosclerosis is a disease of chronic inflammation that is distinguished by the persistence of cholesterol-engorged macrophages in arterial plaques, leading to disease progression and complications.1 These cells are the central mediators of the establishment, progression, and, ultimately, instability of atherosclerotic plaques. Unlike in other tissues, macrophages that accumulate in plaques seem to have a diminished capacity to migrate,2–5 and go from being chemotactic to chemostatic, thereby contributing to a failure to resolve the inflammatory process in arteries set in motion by the retention of atherogenic lipoproteins. Although the regulatory signals that impair this process remain largely unknown, we recently showed that netrin-1, a laminin-like molecule normally expressed during embryonic development to guide the movement of neurons, is secreted by macrophage foam cells and inhibits the emigration of these cells from atherosclerotic plaques.6 In the developing nervous system, netrin-1 can act as either a positive or negative regulator of migration depending on receptor expression by the target cell and can also serve as a cell survival cue.7 We found that netrin-1 differentially regulates cellular constituents of atheroma: netrin-1 blocks the migration of macrophages expressing the uncoordinated-5-B receptor (Unc5b) receptor to chemokines (CCL2, CCL19, CCL21) implicated in the egress of macrophages from plaques, while promoting chemotraction of smooth muscle cells expressing the neogenin receptor.8 These combined effects of netrin-1, macrophage retention and smooth muscle cell recruitment, would be predicted to

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increase plaque size. Notably, targeted deletion of netrin-1 in myeloid cells markedly reduced atherosclerosis burden in \( \text{Ldlr}^{-/-} \) mice and promoted macrophage emigration from plaques. These findings linked netrin-1 in macrophages to the promotion of chronic inflammation; however, the mechanisms regulating expression of this guidance cue in atherosclerotic plaques are poorly understood.

The progression of atherosclerotic lesions is closely associated with microenvironmental changes within lipid- and macrophage-rich areas, particularly shifts in oxygen supply, which can lead to hypoxia. As plaques grow bigger, the oxygen supply is decreased because of both diffusional limitations and the higher demand for oxygen by highly metabolically active cells within the plaque, leading to regions of hypoxia in both human and mouse atheroma. Furthermore, hypoxia has become recognized as a primary impetus of inflammation, and because inflamed lesions often become hypoxic, this further amplifies the inflammatory milieu of the plaque. A recent study reported that netrin-1 is induced by ambient hypoxia in mucosal surfaces of the intestine, where it protects the tissue from transient ischemia by attenuating neutrophil transepithelial migration. In this setting, epithelial expression of netrin-1 was regulated by the hypoxia-inducible transcription factor (HIF) and is a well-known sensor and mediator of hypoxic responses. HIF is a heterodimeric protein that consists of 2 subunits: a constitutively expressed HIF-1 \( \beta \) subunit, and a HIF-1\( \alpha \) or HIF-2\( \alpha \) subunit, whose protein levels are regulated by oxygen concentration. HIF-1\( \alpha \) and -2\( \alpha \) are rapidly degraded by the ubiquitin/proteasome pathway in the presence of oxygen, but under hypoxic conditions these proteins translocate to the nucleus, dimerize with HIF-1\( \beta \), and induce transcription of genes that coordinate cellular adaptations to hypoxia.

In atherosclerosis, HIF-1\( \alpha \) is detected in macrophage-rich and hypoxic regions of human and mouse plaques. HIF-1\( \alpha \) has been reported to regulate several gene programs relevant to atherosclerosis, including angiogenesis, glucose metabolism, apoptosis, nitric oxide metabolism, and the inflammatory response. Furthermore, HIF-1\( \alpha \) in the arterial wall may be stabilized under normoxic conditions by inflammation, particularly by inflammatory lipids that accumulate in plaques, such as oxidized low-density lipoprotein (oxLDL). We thus postulated that hypoxic stress may promote netrin-1 expression in macrophages in atherosclerotic plaques, thereby increasing macrophage retention and chronic inflammation. We show herein that netrin-1 colocalizes with regions of hypoxia and HIF-1\( \alpha \) accumulation in the arterial wall, most prominently in macrophages. In vitro, netrin-1 and its receptor Unc5b are induced in macrophages by hypoxic stressors, including oxLDL, and these responses are dependent on HIF-1\( \alpha \). Overexpression of an active form of HIF-1\( \alpha \) reduces macrophage migration, and this is reversed by blocking netrin-1, providing a mechanism for macrophage chemostasis during hypoxia. Finally, we show that netrin-1 also promotes the survival of macrophages under hypoxic conditions, which combined with its effects on macrophage retention, would be expected to fuel chronic inflammation in the plaque.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Hypoxia Correlates With Netrin-1 Expression in Atherosclerotic Plaques

Because netrin-1 expression has been shown to be upregulated in models of transient ischemia, we investigated whether

