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Increased Wnt5a mRNA Expression in Advanced Atherosclerotic Lesions, and Oxidized LDL Treated Human Monocyte-Derived Macrophages

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Abstract

Objective—Wnt5a is a secreted glycoprotein highly present in atherosclerotic lesions. Uptake of oxidized-low density lipoprotein (ox-LDL) by monocytes/macrophages plays a critical role in atherosclerosis. The objective of this study was to determine if Wnt5a mRNA expression correlates with the severity of atherosclerotic lesions, and if, ox-LDL can induce Wnt5a mRNA in macrophages.

Methods—Wnt5a mRNA in tissue sections from carotid arteries of patients undergoing endarterectomy was quantified via RT-PCR and correlated with plaque severity. Human monocyte-derived macrophages and differentiated THP-1 cells, a human monocytic cell line, were treated with ox-LDL or native-LDL. Subsequently, Wnt5a transcripts were quantified by RT-PCR.

Results—Regions of the arteries with more severe plaques had detectable and significant levels of Wnt5a mRNA, while regions of the arteries containing less vulnerable plaques had low or non-detectable Wnt5a. Ox-LDL, but not native-LDL, induced Wnt5a mRNA in both human monocyte-derived macrophages and differentiated THP-1 cells.

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CONFLICT OF INTEREST
None declared.

Conclusion—Our results demonstrate that the expression of Wnt5a correlates with the severity of atherosclerotic lesions, and that ox-LDL induces Wnt5a mRNA expression in human macrophages. These findings are consistent with the hypothesis that Wnt5a plays a critical role in atherosclerosis progression and that a source of Wnt5a is ox-LDL stimulated macrophages.

Keywords

Cardiovascular disease; atherosclerosis; inflammation; low density lipoprotein; Wnt5a

INTRODUCTION

Atherosclerosis (ATH) is a chronic inflammatory disease and a leading cause of death worldwide [1]. The importance of macrophages in the development of atherosclerotic plaque has been well documented. Indeed, the number of macrophages in the lesions has been shown to increase with disease progression, and macrophage infiltration has been described as one of the major criteria of plaque vulnerability [1–3].

Oxidation of LDL is crucial to the monocyte/macrophage uptake of LDL in the early stages of atherosclerotic plaque development [2, 4]. Oxidized LDL (ox-LDL), a chemoattractant for circulating monocytes, promotes monocyte differentiation to macrophages [2]. Activation of macrophages leads to the production of various pro-inflammatory cytokines [e.g., TNF-α, IL-6, IL-1], chemokines [chemoattractant cytokines like MCP-1], growth factors like VEGF, and matrix degrading enzymes. These events lead to smooth muscle cell migration and proliferation and formation of a fibrous cap. The fibrous cap may eventually rupture due to production of matrix metalloproteinases, which can ultimately lead to deleterious thrombotic events, stroke or coronary heart disease [1, 5, 6].

Wnts are a family of secreted lipid-modified glycoproteins, which upon binding to frizzled (Fz) receptors - a family of seven transmembrane proteins- can lead to various physiological responses. These responses include development and regulation of embryogenesis [7, 8], cell proliferation, migration, survival and differentiation [7]. Wnt5a, a member of the non-canonical pathway, has been suggested to be a macrophage-derived effector molecule. Wnt5a has been implicated in inflammatory diseases such as atherosclerosis and rheumatoid arthritis [9–12]. It has been shown that LPS induces the upregulation of Wnt5a transcripts in human monocytes via Toll-like receptors and the NF-κB signaling pathway [13]. Recently, our group reported that Wnt5a is expressed in the macrophage-rich regions of both murine and human atherosclerotic lesions and that Wnt5a transcripts are upregulated in murine macrophages stimulated with LPS [12], suggesting that Wnt5a expression may be regulated by Toll-like receptor signaling. Combined, these observations led us to hypothesize that Wnt5a signaling in monocytes/macrophages plays a crucial role in the pathogenesis of atherosclerosis.

To further probe the above issues, we first sought to determine if the presence of Wnt5a mRNA transcripts in human atherosclerotic tissue correlates with plaque severity, and we questioned whether ox-LDL-stimulated macrophages could be a source of Wnt5a. In this report we provide evidence of a correlation between Wnt5a expression and plaque severity,

as well as data which suggests that ox-LDL modulates the expression of Wnt5a in macrophages.

