Journal of the American College of Cardiology Vol. 51, No. 13, 2008

Published by Elsevier Inc.
ISSN 0735-1097/08/$34.00
doi:10.1016/j.jacc.2007.12.025

Hypoxia, Hypoxia-Inducible Transcription Factor, and Macrophages in Human Atherosclerotic Plaques Are Correlated With Intraplaque Angiogenesis

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Objectives We sought to examine the presence of hypoxia in human carotid atherosclerosis and its association with hypoxia-inducible transcription factor (HIF) and intraplaque angiogenesis.

Background Atherosclerotic plaques develop intraplaque angiogenesis, which is a typical feature of hypoxic tissue and expression of HIF.

Methods To examine the presence of hypoxia in atherosclerotic plaques, the hypoxia marker pimonidazole was infused before carotid endarterectomy in 7 symptomatic patients. Also, the messenger ribonucleic acid (mRNA) and protein expression of HIF1α, HIF2α, HIF-responsive genes (vascular endothelial growth factor [VEGF], glucose transporter [GLUT]1, GLUT3, hexokinase [HK]1, and HK2), and microvessel density were determined in a larger series of nondiseased and atherosclerotic carotid arteries with microarray, quantitative reverse transcription polymerase chain reaction, in situ hybridization, and immunohistochemistry.

Results Pimonidazole immunohistochemistry demonstrated the presence of hypoxia, especially within the macrophage-rich center of the lesions. Hypoxia correlated with the presence of a thrombus, angiogenesis, and expression of CD68, HIF, and VEGF. The mRNA and protein expression of HIF, its target genes, and microvessel density increased from early to stable lesions, but no changes were observed between stable and ruptured lesions.

Conclusion This is the first study directly demonstrating hypoxia in advanced human atherosclerosis and its correlation with the presence of macrophages and the expression of HIF and VEGF. Also, the HIF pathway was associated with lesion progression and angiogenesis, suggesting its involvement in the response to hypoxia and the regulation of human intraplaque angiogenesis. (J Am Coll Cardiol 2008;51:1258–65) © 2008 by the American College of Cardiology Foundation

Human atherosclerotic plaques demonstrate extensive intraplaque angiogenesis, which is associated with plaque growth and instability (1–3). However, the molecular pathways initiating intraplaque angiogenesis in atherosclerotic lesions have not been revealed. Because angiogenesis is a major consequence of hypoxic tissue, the mere presence of intraplaque angiogenesis suggests the existence of hypoxia in human atherosclerosis. Evidence of hypoxia in human vascular disease is supported by the in vivo detection of hypoxia in macrophage regions in rabbit atherosclerosis (4) and the expression of several hypoxia-induced genes in atherogenesis (5). Nevertheless, hypoxia per se has not yet been demonstrated in human atherosclerosis.

See page 1266

Two well-known sensors and mediators of the hypoxic response are the hypoxia-inducible transcription factors (HIF) 1 and 2. The heterodimer protein consists of

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Manuscript received July 31, 2007; revised manuscript received November 20, 2007, accepted December 10, 2007.

2 subunits: a HIF1β subunit, which is constitutively expressed, and a HIF1α or HIF2α subunit, whose protein levels are highly regulated by the oxygen concentration (6). The HIF1α and -2α proteins are rapidly degraded by the ubiquitin/proteasome pathway in the presence of oxygen. Upon inhibition of an HIFα subunit degradation by hypoxia, the protein translocates to the nucleus, dimerizes with HIF1β, and induces transcription of hypoxia-responsive genes involved in angiogenesis and glucose metabolism (i.e., vascular endothelial growth factor [VEGF], glucose transporter [GLUT]1 and GLUT3, and hexokinase [HK]1 and HK2) (6).

Although it is widely accepted that HIFα expression is mainly regulated at the protein level, quantitative and qualitative differences exist between HIF1α and -2α messenger ribonucleic acid (mRNA) expression (7). In addition, some studies indicate a transcriptional regulation of HIF (8,9). A possible role for HIF in atherosclerosis is supported by the presence of intraplaque angiogenesis and the implication of several known HIF-responsive genes in atherosclerosis, such as VEGF (5,6), endothelin-1 (6,10), and matrix-metalloproteinase-2 (6,11). However, the association of hypoxia and the HIF pathway with human atherosclerosis has not yet been established. In the present study, we tested the hypotheses that hypoxia is present in human atherosclerosis and associated with the HIF pathway and that the HIF pathway is associated with progression and angiogenesis of human atherosclerosis.

Methods

Detection of hypoxia in human atherosclerosis. Hypoxia was detected in human atherosclerotic carotid arteries with the hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1, Natural Pharmacia Inc., Belmont, Massachusetts). The investigation was approved by an external ethics committee, and written informed consent from patients was obtained. Pimonidazole (0.5 g/m^2) was injected intravenously 2 h before carotid endarterectomy of 7 symptomatic patients (Table 1). Four tissue samples were consecutively removed: 1) arterial wall after incision of the artery; 2) atherosclerotic plaque; 3) arterial wall after removing the plaque; and 4) skin prior to wound closure.