Figure 1. Hypoxia correlates with netrin-1 expression in mouse and human atherosclerotic plaques. A and B. Immunofluorescence staining of (A) macrophage CD68 (red) or (B) netrin-1 (red) in aortic root plaques from \( \text{Ldlr}^{-/-} \) mice fed a Western diet for 12 weeks and injected with a hypoxia probe (green) that detects hypoxic regions. Areas of colocalization are shown in yellow in the merged image (arrows). Scale bar, 100 \( \mu \)m. The inset is a x10 zoom of the area of interest. L indicates the lumen. Images are representative of \( n \geq 3 \) mice.
netrin-1 is expressed in hypoxic regions of atherosclerotic plaques. We performed immunofluorescence staining of aortic sinus plaques from Ldlr<sup>−/−</sup> mice fed a Western diet for 12 weeks and injected with a hypoxia probe (pimonidazole hydrochloride). Using double immunostaining on serial sections, we noted that the macrophage marker cluster of differentiation 68 (CD68) colocalized with the hypoxia probe (Figure 1A). Furthermore, netrin-1 was prominently expressed in these regions of the plaque and showed significant colocalization with the hypoxia probe (Figure 1B).

**HIF-1α–Mediated Induction of Ntn1 in Macrophages**

To examine the role of hypoxia in the induction of the netrin-1 gene, *Ntn1*, we incubated bone marrow–derived macrophages (BMDMs) at a level of oxygen of 1%, which has previously been demonstrated in intraplaque regions. Macrophages incubated under these conditions showed a 4-fold increase in *Ntn1* mRNA, as well as the hypoxia-inducible gene *Vegf* (Figure 2A). Similar results were seen under normoxic conditions in macrophages treated with cobalt chloride (CoCl<sub>2</sub>), which mimics hypoxia by stabilizing HIF-1α<sup>−/−</sup> and with dimethyloxalylglycine (DMOG),<sup>18</sup> which increases endogenous levels of this transcription factor by inhibiting the prolyl hydroxylases (Figure 2B). Furthermore, as seen with the chemical inducers of hypoxia, oxLDL, which is believed to promote oxidative stress in the artery wall,<sup>19</sup> also increased both *Ntn1* and *Vegf* mRNA (Figure 2C; Figure 1A in the online-only Data Supplement) and increased netrin-1 protein (Figure 2D) in macrophages.

**Figure 2.** Mediators of hypoxic stress induce netrin-1 (*Ntn1*) in macrophages. A and B, Quantitative polymerase chain reaction (qPCR) analysis of *Ntn1* and *Vegf* mRNA in bone marrow–derived macrophages (BMDMs) (A) exposed to 1% oxygen (hypoxia) or (B) stimulated with dimethylxalyglycine (DMOG; 1 mmol/L) and CoCl<sub>2</sub> (0.1 mmol/L) for 24 hours. C, qPCR analysis of *Ntn1* and *Vegf* mRNA expression in peritoneal macrophages treated with oxidized low-density lipoprotein (oxLDL) with or without hypoxia-inducible transcription factor (HIF-1α) inhibitor (100 μmol/L). D, Western blot of netrin-1 in BMDMs treated with 50 μg/mL oxLDL or 1% oxygen. E, qPCR analysis of *Ntn1* mRNA in BMDMs under normoxic or hypoxic conditions with or without HIF-1α inhibitor (100 μmol/L). F, Netrin-1 promoter–luciferase reporter activity in HEK293 cells treated with oxLDL (50 μg/mL) in the presence/absence of the HIF-1α inhibitor (100 μmol/L). G, qPCR analysis of *Ntn1* mRNA in macrophages isolated from normoxic and hypoxic regions of the plaques of Ldlr<sup>−/−</sup> mice reconstituted with wild-type (WT) or macrophage-specific Hif1a<sup>−/−</sup> (Mac-Hif1a<sup>−/−</sup>) bone marrow (n=4 mice/group). Data in A to F are the mean±SD of triplicate samples in a single experiment and are representative of 3 to 4 independent experiments. *P<0.05.
molecules, we used a mouse J774 macrophage cell line transduced with a retrovirus expressing HIF-1α engineered to be stable in normoxic conditions.13 Gene expression profiling of J774-con and J774-HIF-1α macrophages, using a custom microarray for the analysis of guidance cues of the netrin, slit, semaphorin, and ephrin families and their receptors, showed that overexpression of HIF-1α induced expression of Ntn1 and its chemorepulsive receptor Unc5b but decreased expression of its chemoattractant receptor Dcc (Figure 3A, 3B, and 3D). Notably, J774-HIF-1α cells also showed decreased expression of the adenosine A2a and A2b receptors (Adora2a, Adora2b), which have previously been implicated in the neutrophil response to netrin-1 during transient ischemia.10 To confirm these changes at the protein level, we performed immunofluorescent staining of J774-HIF-1α and control macrophages. Notably, J774-HIF-1α but not J774-Con cells showed abundant netrin-1 and Unc5b staining (Figure 3C), indicating that netrin-1 and Unc5b are regulated by a common mechanism during hypoxia.