MATERIALS AND METHODS

Source and Handling of Human Atherosclerotic Tissue Prior to Analysis

Atherosclerotic plaque material was obtained from 8 human subjects undergoing elective carotid endarterectomy at Riverside Methodist Hospital, Columbus, OH. All samples were obtained and used in compliance with the Institutional Review Board for Human Subjects Committee at Ohio University and Riverside Methodist Hospital. A given tissue sample was divided into fragments that contained well-developed plaques and fragments where the wall of the artery looked less affected based on a gross examination of the tissue; each tissue fragment was processed separately. The samples were immediately fixed with 10% buffered formalin overnight. Subsequently, samples were dehydrated in sequential alcohol/xylene washes and embedded in paraffin.

Hematoxylin and Eosin (H & E) Staining

The tissue blocks were sectioned into 5 μm sections and analyzed via H & E staining. The microanatomy of the tissue section revealed by the H & E staining was used to classify the tissue according to the American Heart Association guidelines as described in the first section of the results. H & E staining was correlated with RT-PCR and immunohistochemistry (see below) for classification and, in an effort to control for variations in the tissue, the three analytical approaches used in this study [H & E, RT-PCR and immunohistochemistry (IHC)] were conducted on tissue sections adjacent to one another.

Human Monocyte Isolation, Differentiation and LDL Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy, fasting volunteers by standard Ficoll–Paque density-gradient centrifugation [14]. Briefly, monocyte-derived macrophages were isolated by adherence to plastic plates in RPMI-1640 Medium supplemented with Penicillin-Streptomycin Stabilized Solution and treated with ox-LDL and native LDL (n-LDL) (both at 50 μg/ml and 100 μg/ml) for 4 hours. Ox-LDL and n-LDL were purchased from Biomedical Technologies Inc. (Stoughton, MA). All experiments involving human subjects were obtained with written consent, and approved by the Institutional Review Board (IRB) at Ohio University.

THP-1 Culture, Differentiation and LDL Stimulation

As described previously [15], THP-1 cells were purchased from American Type Culture Collection (Manassas, VA), and were cultured in RPMI-1640, 10% FCS supplemented with 0.05 mM 2-mercaptoethanol at 37° C, 5% CO_2 in a humidified incubator. For monocyte-to-macrophage differentiation, THP-1 cells were stimulated with 50 ng/ml PMA for 24 hours. Differentiated monocytes were then washed with fresh media twice before growing them for 24 additional hours at 37° C, 5% CO_2 in a humidified incubator. The monolayer was then treated with ox-LDL or n-LDL (both at 50 μg/ml and 100 μg/ml) for 4 hours. Undifferentiated THP-1 cells handled under similar conditions were used as a control.

RNA Extraction and Purification

RNA was extracted from the formalin fixed and paraffin embedded (FFPE) tissues. The paraffin blocks were sectioned into 15-μm thick sections, using a rotary microtome. The first 3 sections were discarded and subsequent sections were removed from the microtome and put in a water bath filled with RNase-free water. Afterwards, each section was mounted on a separate glass slide. Subsequently, the sections were deparaffinized, rehydrated (60°C for 1 hour and then deparaffinized in xylenes and graded alcohol) and the entire tissue section harvested by scraping the contents into a microcentrifuge tube containing 150 μl of proteinase K solution. Total RNA was extracted using the Paradise® Plus Reagent System according to the manufacturer's instructions (Arcturus Bioscience Inc, Mountain View, CA).

Similarly, total RNA was isolated from ox-LDL- or n-LDL-treated cells using the RNeasy® plus mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol. RNA was quantified using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction Analysis

RNA samples from human tissues were treated with DNase, and first strand cDNA synthesis (using 200 ng of total RNA) was performed using the Paradise® Sample Quality Assessment Kit according to the manufacturer's protocol (Arcturus Bioscience Inc.). For quantitative RT-PCR, Taqman™ Gene Expression Assay Endogenous Control for HPRT1 (assay id: Hs99999909_m1) along with gene-specific primers for Wnt5a, (Hs00180103_m1) were used with the Taqman™ Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Human HPRT1 and Wnt5a mRNA transcripts were quantified using MyiQ™ single color real-time quantitative PCR (Biorad, Hercules, CA).