In vivo, pimonidazole is subject to oxidative metabolism and resulting pimonidazole derivatives form protein adducts. Oxygen and pimonidazole compete for electron addition, accounting for the O_2-dependence of pimonidazole adduct formation. Although pimonidazole itself may react with reactive oxygen species (ROS), the antipimonidazole antibody only recognizes hypoxia derivatives. In vivo-formed pimonidazole adducts in hypoxic, but viable cells ($pO_2 \leq 10$ mm Hg ~1% O_2) were detected ex vivo in formalin-fixed, paraffin-embedded tissue by immunohistochemistry with an antibody that only detects hypoxia-derivatives of pimonidazole.

Abbreviations and Acronyms

EC = endothelial cell
GLUT = glucose transporter
HIF = hypoxia-inducible transcription factor
HK = hexokinase
KiEC = proliferating, Ki-67+ endothelial cell
qRT-PCR = quantitative reverse transcription-polymerase chain reaction
ROS = reactive oxygen species
VEGF = vascular endothelial growth factor

Flow cytometry experiments with pimonidazole. Human THP-1 cells (ATCC, TIB-202, Manassas, Virginia) were differentiated into macrophages (Online Appendix) and exposed to an oxygen gradient (0%, 0.2%, 1%, 5%, and 21% O_2; MACS VA500 microaerophilic workstation, Don Whitley Scientific, Shipley, United Kingdom), 100 μmol/l H_2O_2 (Merck, Darmstadt, Germany) or a combination of 0.2% O_2 and H_2O_2 for 15 min, 1 h, and 4 h, all in triplicate. Cells were incubated with and without pimonidazole (100 μmol/l) and stained with fluorescein isothiocyanate-conjugated antipimonidazole (pimo-FITC, 1:1,000; Chemicon, Temecula, Calfifornia). Pimonidazole was quantified in 1×10^4 cells (FACS Calibur) using the excitation geomean at 530 nm (CellQuest, BD-Science, San Jose, California).

Expression of HIF pathway in human atherosclerosis. A total of 10 human caval veins, 15 nondiseased pulmonary or mammary arteries, and 72 atherosclerotic carotid arteries were obtained from patients undergoing vascular surgery (Departments of Surgery, Sittard and Maastricht, the Netherlands) or at autopsy (Department of Pathology, University Hospital Maastricht, Maastricht, the Netherlands) to study the expression pattern of the HIF pathway. Tissue collection was performed in agreement with the "Dutch code of conduct for Observational Research with personal data (2004) and tissue (2001)."

The mean age was 69 ± 11.5 years, and 51% (n = 52) of patients were male. Samples were processed and classified as

Table 1 Clinical Characteristics of Patients Infused With Hypoxia Marker

ID #	Age (yrs)	Gender	BMI (kg/m^2)	Symptoms	Stenosis (%)	Diabetes	Hypertension	Statins	Smoking
1	62	M	29	TIA	80–99	Type 2	–	+	+
2	47	M	31	A. fugax	80–99	—	+	+	+
3	77	M	24	TIA	80–99	—	+	+	–
4	72	F	25	TIA	80–99	—	+	+	+
5	76	F	28	A. fugax	80–99	—	+	+	–
6	59	F	27	Minor stroke	80–99	—	+	+	–

A. fugax = amourosis fugax; BMI = body mass index; TIA = transient ischemic attack.

described previously (12,13) and subdivided as nondiseased, early (intimal thickening or pathological intimal thickening), stable (thin or thick fibrous cap atheroma), or thrombus-containing (luminal thrombus or intraplaque hemorrhage) lesions.

Immunohistochemistry. Sections were stained with primary antibodies against pimonidazole, HIF1α, HIF2α, VEGF, GLUT1, GLUT3, HK1, CD68, Ki-67, CD31, CD34, and activated caspase 3 (Online Table 1), with unrelated immunoglobulin G1 or without the primary antibody (negative controls).

Immunoreactivity in the plaques was scored by one observer (J.C.S) as follows: (0 = none; 1 = mild; 2 = moderate; 3 = extensive). Hematoxylin and eosin-stained sections were used to score fibrosis, thrombus (0 = absent, 1 = present), and angiogenesis (0 = none; 1 = mild; 2 = moderate; 3 = extensive).

In situ hybridization. Ribonucleic acid expression of HIF1α and HIF2α was evaluated in 7-μm sections of 3 carotid arteries with early, stable, or thrombus-containing lesions by in situ hybridization as previously described (14,15).

Microarray. Ribonucleic acid was isolated from 9 early and 6 advanced stable carotid lesions collected at autopsy (12). Samples were individually hybridized to HGU133 2.0 Plus arrays (n = 17, Affymetrix, Santa Clara, California) according to the manufacturer's instructions (Online Appendix). The array represents >54,000 probe sets and 47,000 transcripts (including >38,000 genes). The error model of Rosetta Resolver (Rosetta Biosoftware, Seattle, Washington) was used to determine fold changes between early and stable atherosclerotic lesions, significant when $p < 0.05$. Ingenuity pathway analysis (Ingenuity Systems, Mountain View, California) was performed on genes with a p value of <0.01.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). A comparison of mRNA expression was made between early (n = 5) and stable lesions (n = 5) from autopsy and between stable (n = 4) and thrombus-containing lesions (n = 5) from surgery. Quantitative RT-PCR for HIF1α, HIF2α, VEGF, GLUT1, GLUT3, HK1, HK2, glyceraldehyde-3-phosphate dehydrogenase, and 18S ribosomal ribonucleic acid (Applied Biosystems, Foster City, California) were performed as previously described (12).