To further understand the molecular mechanisms regulating the Unc5b receptor in hypoxic conditions, we incubated macrophages with 1% oxygen or under normoxic conditions with oxLDL or chemical hypoxia mimics. As seen for Ntn1 and Vegf, macrophages incubated under these conditions showed 3- and 6-fold increases in Unc5b mRNA (Figure 4A–4C; Figure ID in the online-only Data Supplement). This increase in Unc5b mRNA was dependent on HIF-1α because macrophages pretreated with a HIF-1α inhibitor show no upregulation of Unc5b in response to oxLDL, low oxygen concentration, CoCl₂, or DMOG (Figure 4C; Figure IE in the online-only Data Supplement). Furthermore, BMDMs incubated with oxLDL or under hypoxic conditions showed increases in Unc5b protein (Figure 4E). To confirm the role of HIF-1α in the transcriptional regulation of Unc5b, we transfected HEK293 cells with an Unc5b promoter-luciferase reporter gene. OxLDL dose-dependently increased luciferase activity in these cells (Figure 4F), and treatment with a HIF-1α inhibitor abrogated this increase in Unc5b promoter activity.

**Figure 3.** Hypoxia induces select guidance cues and receptors in macrophages. A and B, mRNA profiling of J774 macrophages overexpressing a stable form of hypoxia-inducible transcription factor (HIF)-1α (J774-Hif) vs J774 control (J774-Con) cells using a custom quantitative real-time polymerase chain reaction (qRT-PCR) array for the neuronal guidance cue family members (n=3) expressed as (A) a heat map or (B) relative fold change in mRNA expression. C, Immunofluorescence staining of J774-Con and J774-Hif cells for HIF-1α, netrin-1, and uncoordinated-5-B receptor (Unc5b). Scale bar, 10 μm. D, qPCR validation of mRNA expression of Ntn1 and its receptors in J774-Hif compared with J774-con macrophages. Data are mean±SD of triplicate samples in a single experiment and are representative of an experimental n=4. *P<0.05.
Figure 4. Mediators of hypoxic stress induce uncoordinated-5-B receptor (Unc5b) in macrophages. Quantitative polymerase chain reaction analysis of Unc5b, Ntn1, or Vegf mRNA in bone marrow-derived macrophages (BMDM) treated with (A) dimethyloxalylglycine (DMOG; 1 mmol/L) and CoCl₂ (0.1 mmol/L), (B) 1% oxygen, or (C) 50 μg/mL oxidized low-density lipoprotein (oxLDL), and (D) and (E) with or without hypoxia-inducible transcription factor (HIF)-1α inhibitor (100 μmol/L). Western blot of Unc5b in BMDM treated with 50 μg/mL oxLDL or 1% oxygen, or (F) Unc5b promoter-luciferase reporter activity in HEK293 cells treated with (G) oxLDL (50 μg/mL) or (H) DMOG (1 mmol/L) in the presence/absence of an NFkB inhibitor (BAY11-7082). Data are mean±SD of triplicate samples in a single experiment and are representative of 3 independent experiments. *P<0.05.

(Figure 4G). A similar HIF-1α-dependent increase in Unc5b promoter activity was also observed in cells treated with DMOG or CoCl₂ under normoxic conditions (Figure 4H), and this was also dependent on NFkB (Figure 4I). Consistent with these in vitro studies, immunofluorescent staining of mouse atherosclerotic plaques showed colocalization of Unc5b with a hypoxia probe (Figure 5A). Furthermore, macrophages isolated by laser capture microdissection from hypoxic regions of plaques Ldlr⁻/⁻ mice show a 5-fold increase in Unc5b mRNA compared with macrophages from normoxic regions (Figure 5B). By contrast, no increase in Unc5b mRNA was measured in macrophages isolated from hypoxic regions of plaques from Ldlr⁻/⁻Mac-Hif1a⁺/⁻ mice (Figure 5B). Collectively, these data suggest a key role for HIF-1α in the upregulation of both netrin-1 and its receptor Unc5b in plaque macrophages, which would be predicted to promote an autocrine/paracrine signaling loop.

Netrin-1 and Unc5b Are Increased by Hypoxia in Human Monocytes and Atherosclerotic Plaques

To extend these findings in mouse atherosclerotic models to human atherosclerosis, we first treated the human monocytic