First-strand cDNA was synthesized from 600 ng of total cellular RNA using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Quantitative RT-PCR was performed using Taqman™ Gene Expression Assays for each sample using an ABI StepOnePlus (Applied Biosystems, Foster City, CA).

The cycle conditions for both were as follows: hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Relative gene expression was determined by normalizing with HPRT1 using the comparative Ct method.

Immunohistochemistry

To correlate the Wnt5a RNA expression in tissues with the presence of macrophages/foam cells in the tissue, CD68 immunostaining in same tissue samples was performed. Consecutive, 5 μm sections from each block were placed on a single slide for immunostaining. One section served as a negative control (isotype-matched control) for the other section on the slide. Immediately prior to staining, sections were placed in an incubator at 60°C for 1 hour, deparaffinized/rehydrated in xylenes/graded alcohol and blocked with hydrogen peroxide and bovine serum albumin (BSA, Sigma, St. Louis, MO). The sections were stained with murine anti-human CD68, clone KP1 (10 μg/ml, Dako,

Carpinteria, CA) and visualized with the DAKO LSAB+ System-HRP (Dako), according to the manufacturer's instructions. The slides were counter stained with hematoxylin (Harleco, Gibbstown, NJ). Subsequently, DAB enhanced liquid substrate (1:20, Sigma, St. Louis, MO) was added for visualization. In all cases, a species and isotype matched antibody (10 μg/ml) was used as the negative control for the primary antibodies (i.e. a negative control for anti-CD68). All antibodies were diluted in PBS, 1% BSA.

Statistical Analysis

Comparisons of Wnt5a mRNA expression between two groups (less advanced lesions versus advanced lesions) were conducted using single factor ANOVA; P values < 0.01 for Wnt5a was considered statistically significant. Data from *in vitro* studies were analyzed using Student's t-Test for comparison of data amongst groups. Probability values of ≤ 0.02 were considered statistically significant. Data for differentiated and undifferentiated THP-1 cells are given as Mean ± SD.

RESULTS

Categorization of Lesions

We studied 11 samples from eight different patients. The first step was to classify the samples based on microscopic features observed in the tissue sections stained with H&E [16–18]. We made a binary classification of the samples, scoring them as either a less advanced or advanced lesion. The criteria for placing the samples in the two categories were: the presence/absence of a lipid core, the number of foam cells per high powered view, the presence of calcification, and presence of complications such as disruptions of the lesion surface, hematoma or hemorrhage, and/or thrombus. In addition, intimal disorganization and arterial deformity was also considered. Using these criteria, five samples were classified as having areas of less advanced lesions and six samples were classified as having areas with advanced lesions. Note that the tissue taken from three of the patients (donors 2, 3 and 4) contained regions that were categorized as less advanced as well as separate regions that were categorized as more advanced.

Wnt5a RNA Levels are Elevated in Advanced Lesions Relative to Less Advanced Lesions

With the tissue categorized, we next set out to quantify relative levels of Wnt5a transcripts in less advanced and more advanced lesions. For this analysis, we used quantitative RT-PCR to determine the level of Wnt5a transcripts in each sample. The individual results for each sample were averaged within a group to achieve the data presented in Fig. (1). As shown, the level of Wnt5a mRNA transcripts detected in the advanced lesions was significantly higher than that observed in the samples derived from the less advanced group. This result suggests that Wnt5a transcripts correlate with the advanced stage of the plaque.

The variation in the Wnt5a signal is rather high (e.g. CV = 0.745 for the advanced lesion in Fig. 1). As noted above, six of the samples were derived from three subjects whose samples contained both advanced and less advanced lesions. Thus, they are paired samples. To gain further insight into the possible correlation between Wnt5a and the stage of the lesions, we compared the three subjects that have both types of lesions in a pair-wise fashion. Fig. (2A)

shows the H&E stain of the tissue sections from the three subjects. The images on the left (2a, 3a, 4a), are from a region of the artery that exhibits less advanced lesions while the images on the right (2b, 3b, 4b) are from a region of the artery that exhibits advanced lesions. Note that the advanced lesions show large lipid cores, increased thickness of the wall, and calcification. As shown in Fig. (2B), Wnt5a transcripts were only detected in samples from the advanced lesions.