Plaque angiogenesis. A computerized morphometric analysis (Leica QWin V3, Cambridge, United Kingdom) of plaque microvessel density (total count/total plaque area) was performed on 6 carotids with intimal thickening and 6 stable and 5 thrombus-containing lesions of post-mortem carotid arteries. A double-staining with Ki-67 and CD31+CD34 was used to quantify proliferating Ki-67+ endothelial cells (KiECs).

Statistical analysis. All data are presented as mean ± SEM. Quantitative RT-PCR, immunoreactivity, and mean vessel diameter were compared between groups with a Mann-Whitney rank-sum test, and Spearman's ρ correlation coefficient was calculated for ordinal immunohistochemistry variables (version 12.0, SPSS Inc., Chicago, Illinois). Bonferroni's multiple testing correction was performed, and results were considered significantly different when $p < 0.05$.

Results

Hypoxia present in human carotid atherosclerosis. The presence of hypoxia in human carotid atherosclerosis was demonstrated by pimonidazole immunoreactivity in all patients injected with pimonidazole (Fig. 1). Hypoxia was present throughout the plaque (n = 27 of 31 segments) of

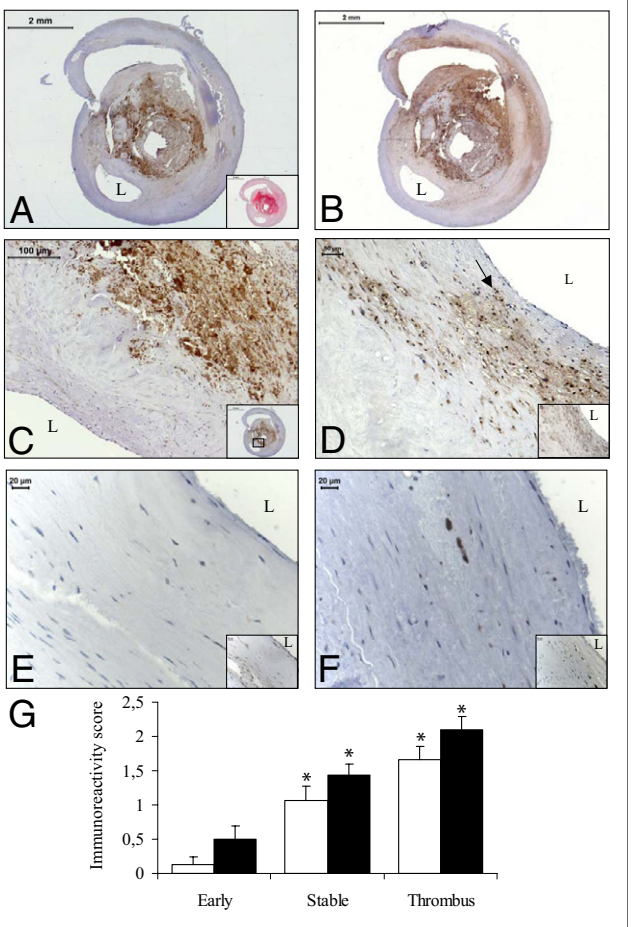

Figure 1 Hypoxia Detection With Pimonidazole in Human Carotid Endarterectomy

(A) Hypoxia (pimonidazole immunoreactivity) is present in the center of an advanced human carotid atherosclerotic plaque, but not in the media. **Inset** shows hematoxylin and eosin staining. **(B)** A serial section of **panel A** shows that CD68-positive macrophages colocalize with hypoxia. **(C)** Macrophage regions of the lesion from **panel A** show extensive hypoxia, whereas the cap shows mild or no hypoxia. **(D)** Hypoxia is present in CD68-positive macrophages **(inset)** at 20 to 30 μm from the lumen. **(E)** Hypoxia is absent in CD68-positive macrophages **(inset)** of a plaque shoulder segment (pathological intimal thickening). **(F)** Hypoxia is present in an atherosclerotic plaque segment with intimal thickening, more specifically in a few CD68-positive macrophages **(inset)**. **(G)** The immunoreactivity score of hypoxia **(open bars)** and CD68 **(solid bars)** is increased in stable and ruptured atherosclerotic lesions versus early lesions (*$p < 0.05$ vs. early; stable vs. ruptured is not significant). L = lumen.