Figure 5. Uncoordinated-5-B receptor (Unc5b) is expressed in hypoxic-rich regions in mouse and human atherosclerotic lesions. Immunofluorescence staining of (A) Unc5b (red) in aortic root plaques from Ldlr⁻/⁻ mice fed a Western diet for 12 weeks and injected with a hypoxia probe (green) to detect hypoxic regions. Areas of colocalization are shown in yellow in the merged image (arrows). Scale bar, 100 μm. The inset is a ×10 zoom of the area of interest. L indicates the lumen. Images are representative of n=3 mice. B, Quantitative polymerase chain reaction analysis of Unc5b mRNA in macrophages isolated from normoxic and hypoxic regions of the plaques of Ldlr⁻/⁻ mice reconstituted with wild-type (WT) or macrophage-specific Hif1a⁺/⁻ (Mac-Hif1a⁺/⁻) bone marrow (n=4 mice/group). Data are mean±SD, *P<0.05.
cell line THP-1 with oxLDL under normoxic conditions or subjected the cells to hypoxic conditions by incubating them in 1% oxygen. As seen in mouse macrophages, these conditions induce 4- to 5-fold increases in NTNI (Figure 6A) and UNC5B (Figure 6B) mRNA. The upregulation of NTNI and UNC5B by oxLDL or hypoxia was reduced by treating cells with an inhibitor of HIF-1α (Figure 6A and 6B). We next stained human carotid endarterectomy sections for netrin-1, Unc5b, and HIF-1α. HIF-1α was detected in the central region of human plaques, in areas maximally distant from the blood supply (Figure 6C and 6D) and staining for netrin-1 and Unc5b colocalized with HIF-1α in these regions. These data suggest a common mechanism of HIF-1α–dependent regulation of netrin-1 and Unc5b in macrophages in mouse and human atherosclerosis.

**Netrin-1 Reduces Macrophage Migration and Promotes Survival**

Because netrin-1 secreted during hypoxia may act in an autocrine/paracrine manner on Unc5b-expressing macrophages, we next examined the functional consequences of this regulation. To understand the role of netrin-1 in regulating macrophage migration under hypoxic conditions, we measured the chemotaxis of control or CoCl2-treated BMDM to monocyte chemotactic protein (MCP-1 or CCL2) in the presence of recombinant Unc5b-FC to block the effects of netrin-1. Macrophages treated with CoCl2 were strongly impaired in their ability to migrate to MCP-1 compared with control macrophages (Figure 7A). Notably, treatment of macrophages with Unc5b-FC reversed this effect, restoring macrophage migration to MCP-1. Similarly, overexpression of stable HIF-1α, which induces netrin-1 expression, reduced the migratory capacity of J774 macrophages in the absence of exogenous chemokine (Figure 7B), whereas J774-HIF cell treated with Unc5b-FC exhibited migration levels comparable with control J774 macrophages. Together these data indicate that upregulation of netrin-1 and Unc5b by hypoxic stress promotes macrophage chemostasis.

In addition to regulating migration, netrin-1 has also been reported to be a survival factor for neurons.20,21 To test whether netrin-1 inhibits macrophage apoptosis under hypoxic conditions, we measured apoptosis of growth factor–deprived BMDM under normoxic and hypoxic conditions. Primary BMDM cultivated in the absence of L929 conditioned media undergo apoptosis under normoxic conditions, with a 5-fold increase in transferase dUTP nick end labeling (TUNEL) staining observed after 3 days (Figure 7C). However, these cells are largely protected from this apoptosis, as shown by reduced TUNEL staining when cultured under hypoxic conditions, and this protection from apoptosis is reversed in the presence of a HIF-1α inhibitor. To determine whether HIF-1α induction of netrin-1 contributes to macrophage survival under hypoxic conditions, we repeated this experiment in the presence of recombinant Unc5b-FC to block the effects of netrin-1 or a control FC fragment. Under hypoxic conditions, growth factor–deprived macrophages treated with control FC

**Figure 6.** Netrin-1 (Ntn1) and uncoordinated-5-B receptor (Unc5b) are induced by hypoxic stress in human monocytes and colocalize with hypoxia-inducible transcription factor (HIF)-1α in human atheroma. Quantitative polymerase chain reaction analysis of (A) Ntn1 and (B) Unc5b mRNA in human THP-1 cells treated with 50 μg/mL oxidized low-density lipoprotein (oxLDL) under normoxic conditions or exposed to 1% oxygen (hypoxia) for 24 hours, in the presence or absence of a HIF-1α inhibitor (100 μM/L). Data are mean±SD of triplicate samples in a single experiment and are representative of 3 independent experiments, *P<0.05. C and D, Immunofluorescence staining of human carotid plaques for (C) netrin-1 (red), (D) Unc5b (red), and HIF-1α (green). DAPI nuclear stain is in blue. Areas of colocalization are shown in yellow in the merged image (arrows). Scale bar of inset images, 500 μm. Brightfield image on left indicates the region of staining (boxed area) which is in areas maximally distant from the blood supply. Staining is representative of plaques from 5 subjects.
tissues often become hypoxic, fueling a continuous cycle of intertwined: hypoxia can induce inflammation, and inflamed It is now widely accepted that hypoxia and inflammation are characteristic features of atherosclerosis, in which macrophages accumulate prominently in arterial plaques. Our data provide a molecular mechanism for macrophage persistence during hypoxic stress induced by low oxygen concentration or treatment with oxLDL (a key inflammatory component of plaques that increases oxidative

