Wnt5a is expressed in murine and human atherosclerotic lesions

Mark A. Christman II,1 Douglas J. Goetz,1 Eric Dickerson,3 Kelly D. McCall,5 Christopher J. Lewis,6 Fabian Benencia,4 Mitchell J. Silver,2 Leonard D. Kohn,3,4 and Ramiro Malgor4

1Department of Chemical and Biomolecular Engineering, Ohio University, 2Mid West Cardiology Research Foundation, Columbus, Ohio, 3Edison Biotechnology Institute, Ohio University, 4Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University, 5Department of Specialty Medicine, Ohio University, and 6Department of Biological Sciences, Molecular and Cellular Biology Graduate Program, Ohio University, Athens, OH

Submitted 24 August 2007; accepted in final form 22 April 2008

Atherosclerosis is an inflammatory disease involving several cell types including macrophages and smooth muscle cells (10, 19). Initially, monocytes adhere to the luminal surface of dysfunctional endothelium present at sites of the developing lesion. Subsequently the monocytes cross the endothelium and migrate into the intima where they differentiate into activated macrophages and ultimately become foam cells. The activated macrophages release a variety of cytokines, chemokines, and mitogens [e.g., TNF-α, IL-1, MCP-1, VEGF] (10, 20) that can act in an autocrine and/or paracrine manner. These effects include the initiation of intracellular signaling, which, in turn, leads to the generation of another set of autocrine/paracrine factors. A key outcome of this process is smooth muscle cell proliferation and migration from the media to the intima. In this fashion, the development and progression of atherosclerotic lesions is governed by a complex network of intracellular signaling cascades that are intertwined with intercellular communication. Although a host of these pathways and factors have been discovered and characterized, many have not yet been identified.

In this context, Toll-like receptors (TLR) are a family of transmembrane proteins involved in the recognition of (foreign) pathogen-associated molecular patterns. TLR signal transduction pathways control the innate immune response and play a role in inflammation (1, 24). There is a growing body of evidence that suggests that aberrant TLR expression or signaling is involved in pathological inflammation (1, 6, 24). Specifically, TLR-4, a TLR that recognizes lipopolysaccharide (LPS), has been implicated in atherosclerosis (7, 17, 31).

Wnts are a highly conserved family of secreted glycoproteins that play a key role in normal [e.g., embryonic development (3, 23)] as well as pathological processes [e.g., carcinogenesis (22) and chronic inflammation (21)]. Wnts activate at least two distinct signaling pathways, i.e., the canonical and noncanonical pathways (3, 9, 27, 28). Wnt2, 5a, 7a, and 10b have been implicated in vascular biology, specifically angiogenesis, vessel remodeling, and transcortical migration of monocytes (5, 26, 30). These observations suggest that Wnts might be involved in atherosclerosis. Of particular interest is Wnt5a that, through the noncanonical signaling pathway, has been implicated in cell migration, suggesting that it might be involved in angiogenesis. Noncanonical signaling in general transduces through the JNK/planar cell polarity or the calcium-releasing pathways and regulates cell movement (18, 29).

Recently Blumenthal et al. (2) linked TLR-4, Wnt5a, and pathological inflammation. Specifically, these investigators (2) reported Wnt5a expression in human macrophages derived from Mycobacterium tuberculosis-infected patients and demonstrated that activation of the TLR-4 signaling cascade in human macrophages induces Wnt5a expression. Combined, these considerations and observations led us to hypothesize that Wnt5a plays a role in atherosclerosis. To investigate this hypothesis, we characterized Wnt5a expression in murine and human atherosclerotic lesions. We report, for the first time, that Wnt5a is present within murine and human atherosclerotic lesions and that this expression is coincident with TLR-4.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Source and preparation of murine and human atherosclerotic tissue. Apolipoprotein E-deficient (ApoE−/−) mice (B6.129P2-Apoetm1Unc/J strain, female, 6 wk old, 14–16 g), and C57BL/6 were obtained from Jackson Laboratory (Bar Harbor, ME). All handling and animal experimentation followed protocols approved by the Ohio University Institutional Animal Care and Use Committee. The mice were euthanized (exsanguination) at an age of 14 wk, i.e., 8 wk after the start of the experiment and subsequently perfused (10 min) with Dulbecco’s phosphate-buffered saline pH 7.4 (DPBS) followed by 10% buffered formalin. The hearts including the aortas were harvested and fixed in 10% buffered formalin overnight. The next day, the hearts were cut into three segments and the segments running from the aortic sinus to the beginning of the aortic arch were used for evaluation. Care was taken to cut along a plane that was perpendicular to the axis of the aorta to yield a uniform cross section as previously described (16). Tissue was then dehydrated in sequential alcohol-xylene washes and embedded in paraffin. Blocks were later cut into 6-μm-thick sections. Each pair of consecutive sections was placed on a single slide. One section served as a negative control for the other section on the slide. Immediately prior to immunohistochemical analysis, sections were placed in an incubator at 60°C for 1 h and then deparaffinized in xylenes and graded alcohol.