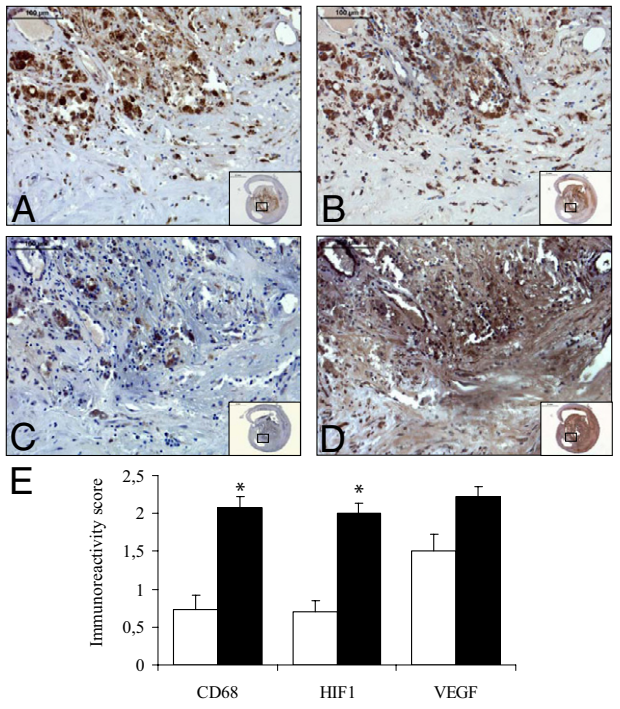

Figure 2 **Hypoxia Colocalizes With CD68, HIF, and VEGF**

(A) Hypoxia detected by pimonidazole immunoreactivity in human carotid atherosclerosis. Serial sections show colocalization with CD68-positive macrophages **(B)**, HIF1α **(C)**, and VEGF **(D)**. **(E)** The immunoreactivity score of CD68, HIF1α, and VEGF is increased when hypoxia is present **(solid bars)** versus absent **(open bars)** in human carotid atherosclerosis (*$p < 0.05$). HIF = hypoxia-inducible transcription factor; VEGF = vascular endothelial growth factor.

5 patients containing advanced atheromas with intraplaque hemorrhage or a luminal thrombus. In 2 patients, hypoxia was present in only 1 segment per plaque (n = 2 of 12). One lesion was a stable fibrocalcified plaque with no or minor inflammation, and the other showed almost undetectable intraplaque hemorrhage (data not shown). Because the use of pimonidazole was previously shown to detect hypoxia in normal skin (16), staining of a skin biopsy confirmed that the pimonidazole injection was successful in all patients (Online Fig. 1). Antibody specificity was confirmed by a complete lack of immunoreactivity in 5 patients without pimonidazole administration (Online Fig. 1).

Hypoxia immunoreactivity colocalized with CD68-positive macrophage foam cells (Figs. 1B, 2A, and 2B) but was also mildly present in smooth muscle cells. Pimonidazole is only metabolized in viable cells, explaining the lack of staining in necrotic or acellular areas. Hypoxia strongly correlated with CD68, angiogenesis and the presence of a thrombus, but not with the presence of fibrosis (Table 2) or apoptosis (data not shown). Two hypoxic gradients were observed as illustrated by Figure 3. Cross-sectionally, hypoxia was very strong in macrophage clusters surrounding the plaque core, but was absent in a 100- to 250-μm rim bordering the lumen (Figs. 1A and 1B) and in the media (Figs. 1A and 1C). However, some isolated macrophages located 20 to 30 μm from the lumen were hypoxic (Fig. 1D). Longitudinally, hypoxia was most intense in parts of the plaque containing an advanced atheroma with a thrombus and inflammation (Figs. 1A and 1G) and mostly absent in shoulder regions with (pathological) intimal thickening despite inflammatory infiltration (Figs. 1E and 1G). However, in a minority of shoulder segments with (pathological) intimal thickening hypoxia was present, but only in macrophages (Fig. 1F). Hypoxia was present in all longitudinal parts of the plaque, predominantly in macrophages, although not all macrophages were hypoxic (Table 3, Fig. 1G).

Hypoxia detection is not influenced by surgery-induced ischemia or ROS. To exclude that pimonidazole immunoreactivity in the atherosclerotic plaque was the result of surgery-induced ischemia, arterial wall segments were collected at 2 time points: directly after incision of the carotid artery and directly after excision of the plaque. Pimonidazole immunoreactivity in these 2 pieces was not different (Figs. 4A and 4B), suggesting that hypoxia and pimonidazole adducts were already present in the plaques before surgery.

Although the antipimonidazole antibody only recognizes hypoxia derivatives, pimonidazole itself may react with ROS. Hypoxia specificity was confirmed in human THP-1 macrophages exposed to hypoxia and/or H_2O_2. Exposure to H_2O_2 induced intracellular antioxidant activity without influencing cell viability (data not shown). Only hypoxic exposure up to 1% O_2 showed pimonidazole-immunoreactive cells (data not shown). Immunoreactivity of combined exposure to hypoxia and H_2O_2 was similar to single hypoxic exposure, showing that the concentration of pimonidazole available for hypoxia detection was not limited by H_2O_2-derivatives of pimonidazole (Fig. 4C).