Immunostaining in consecutive sections for CD68 revealed that the reactivity for this macrophage marker is higher in the advanced lesions compared to the less advanced lesions (Fig. 3). Nonspecific IgG, used as an isotype-matched negative control for the CD68 antibodies, gave negligible background staining for both tissue types.

Ox-LDL Induces the Expression of Wnt5a in Human Monocyte-derived Macrophages

The above results suggest that macrophages could be a source of Wnt5a in atherosclerotic lesions. To begin to address this issue, we evaluated if ox-LDL could stimulate Wnt5a mRNA expression in human macrophages *in vitro*. Human monocyte-derived macrophages from eight healthy donors were treated, separately, with either 50 or 100 μg/ml of ox-LDL or n-LDL. Four hrs. later, the RNA from each group was isolated and analyzed by quantitative RT-PCR. This analysis revealed that ox-LDL induced Wnt5a mRNA expression in human monocyte-derived macrophages (Fig. 4). In contrast, n-LDL did not induce Wnt5a mRNA expression (Fig. 4). Additionally, ox-LDL, but not n-LDL, also induced IL-6 mRNA expression in human monocyte-derived macrophages" (data not shown).

Ox-LDL Induces the Expression of Wnt5a in THP-1 Macrophages

To further evaluate the ox-LDL influence on Wnt5a mRNA expression in macrophages, we tested the effect of ox-LDL on the human monocytic cell line THP-1. THP-1 cells were differentiated to macrophages via PMA treatment for 48 hours. PMA treated THP-1 cells were subsequently stimulated with 50 or 100 μg/ml of ox-LDL or n-LDL for 4 hrs. RT-PCR revealed that ox-LDL, but not n-LDL, induced Wnt5a mRNA expression in a dose dependent manner (Fig. 5). Ox-LDL induced little, if any, Wnt5a mRNA expression in undifferentiated THP-1 cells suggesting that differentiation to a macrophage phenotype is critical for ox-LDL stimulation of Wnt5a. We also found that, ox-LDL, but not n-LDL, induced IL-6 mRNA expression in PMA differentiated THP-1 cells (data not shown).

DISCUSSION

The pathogenesis of atherosclerosis involves a variety of cell types with extensive intercellular communication, however this process is not completely understood. In our previous study we revealed, via immunohistochemistry, that Wnt5a protein is present in human and murine atherosclerotic lesions. Futher, Wnt5a signaling in macrophages has been suggested to play a role in other inflammatory disorders such as sepsis, tuberculosis and rheumatoid arthritis [10, 11, 13, 19]. This led us to hypothesize that Wnt5a may play a role in the development and progression of atherosclerosis.

In this study we reveal the expression of Wnt5a mRNA in human atherosclerotic lesions, and that the expression is higher in lesions with advanced histopathological characteristics in

comparison to areas with less advanced disease. Our findings also show that in advanced lesions the expression of CD68 is higher than in areas of the artery with less advanced disease. This result agrees with other reports of increased numbers of macrophages in vulnerable plaques [3]. In addition, we found that ox-LDL, a key player in atherosclerosis, can induce the expression of Wnt5a transcripts in human macrophages, and that IL-6 transcripts were also induced by ox-LDL, as suggested by previous studies [20], but not to the level seen for Wnt5a (data not shown).

It is well established that macrophages resident in the arterial wall internalize ox-LDL, via the scavenger receptors, to become foam cells. Early foam cells represent the beginning of atherosclerotic plaque development. As additional foam cells accumulate, a fatty streak is formed leading to an intensified inflammatory response [1, 5]. IL-6, TNF-α, VEGF, IL-1, among others, are known to be secreted by foam cells. These factors stimulate the smooth muscle cells to proliferate and migrate into the intima leading to further plaque development and the formation of a fibrous cap [1, 4]. While these and other atherosclerotic mediators and pathways have been identified and well characterized, information regarding the potential role of Wnt5a is lacking.