Human atherosclerotic plaque material was obtained during elective carotid endarterectomy and carotid stenting procedures performed at Riverside Methodist Hospital, Columbus, OH. Normal human umbilical cord was used as a negative control (obtained from Dr. Scott Jenkinson, Ohio University). Human samples were used in compliance with the Institutional Review Board for Human Subjects Committee at Ohio University. Samples were fixed with 10% buffered formalin overnight, subsequently dehydrated, embedded, sectioned, and prepared for immunohistochemical analysis as described above for the mouse tissue.

Immunohistochemistry. The R&D tissue staining kit (catalog number CTS008) was used to detect murine Wnt5a and TLR-4 and human TLR-4. This kit is based on the formation of the avidin-biotin complex.
and uses a horseradish peroxidase (HRP)-3,3'-diaminobenzidine (DAB) enzyme-substrate system for visualization. ApoE–/– mice sections were immunostained with goat anti-mouse Wnt5a or TLR-4 (2 μg/ml, R&D Systems, Minneapolis, MN) and human sections were immunostained with biotinylated goat anti-human TLR-4 (2 μg/ml, R&D Systems). In the latter case, the biotinylated secondary antibody was not used. Subsequent to immunostaining, slides were counterstained with hematoxylin (Harleco, Gibbstown, NJ). Mouse macrophages were detected with F4/80 (M3000, Sigma, St. Louis, MO). Subsequently, DAB-enhanced liquid substrate (1:20, Sigma) was added to develop the color and the slides were counterstained with hematoxylin (Harleco).

Human Wnt5a and human macrophages were detected as follows. Subsequent to blocking with hydrogen peroxide and BSA (Sigma), human sections were stained with rabbit anti-human Wnt5a (2 μg/ml, Santa Cruz Biotechnology) or murine anti-human CD68, clone KP16 (10 μg/ml, DakoCytomation, Carpinteria, CA). Wnt5a was detected by treating the slides with a biotinylated-goat anti-rabbit IgG secondary antibody (1:200, Zymed Laboratories, Carlsbad, CA) followed by an ultrasensitive streptavidin-peroxidase polymer (1:500, Sigma, St. Louis, MO). Subsequently, DAB-enhanced liquid substrate (1:20, Sigma) was added to develop the color. Anti-human CD68 was visualized with DAKO LSAB System-HRP (DakoCytomation), according to the manufacturer’s instructions. The slides were counterstained with hematoxylin (Harleco).

In all cases, normal species and isotype-matched IgG at the same concentration was used as the negative control for the primary antibodies (i.e., a negative control for anti-Wnt5a, anti-TLR-4, anti-macrophage). Note that the negative control and the test sections were on the same slide and were consecutive sections from the tissue. All antibodies were diluted in DPBS, 1% BSA.