Colocalization of hypoxia, HIF, and macrophages. Hypoxia typically stabilizes HIF protein, and the colocalization and correlation of hypoxia with HIF1α (Fig. 2C), HIF2α (data not shown), and VEGF (Fig. 2D) suggests this also occurs in atherosclerosis (Table 3). Analogous to hypoxia immunoreactivity, nuclear HIF1α (Figs. 5C and 5D) and

Table 2 **Spearman Correlation Coefficients of Hypoxia Immunoreactivity With Characteristics of Carotid Atherosclerosis**

	Thrombus	Fibrosis	CD68	Angiogenesis	HIF1α	HIF2α	VEGF
Spearman's ρ	0.60*	0.07	0.70*	0.62*	0.83*	0.75*	0.49*

*Bonferroni corrected $p < 0.05$.
HIF = hypoxia-inducible transcription factor; VEGF = vascular endothelial growth factor.

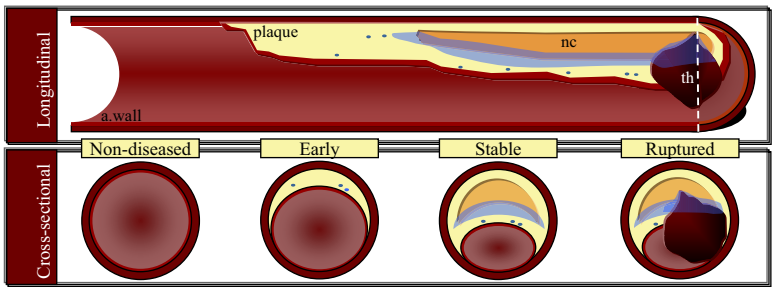

Figure 3 Schematic Illustration of Hypoxia in Atherosclerosis

Illustration of the presence of hypoxia **(blue)** in a longitudinal and cross-sectional representation of atherogenesis. a.wall = arterial wall; nc = necrotic core; th = thrombus.

HIF2α (Figs. 5G to 5H) immunoreactivity and mRNA (Figs. 6B and 6C) were predominantly detected at sites of inflammation. Strong correlations of CD68 immunoreactivity were found with HIF1α (ρ = 0.70, p = 0.007) and HIF2α (ρ = 0.63, p = 0.02).

Hypoxia-inducible transcription factor pathway mRNA and protein are associated with progression from early to advanced atherosclerosis. Microarray, qRT-PCR, and semiquantitative immunohistochemistry showed that mRNA and protein expression of HIF1α, HIF2α, and their targets increased from early to advanced human atherosclerosis. Microarray pathway analysis revealed the significant differential expression of canonical pathways "HIF signaling" (7 of 70 genes, p = 0.02) and "VEGF signaling" (15 of 90 genes, p = 0.02) between early and advanced human atherosclerosis. At the single gene level, HIF1α, VEGF, GLUT3, and HK2 mRNA were significantly up-regulated between early and stable lesions: 1.4-, 1.8-, 2.0-, and 2.3-fold, respectively (Fig. 7A). Microarray results were validated with qRT-PCR with the use of a different set of patient samples, explaining that only VEGF mRNA was significantly upregulated on qRT-PCR (Fig. 7B). However, the same trends were shown using microarray and qRT-PCR. On the other hand, protein expression levels of HIF and HIF-responsive genes were significantly increased with progression of atherosclerosis from early to advanced plaques, except for HK1 (Fig. 8A). The lack of corresponding GLUT1 and GLUT3 mRNA and protein levels may be explained by previous observations of increased translation efficiency and protein stability, respectively. No significant differences of HIF and HIF-responsive genes were seen between the 2 advanced lesion types, stable or thrombus-containing lesions, either on the mRNA level or on the protein level (Figs. 7C and 8A).

Angiogenesis in human carotid atherosclerosis paralleled HIF and VEGF expression. Intraplaque microvessel density increased from early to advanced lesions (p = 0.01) but was not different between stable or thrombus-containing lesions (Fig. 8E). In addition, strong correlations were found between microvessel density and HIF1α (ρ = 0.80, p = 0.001), HIF2α (ρ = 0.77, p = 0.001), and VEGF (ρ =

Table 3 Percentage of Segments Showing Hypoxia or CD68 Immunoreactivity

Lesion type (n)	Hypoxia, % (n)	CD68, % (n)
Early (8)	13 (1)	50 (4)
Stable (14)	71 (10)	93 (13)
Thrombus (21)	90 (19)	95 (20)

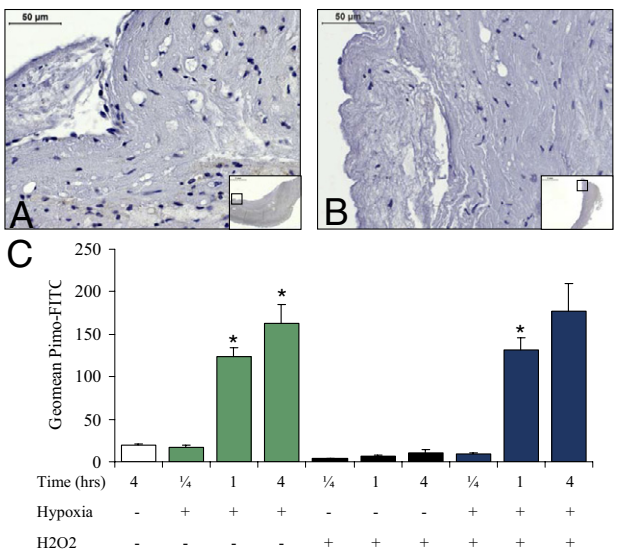

Figure 4 Hypoxia Detection in Arterial Wall and Human THP-1 Macrophages After Hypoxic and/or H_2O_2 Exposure