It is important to highlight that in this report we have only provided evidence that Wnt5a *mRNA* expression is increased in THP-1 cells and monocytes stimulated with ox-LDL in cell culture. This is because our efforts to determine Wnt5a *protein* expression via Western blotting, immunofluorescence and ELISA in lysates and supernatants of cultured THP-1 cells and freshly isolated monocytes stimulated with ox-LDL have been inconclusive to date. It is noteworthy that numerous studies have shown the detection of Wnt5a protein in cultured cells to be problematic [13, 21–25]. That said, the results at the mRNA level presented in this study are consistent with the hypothesis that ox-LDL stimulated macrophages are an important source of Wnt5a in atherosclerosis. Although we were unable to detect Wnt5a protein in our *in vitro* experiments, we have already shown the presence of Wnt5a protein in the macrophage-rich regions of both human and murine atherosclerotic lesions [12] in a previous report, a finding which supports our *in vitro* findings at the RNA level presented herein.

There are many possible roles for Wnt5a in atherosclerosis. Wnt5a could induce, in an autocrine or paracrine manner, upregulation of various inflammatory cytokines (e.g. IL-6, TNF-α) as well as Wnt5a itself [9, 19]. Additionally, since Wnt5a is known to play a role in cell migration, it is possible that Wnt5a could act directly on resident and neighboring cells (e.g. smooth muscle cells) to induce cell migration into or within the plaque. Finally, Wnts could actually be helpful in preventing atherosclerosis (e.g. Wnts have been shown to play a role in controlling cholesterol homeostasis [26]). Clearly, the exact role for Wnt5a and Wnts in atherosclerosis remains to be determined, however our data suggest that Wnt5a may be involved in the pathogenesis of atherosclerosis rather than protection from it.

In conclusion, we have found that the expression of Wnt5a mRNA correlates with severity of atherosclerotic lesions and that ox-LDL induces Wnt5a mRNA expression in human macrophages. Combined, these results support the hypothesis that Wnt5a signaling in macrophages plays a role in the pathogenesis of atherosclerosis.

Acknowledgments

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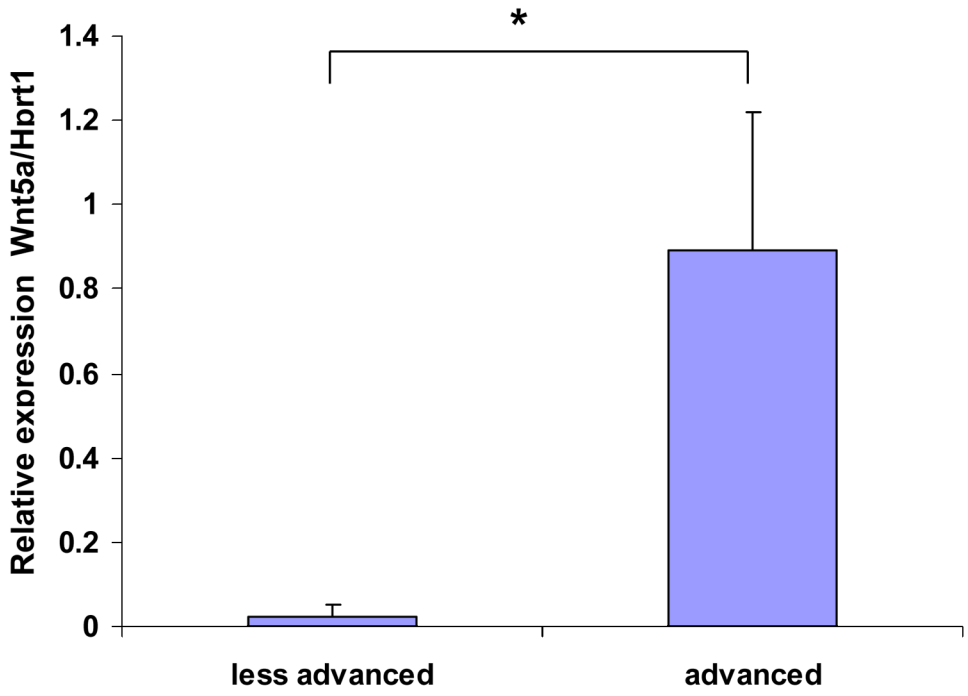

Fig. 1.