RT-PCR. RAW264.7 mouse macrophages (ATTC American Type Culture Collection, Manassas, VA) were grown in DMEM (GIBCO, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (HyClone, Fisher Scientific). Cells were treated with LPS (0, 5, 10, 20, or 50 ng/ml) for 4 h at 37°C. Total RNA was isolated using Trizol (Invitrogen). Residual genomic DNA was removed from total RNA by using a DNA-free kit (Ambion, Austin, TX) according to the manufacturer’s instructions. One microgram of total RNA was then used to synthesize cDNA using the Advantage RT-for-PCR kit (BD Biosciences, Palo Alto, CA) according to the manufacturer’s protocol. A total of 50 ng of cDNA was subsequently used for PCR of Wnt5a. The 5' and 3' primers used for mouse Wnt5a were, respectively, 5'-GGT GCC ATG TCT TCC AAG TT-3' and 5'-ATC ACC ATG CCA AAG ACA GA-3'. Mouse GAPDH was used as a control. The 5' and 3' primers used for amplification of mouse GAPDH were, respectively, 5'-ATG TCA GAT CCA CAA CGG ATA CAT-3' and 5'-ACT CCC TCA AGA TTG TCA GCA AT-3'. The PCR reaction conditions for Wnt5a and GAPDH were as follows: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final cycle of 72°C for 7 min.

For quantitative PCR, cells were treated for 4 h with LPS as above, followed by total RNA isolation with Trizol (Invitrogen). cDNA was synthesized with the High Capacity RT kit (Applied Biosystems) using 2 μg of total RNA in 40 μl RT reactions. Quantitative PCR was then performed with 450 ng of cDNA in duplicate using the Stratagene MX3000p. Primer and probe sets for both Wnt5a and Hprt1 were TaqMan Gene Expression Assays (Applied Biosystems). Wnt5a expression levels were normalized to Hprt1 and determined by the comparative ΔΔCt method (11).

Fig. 4. Immunohistochemical analysis reveals lack of Wnt5a and TLR-4 reactivity in aorta isolated from a C57BL/6 mouse, and reactivity focal to lesions in ApoE–/– mouse fed a low-fat diet. A tissue section from the root of the aorta isolated from C57BL/6 mouse fed a low-fat diet for 8 wk treated with goat anti-mouse Wnt5a (A) or goat anti-mouse TLR-4 (B). A tissue section from the root of the aorta isolated from ApoE–/– mouse fed a low-fat for 8 wk treated with goat anti-mouse Wnt5a (C) or goat anti-mouse TLR-4 (D).
RESULTS

Morphology of the human and ApoE^{-/-} lesions used in this study. Figure 1A shows a typical section from the root of the aorta of an ApoE^{-/-} mouse after 8 wk on an atherogenic (high-fat) diet. This section is representative of the sections used throughout the study. As described by others (14, 25, 31), multiple lesions are present with resident macrophages in the intima, resulting in the protrusion of the endothelium into the vessel lumen. This extensive lesion development is in contrast to what is observed in aortas isolated from ApoE^{-/-} mice fed a chow (low-fat) diet for 8 wk (Fig. 1B) and wild-type C57BL/6 mice fed an atherogenic diet for 8 wk (Fig. 1C). Figure 1D shows a representative section of human atherosclerotic tissue used in this study. Within the intima of the human sample a necrotic region with calcium deposits and a fibrous cap is present. Accumulation of macrophages within the intima is observed near the shoulder of the lesion. Ten human samples were collected and four of these had macrophage-rich regions. The macrophage-rich regions of these four samples were used in the analysis.

Wnt5a is expressed in the macrophage region of murine and human atherosclerotic lesions. Aortic lesions from 11 ApoE^{-/-} mice on an atherogenic diet for 8 wk were analyzed by immunohistochemical analysis to access the expression of Wnt5a. A tissue section was stained with an antibody to Wnt5a. A separate serial section, no more than 7 μm from the section treated with Wnt5a, was treated with an isotype-matched IgG and served as a negative control. A typical result is shown in Figs. 2, A and B and 3, A and B. In Figs. 2A and 3A, a positive reaction for Wnt5a is clearly present. In contrast, the section treated with the isotype-matched IgG exhibits little, if any, reactivity (Figs. 2B and 3B). An identical analysis was performed on three section pairs per mouse. Similar results were observed in all sections from all 11 mice (i.e., similar results were observed in 33 side-by-side comparisons derived from 11 different mice).