Discussion
It is now widely accepted that hypoxia and inflammation are intertwined: hypoxia can induce inflammation, and inflamed tissues often become hypoxic, fueling a continuous cycle of inflammation. Hypoxia and chronic inflammation are characteristic features of atherosclerosis, in which macrophages accumulate prominently in arterial plaques. Our data provide a molecular mechanism for macrophage persistence during hypoxia through the upregulation of netrin-1, a neuroimmune guidance cue that inhibits chemokine-directed migration of macrophages in vitro and in vivo, including in atherosclerotic plaques. We show that netrin-1 and its receptor Unc5b are expressed in macrophages in hypoxic regions of mouse and human atherosclerotic lesions. Furthermore, HIF-1α, a transcription factor that mediates cellular adaptations to hypoxia, regulates the expression of both netrin-1 and Unc5b, leading to macrophage chemostasis and protection from apoptosis under hypoxic conditions. Thus, the regulation of the netrin-1-Unc5b axis under hypoxic conditions would be predicted to promote the accumulation of these highly metabolically active cells, further amplifying oxidative stress and chronic inflammation in atherosclerosis.

Recent studies indicate that hypoxia plays a key role in the progression of atherosclerotic plaques to advanced stages by increasing lipid accumulation, inflammation, and angiogenesis. The transcription factor HIF-1α is a central regulator of these cellular programs, which help hypoxic cells adapt to their hostile environment. Previous studies from our group reported that similar to human atheroma, hypoxic regions are present in mouse atherosclerotic plaques and show increased accumulation of HIF-1α. In vitro, such hypoxic conditions alter macrophage lipid metabolism by increasing macrophage foam cell formation through both induction of sterol synthesis and suppression of cholesterol efflux through a mechanism involving HIF-1α. Lipid-laden macrophage foam cells are known to have a diminished capacity to migrate thereby promoting macrophage retention in plaques, and in the present study, we identify hypoxia and stabilization of HIF-1α as factors that contribute to this retention by promoting macrophage chemostasis and survival. Hypoxic stress induced by low oxygen concentration or treatment with oxLDL (a key inflammatory component of plaques that increases oxidative
strong upregulated macrophage expression of netrin-1 and its receptor Unc5b. This neuroimmune guidance cue-receptor interaction was recently shown to block macrophage migration to chemokines implicated in the egress of macrophages from plaques or other inflammatory sites (eg, CCL19, CCL2).23–24 Furthermore, targeted deletion of netrin-1 in macrophages reduced atherosclerosis burden in Ldlr−/− mice and restored macrophage emigration from plaques.6 Notably, we now show that macrophages overexpressing HIF-1α or treated with a hypoxia mimic have impaired migratory capacity, and this hypoxia-induced macrophage chemotaxis is reversed by blocking netrin-1. These data suggest that strategies that target hypoxia, thereby reducing netrin-1 and Unc5b, may facilitate macrophage emigration from the plaque and the resolution of inflammation.

In addition to regulating cell migration, as noted above, we now also show that netrin-1 plays an important role in promoting macrophage survival during hypoxic stress. HIF-1α has been reported to promote the survival of neutrophils in hypoxic conditions,25 and we show a similar role for HIF-1α in protecting macrophages from apoptosis during hypoxia. Notably, inhibition of netrin-1 in macrophages in vitro under these conditions resulted in a loss of protection from apoptosis. Although the effect of netrin-1 on leukocyte survival has not been investigated previously, forced expression of deleted netrin-1 has been reported to suppress apoptosis in renal tubular epithelial cells in ischemia/reperfusion injury.26 Deleted in colorectal cancer and UNC5 are thought to initiate cell death in the absence of their ligand, as has been described for other dependence receptors, including the neurotrophin receptor p75NTR and the androgen receptor.31–34

Although the HIF-1α–induced upregulation of netrin-1 and Unc5b in macrophage foam cells in atherosclerotic plaques would be predicted to foster inflammation by both blocking macrophage emigration and sustaining the survival of these trapped cells, other studies report protective roles for HIF-1α and netrin-1 during inflammation. For example, during transient ischemia in the gut, upregulation of netrin-1 on epithelial cells by HIF-1α attenuates neutrophil recruitment and protects the tissue from hypoxia-induced inflammation.10 In this model, neutrophil responses to netrin-1 required the adenosine A2B receptor to limit hypoxia-induced inflammation, but not Unc5b, which we found to be the active receptor in macrophages. In fact, using a custom microarray to measure expression of members of the netrin family and its receptors, we found in macrophages that HIF-1α upregulates netrin-1, Unc5a, and Unc5b, but highly downregulates other netrin-1 receptors, including the adenosine A2B receptor and deleted in colorectal cancer. Consistent with this, Unc5b was detected in macrophages in hypoxic regions of the plaque and colocalized with HIF-1α. In vitro, regulation of the Unc5b promoter was also found to depend on NFκB, a master transcriptional regulator of inflammatory genes, which has been shown to upregulate HIF-1α.35 In this context, increased secretion of netrin-1 and upregulated Unc5b expression by macrophages would be predicted to inactivate emigration and enhance the survival of these immune cells, thereby promoting chronic inflammation. Although not investigated here, netrin-1 can be expressed by endothelial cells,36 and we have previously demonstrated that in this context netrin-1 regulates monocyte adhesion to endothelial cells.27 Because hypoxia may also affect endothelial cells in atheromata, it may potentially alter the expression of netrin-1 on the endothelium. Thus, as in the nervous system where netrin-1 can have both positive and negative effects on axonal migration, netrin-1 may play multifunctional roles in regulating inflammation depending on the site of its expression (ie, plaque versus endothelium or epithelium) and the receptors it engages.