(A) Hypoxia (pimonidazole immunoreactivity) is almost absent in the arterial wall collected right after carotid incision, as well as right after excision of the atherosclerotic plaque **(B)**. **Insets** show origin of magnification. **(C)** Pimonidazole was detected in human THP-1 macrophages in 0.2% O_2 with fluorescence-activated cell sorting analysis **(green bars)**, but was undetectable in 21% O_2 **(open bars)** or after H_2O_2 stimulus **(black bars)**. No significant differences were found between single O_2 and O_2 + H_2O_2 exposure **(blue bars)**. *p < 0.05 versus 21% O_2. Pimo-FITC = fluorescein isothiocyanate-conjugated antipimonidazole.

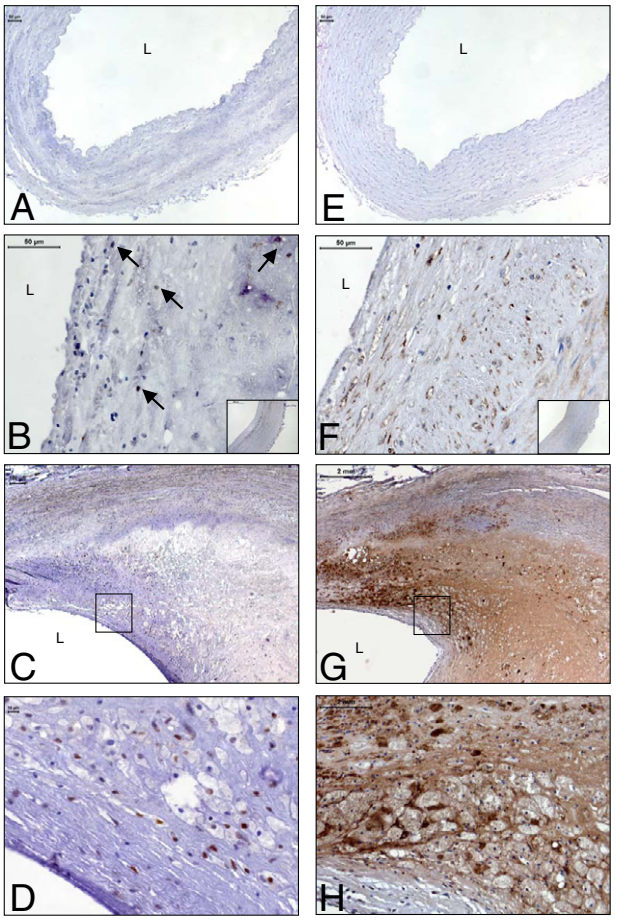

Figure 5 **Immunoreactivity of HIF Pathway in Nondiseased and Atherosclerotic Carotid Artery**

Nuclear staining of HIF1α **(A to D)** and HIF2α **(E to H)** is absent in the human mammary artery (**A** and **E**, respectively) and increased from early (**B** and **F**, respectively) to advanced carotid lesions (**C** and **G**, respectively). Macrophages in advanced lesions demonstrated strong HIF1α **(D)** and HIF2α **(H)** expression. Nuclear immunoreactivity (functional protein levels) of HIF1α and HIF2α are similar. Abbreviations as in Figures 1 and 2.

0.66, p = 0.01). Although abundant proliferating macrophages and smooth muscle cells were observed, hardly any KiECs were detected in plaque microvessels: 3 KiECs in 5 thrombus-containing lesions. However, microvessel ECs were not apoptotic either, as indicated by the absence of activated-caspase 3 (data not shown).

Discussion

This study convincingly demonstrates the direct presence of genuine hypoxia in the macrophage-rich center of human carotid atherosclerosis using the hypoxia marker pimonidazole. Hypoxia strongly correlated with CD68-positive macrophages, which is similar to previous studies in rabbit atherosclerosis (4), but also with the presence of angiogenesis and a thrombus. The latter effect is most likely mediated by macrophages, as these infiltrate into the thrombus, resolving the clot.

Hypoxia detection was not due to an artifact of surgery or ROS. First, not all cells nor every part of the plaque was hypoxic. Hypoxia was mild or absent in the media and close to the main artery lumen, although smooth muscle cells are assumed to be metabolically active. Second, hypoxia detection was similar between vascular specimens collected either immediately after carotid clamping or just before clamp release, arguing that hypoxia was present in the plaque prior to clamping. In addition, macrophage in vitro data confirmed the hypoxia-selective detection of pimonidazole by its antibody. As previously shown (17), the antibody did not cross-react with ROS-derivatives of pimonidazole, nor was the pimonidazole dose a limiting factor.