A recent study has linked TLR-4 expression with Wnt5a in pathological inflammation (2) and other studies have reported high TLR-4 expression in atherosclerotic macrophages. These previous observations combined with the results presented in Figs. 2, A and B and 3, A and B led us to investigate the expression of TLR-4 in a manner identical to that for Wnt5a. In Figs. 2C and 3C, a positive reaction for TLR-4 is clearly present. In contrast, the section treated with the isotype-matched IgG exhibits very little reactivity (Figs. 2D and 3D). An identical analysis was performed on three section pairs per...
mouse. Similar results were observed in all sections from all 11 mice (i.e., similar results were observed in 33 side-by-side comparisons derived from 11 different mice).

Immunohistochemical staining of sections isolated from C57BL/6 mice that did not develop atherosclerotic lesions revealed that murine aortas without overt lesions exhibit little, if any, reactivity to antibodies to Wnt5a and TLR-4 (Fig. 4, A and B). Immunohistochemical staining of sections isolated from ApoE<sup>−/−</sup> mice fed a low-fat diet for 8 wk that do have limited lesion development revealed reactivity to anti-Wnt5a and anti-TLR-4 (Fig. 4, C and D) that was focal to the lesions. Analysis of the morphology of the regions that stained positive for Wnt5a and TLR-4 suggested that the reactivity occurred in regions that were rich with macrophages. To further investigate the lineage of the reactive region, immunohistochemical analysis was performed using an anti-F4/80 antibody. In Figs. 2E and 3E a positive reaction with the anti-F4/80 antibody is clearly present whereas the section treated with an isotype-matched IgG exhibits limited reactivity (Figs. 2F and 3F).

Combined, these results strongly suggest that 1) Wnt5a is expressed in murine atherosclerotic plaques, 2) the observed expression occurs in macrophage-rich regions, and 3) the expression is colocalized with TLR-4.

The above findings motivated us to conduct a similar analysis of atherosclerotic tissue isolated from humans. The regions of carotid lesions from four separate patients that appeared to be rich in macrophages were analyzed by immunohistochemical analysis to access the expression of Wnt5a and TLR-4 in a manner similar to that described above for the murine tissue. Typical results are shown in Fig. 5. In Fig. 5, A and B, a positive reaction for Wnt5a is clearly present whereas the tissue stained with the isotype-matched IgG exhibits little, if any, reactivity (Fig. 5C). In Fig. 5, D and E, a positive reaction for TLR-4 is clearly present whereas the section treated with the isotype-matched IgG exhibits very little, if any, reactivity (Fig. 5F). Note that we also analyzed arterial sections from a human umbilical cord and found no difference in the reactivity of Wnt5a and TLR-4 relative to isotype-matched IgG in the vessel wall.

As stated above, we chose to analyze tissue sections that appeared to be rich in macrophages. Figure 5B reveals that the Wnt5a staining is localized to what morphologically appears to be macrophages. To further investigate the lineage of the reactive region, immunohistochemical analysis was performed using an anti-macrophage antibody. In Fig. 5, G and H, a positive reaction with the anti-CD68 antibody is clearly present in this region whereas the section treated with the isotype-matched IgG exhibits very little, if any, reactivity (Fig. 5I), strongly suggesting that this region is indeed rich in macrophages. Combined, these results strongly suggest that 1) Wnt5a is expressed in human atherosclerotic plaques, 2) the observed expression occurs in macrophage-rich regions, and 3) the expression is colocalized with TLR-4.

**DISCUSSION**

The above observations demonstrate, to our knowledge for the first time, that Wnt5a is expressed in the macrophage-rich regions of murine and human atherosclerotic lesions. Wright et al. (30) reported Wnt5a mRNA expression in cultured primary endothelial cells and smooth muscle cells. Our own preliminary studies, probing for the presence of Wnt5a in non-macrophage-rich regions of developed human plaques, suggest that Wnt5a is present in regions dominated by smooth muscle cells (data not shown). Thus the expression of Wnt5a may not be restricted to macrophages in atherosclerosis. We are currently working to determine a full description of the temporal and spatial distribution of Wnt5a in atherogenesis.