Together, these studies strongly suggest that netrin-1 and its receptor, Unc5b, initially characterized in the specialized migration of neurons during development, also regulate macrophage trafficking and accumulation during hypoxia in atherosclerosis. These findings also directly implicate HIF-1α regulation of netrin-1 in promoting macrophage accumulation and survival in atherosclerotic plaque and provide further evidence of a role for hypoxia in sustaining inflammation.

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Disclosures

None.

References


**Significance**

Our data show that netrin-1 and its receptor Unc5b, initially characterized in the specialized migration of neurons during development, are expressed in macrophages in hypoxic regions of mouse and human atherosclerotic lesions. Furthermore, HIF-1α, a transcription factor that mediates cellular adaptations to hypoxia, regulates the expression of both netrin-1 and Unc5b, leading to macrophage chemostasis and protection from apoptosis under hypoxic conditions. The regulation of the netrin-1-Unc5b axis under hypoxic conditions would be predicted to promote the accumulation of these highly metabolically active cells, further amplifying oxidative stress and chronic inflammation in atherosclerosis. Collectively, these findings implicate HIF-1α regulation of netrin-1 in promoting macrophage accumulation and survival in atherosclerotic plaques and provide further evidence of a role for hypoxia in sustaining inflammation.
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Supplement information

Hypoxia induces netrin-1 and Unc5b in atherosclerotic plaques: a mechanism for macrophage retention and survival

Supplemental Figure I. (a) qPCR analysis of Ntn1 and Vegf mRNA in BMDMs treated with oxLDL for 24 hours. (b) qPCR analysis of Ntn1 mRNA in BMDMs treated with DMOG (1 mmol/L) or CoCl2 (0.1 mmol/L) with or without HIF-1α inhibitor (100 µmol/L). (c) Netrin-1 promoter-luciferase reporter activity in HEK293 cells treated with DMOG (1 mmol/L) in the presence/absence of the HIF-1α inhibitor (100 µmol/L). (d) qPCR analysis of Vegf, Ntn1 and Unc5b mRNA in peritoneal macrophages stimulated with 50µg/ml oxLDL for the indicated times. (e) qPCR analysis of Unc5b mRNA expression in BMDMs stimulated with DMOG (1 mmol/L) and CoCl2 (0.1 mmol/L) with or without HIF-1α inhibitor (100 µmol/L). Data are mean ± s.d. of triplicate samples in a single experiment and are representative of 3-4 independent experiments. *P<0.05.
Materials and Methods

Reagents
OxLDL was prepared by as we described by first dialyzing LDL (0.25 mg/ml) in PBS for 24 hours at 4°C and then incubating with 5µM CuSO₄ for 6 hours at 37°C. To stop the reaction, 0.2 µM EDTA and 50 µM BHT were added. The extent of oxidation of oxLDL was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to the manufacturer’s instructions (CellBiolabs) and was between 15-20 nmol/mg. Cobalt chloride (CoCl₂) and dimethylxoyllyglycine (DMOG) were from Sigma Aldrich (15862 and D3695, respectively). The NFκB inhibitor (Bay11-7082) and HIF-1α inhibitor (400083) were from Calbiochem.

Cell culture
L929 conditioned medium was used as a source of macrophage colony stimulating factor for the differentiation of primary bone marrow derived macrophages (BMDM). Briefly, L929 cells were grown to confluence in DMEM supplemented with 10% FBS, media was changed to DMEM supplemented with 2% FBS and the conditioned media was harvested 3 days later and filtered through a 0.2 µm filter. Bone marrow was flushed from the tibias and femurs of 6-8 week old C57BL/6 mice as we described², and cultured in DMEM supplemented with 15% L929-conditioned media, 10% FBS and 1% penicillin/streptomycin for 7 days. BMDM or human THP-1 monocytes were treated with 50 µg/ml oxLDL or subjected to hypoxic conditions in a Billups-Rothenberg modular incubator chamber at 1% O₂, 5% CO₂ and 94% N₂ for 24 hours at 37°C. J774 control (J774con) and HIF-1α overexpressing (J774Hif) macrophages² were cultured in 5.5 mmol/L glucose containing DMEM supplemented with 15% FBS and 1% penicillin/streptomycin.