Hypoxia occurs when oxygen supply is decreased and/or demand is increased. Hypoxia of the vessel wall could arise when the intimal thickness exceeds the maximal oxygen diffusion distance of ~100 to 250 μm (18), reducing oxygen supply. In fact, the intimal thickness of the advanced, carotid lesions analyzed was significantly greater (1,500 $\pm$ 350 μm) than the oxygen diffusion distance, and in most lesions a hypoxia-negative rim of 100 to 250 μm borders the lumen. Hypoxia may also develop from an increased oxygen demand, resulting from the high oxygen demand of metabolically active inflammatory cells (19). Hypoxia is indeed typically present in macrophage foam cells, but not all macrophages are hypoxic. However, some subluminal (20- to 30-μm) foam cells were already hypoxic, despite their location well within the oxygen diffusion distance. Thus, the hypoxia threshold seems mostly dependent on the inflam-

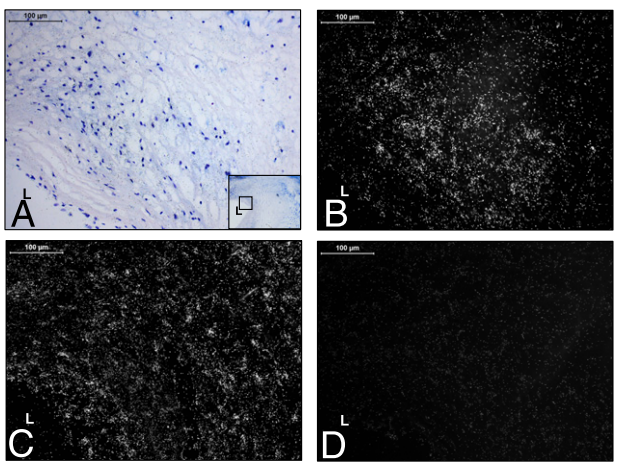

Figure 6 **Localization of HIF1α and HIF2α mRNA in Advanced Carotid Lesion**

(A) Bright field images of human carotid with an advanced atherosclerotic plaque corresponding to dark field images **(B and C)**. Expression of HIF1α **(B)** and HIF2α **(C)** messenger ribonucleic acid (mRNA) **(white dots)** was observed in macrophages surrounding the core and in the shoulder regions of advanced atherosclerosis. **(D)** No signal is observed using sense probes (negative control) for HIF1α. Abbreviations as in Figures 1 and 2.

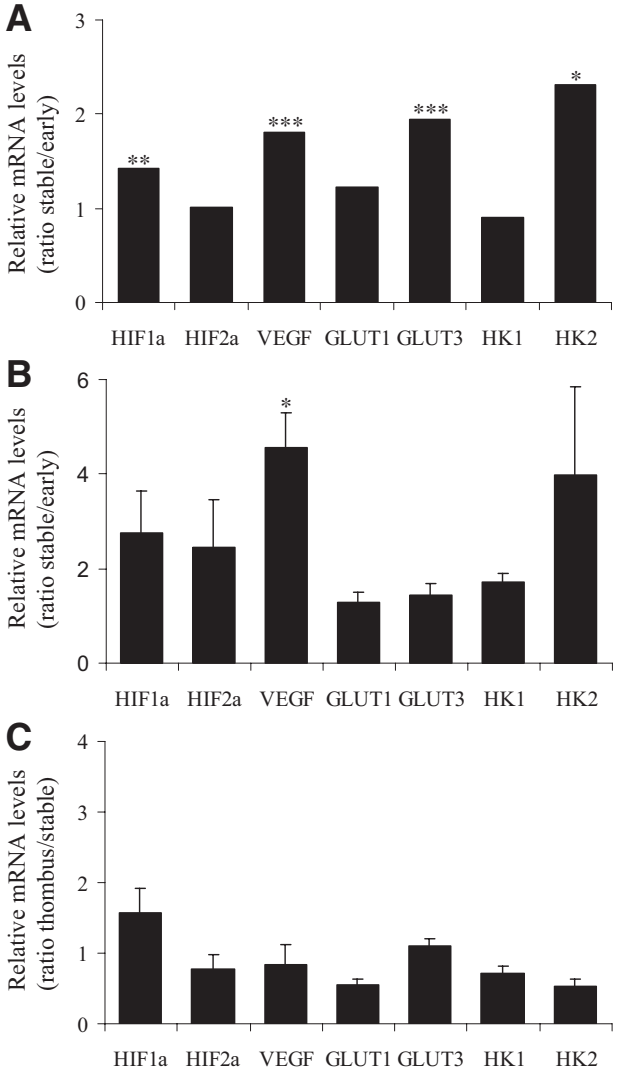

Figure 7 **Relative Expression Levels of HIF Pathway mRNA**

(A) The expression ratio determined between 9 early and 6 stable lesions by microarray analysis was significantly different for HIF1α, VEGF, GLUT3, and HK2. *$p < 0.01$; **$p < 0.001$; ***$p < 0.00001$. **(B)** Quantitative reverse transcription polymerase chain reaction was used to compare the expression ratio between 5 early and 5 stable lesions collected at autopsy (*$p < 0.05$) and **(C)** between 4 stable and 5 thrombus-containing lesions collected at surgery. GLUT = glucose transporter; HK = hexokinase; mRNA = messenger ribonucleic acid; other abbreviations as in Figure 2.

matory micro-environment, although our observations support a (minor) contribution for a decreased oxygen supply.