The presence of Wnt5a in atherosclerotic regions raises the question as to its role. In this regard, it is insightful that aberrant Wnt5a signaling has been shown to act on fibroblast-like cells in rheumatoid arthritis (21), a disease of pathological inflammation. Additionally, Wnt5a has been implicated in cell proliferation and migration in malignant diseases including melanoma (29), colorectal cancer (22), and thyroid cancer (12). These observations, combined with the fact that smooth muscle
cells are germane to the development of atherosclerotic plaques (19, 20), make it reasonable to speculate that Wnt5a acts on smooth muscle cells, perhaps providing a signal for their proliferation and/or migration. Our laboratory is currently investigating the expression of Frizzled 5 (Fzd5), the most well known receptor for Wnt5a, on smooth muscle cells in atherosclerotic lesions. There are, however, other receptors for Wnt5a (e.g., Frizzled 4) (8, 13, 15). A recent article suggests that the signaling output of Wnt5a is a function of the receptor that engages Wnt5a (13). Thus insight into the potential role of Wnt5a in atherosclerosis will come from an examination of the expression of the different receptors for Wnt5a (e.g., Fzd5, Fzd4) in atherosclerotic lesions.

Our finding that TLR-4 is expressed in the macrophage-rich regions of atherosclerotic tissue confirms previous reports. The present study extends the previous work by demonstrating coincident TLR-4 and Wnt5a expression. This observation suggests interplay between Wnt5a and TLR-4. Recently, Blumenthal et al. (2) reported Wnt5a expression in human macrophages upon stimulation with LPS, a known trigger of the TLR-4 signal transduction cascade, linking TLR-4 with Wnt5a. In support of Blumenthal et al.’s findings, we observed that stimulation of RAW264.7 murine macrophages with LPS induces Wnt5a RNA expression (Fig. 6). Thus there is a growing body of evidence that suggests an interaction between TLR-4 and Wnt5a, specifically that activation of the TLR-4 signal cascade leads to expression of Wnt5a. The substantial number of reports linking TLR-4 to atherosclerosis (4, 17, 31) combined with the observation that TLR-4 can induce Wnt5a expression (Fig. 6) suggests that TLR-4 signal transduction cascade, linking TLR-4 with Wnt5a. For example, although the presence of Wnt5a in the macrophage-rich regions and the ability of RAW264.7 cells to express Wnt5a mRNA in response to a TLR-4 trigger, i.e., LPS, suggest that macrophages may be the source of Wnt5a in the atherosclerotic plaques, further studies are required to more definitively draw this conclusion. These studies include Wnt5a RNA in situ hybridization of the tissue sections and quantitative RT-PCR analysis of mRNA obtained from freshly isolated macrophages stimulated with LPS. Since TLRs are involved with a host of diseases, including autoimmune and inflammatory diseases (1, 6), studies aimed at elucidating the relationship between TLR-4 and Wnt5a will not only give insight into the mechanisms of atherosclerosis but also insight into inflammatory diseases in general.

In summary, we have found high expression of Wnt5a in the macrophage-rich regions of murine and human atherosclerotic lesions/plaques. The expression of Wnt5a is colocalized with TLR-4 suggesting an interaction between TLR-4 and Wnt5a in atherosclerosis. These findings demonstrate that Wnt5a may be a key player in the complex signaling networks that govern atherogenesis.

ACKNOWLEDGMENTS

We thank Dr. Scott Jenkinson (Ohio University) for providing us with the human umbilical cord used in this study.

GRANTS

This work was supported in part by an Established Investigator award from the American Heart Association (D. J. Goetz) and the Interthyroid Research Foundation (L. D. Kohn).

REFERENCES

12. McCall KD, Harri N, Lewis CJ, Malgor R, Kim WB, Saji M, Kohn AD, Moon RT, Kohn LD. High basal levels of functional toll-like receptor 3 (TLR3) and noncanonical Wnt5a are expressed in papillary thyroid cancer and are coordinately decreased by phenylmethimazole together with cell proliferation and migration. Endocrinology 148: 4226–4237, 2007.