Quantitative RT-PCR
Total RNA was isolated using TRIzol reagent (Invitrogen) and RNA (0.5–1 µg) was reverse transcribed with an iScript cDNA Synthesis kit according to the manufacturer’s instructions (Bio-Rad). RT-PCR analysis was performed using iQ SYBR green Supermix (Biorad) and a Mastercycler Realplex (Eppendorf) as we described³. The change in mRNA expression was calculated by the comparative change-in-cycle-method (ΔΔCT) relative to GAPDH mRNA levels. The following primer sets were used:
Mouse Ntn1 5’CAGCCTGATCTTGGTCTCGG3’, 3’GCGGGTTATTTGAGGTCGGTG5’,
Mouse Unc5b 5’CTGGGGACCGGGAAAGAAC3’, 3’CTGATGGGTAGGAGTCTGGG5’,
Mouse Vegf 5’CTCCGCTCTGAAACAAGGCT3’, 3’GCACATAGAGAGAATGAGCTTCC5’
GAPDH 5’AGGTCGGTGGAACCGATTG3’, 3’TGTAGACCATGTAGTTGAGGTCA5’
Human NTN1 5’CTCACACTGTCCCTCGGCAAGAAGT3’,
3’CTCCAGTCGTCCTCGTCTCTCGTC5’, UNc5B
5’CAGCCTTAAGGGTCAAGGTCTACAGCTC3’, 3’TGACTGGAAGCTCTCTCCAAGACC5’.
RT2 Custom Profiler PCR Arrays (Qiagen) were obtained for neuronal guidance molecules and their receptors (Supplemental Table 1). 1 µg of total RNA was reverse transcribed and quantitative RT-PCR analysis was performed according to manufacturer’s protocol. Data analysis was performed using the manufacturer’s integrated web-based software package of the PCR Array System using ΔΔCT based fold-change calculations.

Western Blotting: Protein was extracted in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40) and 60 µg of protein were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, blocked in TBST-5% milk and blotted for Netrin-1 (R&D Systems Inc) (1:500 dilution), Unc5b (Millipore) (1:1000 dilution), GAPDH (1:10000 dilution). Horseradish peroxidase conjugated secondary antibodies followed by SuperSignal West Pico Chemiluminescent Substrate (Pierce 34080) were used and the signals were detected by using LI-COR Odyssey Fc Imaging system.

Immunoflurescent staining
J774con or J774Hif were plated on coverslips and fixed in 4% paraformaldehyde and permeabilised with 0.1% TritonX100 in PBS. Non-specific binding was blocked with 5% BSA for 30 minutes and then coverslips were incubated with anti-HIF-1alpha (Novus Biologica, NB100-479) and anti-netrin-1 chicken polyclonal (Abcam, ab39370), or mouse monoclonal anti-Unc5b antibody (Abcam, ab54430). Secondary fluorescent antibodies used were AlexaFluor 568-anti-rabbit, Alexafluor 488-anti-chicken antibody, or Alexafluor 488-anti-mouse antibodies (Molecular Probes, A11011, A11039, A11008, respectively). Experimental negative controls were incubated in PBS with isotype matched control antibodies (Santa Cruz Biotechnology). Cells were visualized under x40 using a Nikon Eclipse microscope.