As hypoxic cells typically activate HIF, we studied whether HIF was present in hypoxic atherosclerosis. Although several nonhypoxic stimuli are known to induce HIF protein, such as lipopolysacharide (20) and angiotensin II (8), hypoxia is the most obvious stimulator of HIF-induced angiogenesis to restore oxygen load. Indeed, in human atherosclerosis hypoxia colocalized and correlated with HIF, VEGF, and intraplaque angiogenesis. Recent studies showed no association of HIF immunoreactivity with coronary intraplaque angiogenesis (21) and an inverse correlation with carotid and femoral intraplaque angiogenesis (22). These results are unexpected, considering the established positive correlation between HIF and angiogenesis in tumors (6), in rabbit atherosclerosis in vivo, and in our study (23). Nonetheless, the strong correlation of HIF immunoreactivity with inflammation and VEGF has been confirmed (22).

We hypothesize that hypoxia in macrophages stimulates HIF and angiogenesis in the progression of human atherosclerosis. In addition to a proangiogenic effect, hypoxia in macrophages has been described to increase cytokine production (19), low-density lipoprotein oxidation (24), and lipid loading (25) in vitro, processes that are all associated with the development of macrophage foam cells, a lipid and/or necrotic core, and hence, with destabilization of a plaque (1). Thus, we speculate that hypoxic macrophages contribute to atherosclerotic plaque instability.

Study limitations. However, because of the cross-sectional, observational study design, it remains unclear at

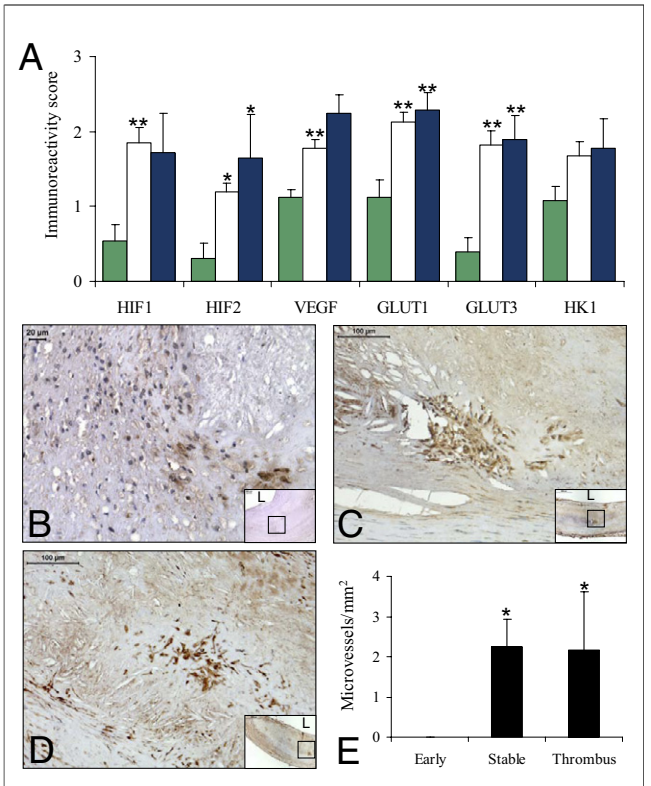

Figure 8 **Semiquantification of HIF Pathway Immunoreactivity in Carotid Atherosclerosis**

(A) Immunoreactivity of HIF pathway proteins was significantly increased from early (**green bars**, n = 5) to stable lesions (**white bars**, n = 5) for HIF1α, HIF2α, VEGF, GLUT1, and GLUT3 but similar in stable and thrombus-containing lesions (**blue bars,** n = 5). *$p < 0.05$ versus early; **$p < 0.01$ versus early. **(B)** Macrophages in advanced lesions demonstrated strong immunoreactivity of HIF-responsive genes: VEGF **(B)**, GLUT3 **(C)**, and HK1 **(D)**. **(E)** Microvessel density was determined in 6 human carotid arteries with early (intimal thickening), 6 stable, or 5 thrombus-containing atherosclerotic lesions. *$p < 0.01$ versus early. L = lumen; other abbreviations as in Figures 2 and 7.

what plaque stage hypoxia first occurs and whether hypoxia is a cause or consequence of atherogenesis. The HIF expression study precludes establishing an association between hypoxia and atherogenesis, as upstream stimuli other than hypoxia may explain HIF stabilization in atherosclerosis. Other limitations of this study are the lack of clinical characteristics in the HIF expression study and of quantitative protein data (e.g., western blot).

Conclusions

This study is the first to directly demonstrate hypoxia in human advanced atherosclerotic lesions. Hypoxia correlated with the presence of macrophages, angiogenesis, thrombus, and the expression of HIF and VEGF. Also, the HIF pathway and microvessel density were associated with lesion progression and angiogenesis, suggesting the involvement of the HIF pathway in the response to hypoxia and the regulation of human intraplaque angiogenesis.

Acknowledgments

The authors thank Marie-Thérèse Morin, Chantal Pottgens, Petra Aarts, Roselinde van Os, and Roger van de Wetering for excellent technical assistance and Erik Biessen and Sylvia Heeneman for critically reading the manuscript.

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APPENDIX

For additional Methods information and a supplementary table and figure, please see the online version of this article.