**Mouse Atherosclerosis**

C57BL/6 and Ldlr⁻/⁻ mice were from Charles River Laboratories and maintained in a pathogen-free facility. Experimental procedures were done in accordance with the USDA Animal Welfare Act and the PHS Policy for the Human Care and Use of Laboratory Animals and New York University School of Medicine’s Subcommittees on Research Animal Care and Use. Eight-week old Ldlr⁻/⁻ mice from Jackson Laboratories were fed a Western diet (WD; 21% [wt/wt] fat, 0.3% cholesterol; Research Deyet) for 12 weeks. Mice were injected prior to sacrifice with a hypoxia probe (hpi, Hypoxia probe, Inc) at a dose of 60mg/kg i.p. Mice were anesthetized with tribromoethanol (0.4 mg/g i.p.) and ex-sanguninated by cardiac puncture. Aortas were flushed with PBS and perfused with 10% sucrose. Aortic roots were embedded in OCT medium, snap frozen and cross-sections (7 µm thick) were taken for analysis. For immunofluorescent staining, sections were fixed with ice cold acetone for 10 minutes and non-specific binding sites were blocked with 5% BSA for 30 minutes at room temperature. Thereafter, either anti-pimonidazole antibody (clone 4.3.11.3, MAb1) or anti-netrin-1 antibody (Calbiochem, ab-2), or anti-Unc5b antibody (Abcam, ab54430) or anti-CD68 (Lifespan biosciences, LS-C33253) were applied to the sections. After PBS washes, sections were incubated with anti-mouse biotin followed by FITC-conjugated streptavidin to detect the hypoxia probe. The segments were stained with DAPI to detect nuclei and mounted with Dako fluorescent mounting medium (Dako, S3023). The slides were visualized under Nikon Eclipse microscope and images captured under x20 objective. For mice with conditional deletion of HIF1a, lethally irradiated 8 week old Ldlr⁻/⁻ mice were reconstituted with bone marrow (3×10⁵ cells) from Hif1a⁺/⁺/LysMcre/- (Mac-Hif1a⁻/⁻) and Hif1a⁺/⁺ (WT) donor mice. After four weeks recovery, mice were fed a Western diet for 20 weeks and injected with a hypoxiaprope-1 (pimonidazole HCl, hpi-Hypoxia probe, Inc; 1.5 mg/25g mouse) i.v. prior to sacrifice. Laser capture microdissection (Arcturus Bioscience, Mountain View, CA) was carried out on 6-µm frozen sections that were dehydrated in ethanol and xylene and air-dried. At 100-µm intervals, sections were immunostained with a FITC-Hypoxyprobe-1 antibody (Hypoxiaprope-1 plus kit, hpi-Hypoxia probe, Inc) and CD68 primary antibody (ABD Serotec, Raleigh, NC) to identify hypoxic macrophages and normoxic macrophages. These guide sections were used as templates for the isolation of hypoxic and normoxic macrophages for the next five serial sections. RNA was isolated using the PicoPure RNA Isolation Kit (Life technologies, Grand Island, NY) and the concentration and quality of RNA was determined by Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA). RNA was converted to cDNA and amplified using the WT-Ovation Pico RNA Amplification kit (NuGEN, San Carlos, CA) and qRT-PCR was carried out as described above.

**Human atherosclerosis**

The use of human endarterectomy carotid tissue was approved by the New York University School of Medicine IRB. For immunofluorescent staining, sections from 5 subjects were fixed with ice-cold acetone for 10 minutes and permeabilized for 15 minutes with 0.2% Triton X-100 in PBS. Non-specific binding sites were blocked with 5% BSA for 30 minutes at room temperature. Thereafter, either goat anti-netrin-1 antibody (R&D Systems, AF 1109), or mouse monoclonal anti-Unc5b antibody (Abcam, ab54430) or rabbit polyclonal anti-HIF1-a (Novus Biologicals, NB100-479) were applied to the sections. After PBS washes, Alexafluor 568 or 488-coupled secondary antibodies (Molecular Probes, A11057, A11034, A11004) were applied for one hour.
each at room temperature. The segments were stained with DAPI to detect nuclei and mounted with Dako fluorescent mounting medium (Dako, S3023). The slides were visualized under Nikon Eclipse microscope and images captured under x20 objective.

**Promoter luciferase assays**

Human NTN1 or UNC5B promoter-luciferase reporter plasmids (Switchgear Genomics) were transfected into HEK293T using lipofectamineTM 2000 (11668-019, Invitrogen). After transfection, media was changed and cells were treated with oxLDL (50 µg/ml) or CoCl2 (0.1 mM), or DMOG (1 mmol/l) for 24 hours, in the presence or absence (PBS control) of inhibitors of NFκB (10 µmol/L Bay11-7082) or HIF1a (100 µmol/L HIF-1α inhibitor). Luciferase activity was measured using the DUAL-Glo Luciferase Assay system (E1910, Promega) and normalized to a constitutively expressed renilla reporter or to protein concentration.

**Migration assays**

Macrophage chemotaxis was measured using the Real-Time Cell Invasion and Migration xCelligence Assay System with monitoring every 5 min (Roche Applied Science). BMDM migration towards MCP-1 (100 ng/ml; R&D Systems) was measured in the presence or absence of oxLDL (50 µg/ml) and/or recombinant rat UNC5b-Fc chimera (1006-UN; R&D Systems) to block the effects of netrin-1. In a subset of experiments, cells were pre-treated with 0.1 mmol/L CoCl2 for 1 h prior to migration. J774 macrophage migration was carried out in the presence or absence of UNC5b-Fc chimera. Results of chemotaxis assays are representative of at least three independent experiments performed on triplicate samples.

**Apoptotic assays**

To induce apoptosis, BMDMs were grown in the absence of L929 conditioned media (CM) for 3 days under normoxic or hypoxic conditions. In some assays, cells were treated with 100 µmol/L HIF-1α inhibitor or Unc5b-FC fragment or control IgG. After treatment, apoptotic cells were labeled with PE-active caspase-3 (557091, BD pharimingen) or by the TUNEL method using an in situ cell detection kit (Roche Diagnostics). Active Caspase-3, TUNEL and DAPI staining were analyzed with a Nikon Eclipse fluorescent microscope. Only TUNEL positive cells that colocalized with DAPI stained nuclei were considered apoptotic.

**Statistical analysis**

The difference between two groups was analyzed by two-tailed Student’s t-test or for multiple comparisons, by one-way analysis of variance. A P value of <0.05 was considered significant.

**References:**