Wnt5a transcripts are significantly higher in advanced versus less advanced lesions. Quantitative RT- PCR analysis of Wnt5a transcription levels in mRNA extracted from less advanced and advanced atherosclerotic lesions. Results are expressed as mean ± SEM, n = 6 for advanced lesions; n= 5 for less advanced lesions. Each sample (n) was analyzed in duplicate and averaged to obtain a single result for that sample * $P < 0.01$.

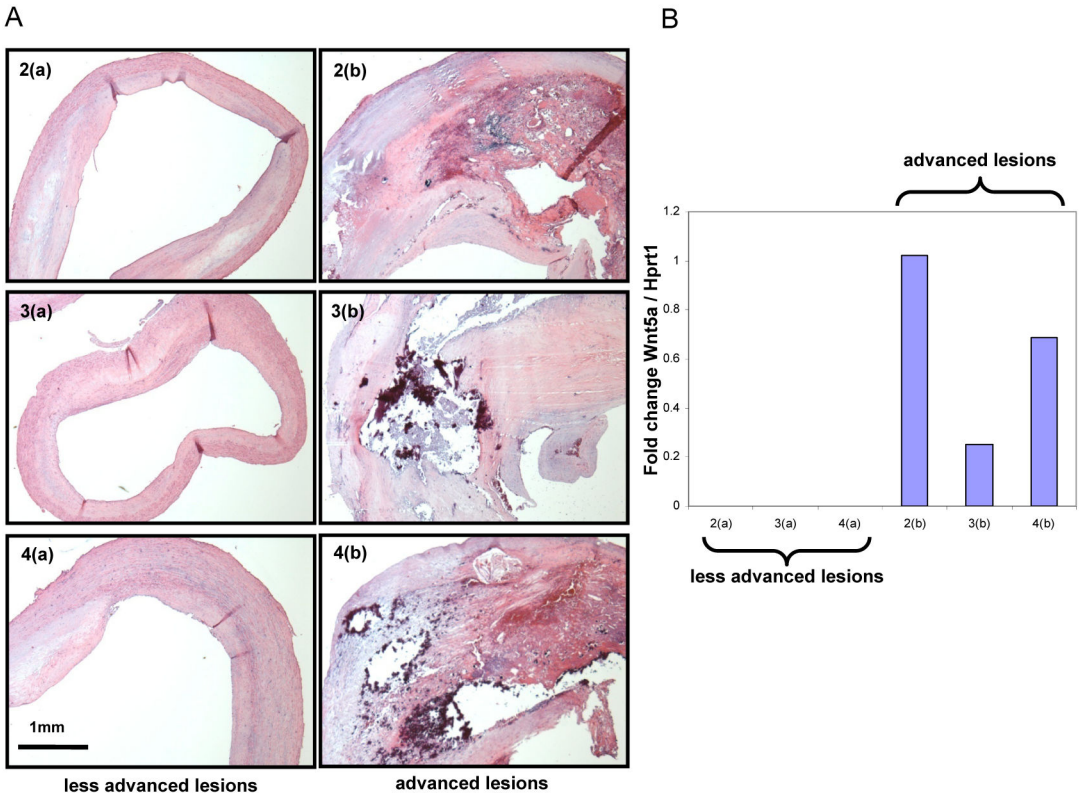

Fig. 2.

Quantitative RT- PCR analysis of paired tissue samples reveals that Wnt5a transcripts are significantly higher in advanced versus less advanced lesions. Separate sections (advanced and less advanced sections) from the same patient, i.e. paired samples, were used in a RT-PCR analysis to determine the relative expression of Wnt5a. (**A**) H&E staining of human carotid artery samples taken from 3 different subjects (paired samples from patients 2, 3 and 4) at 20x magnification. The panels on the left (2a, 3a, 4a) reveal the less advanced areas, while the panels on the right (2b, 3b, 4b) reveal the more advanced areas for the same artery. Bar = 1mm. (**B**) Quantitative RT- PCR analysis of Wnt5a transcripts from mRNA derived from the tissue presented in (A). The three results on the left side of the graph correspond to the tissue on the left in (A), i.e. the less advanced areas, and the three results on the right side of the graph correspond to the tissue sections on the right of (A), i.e. the more advanced areas. Wnt5a transcripts were only detected in the advanced lesions.

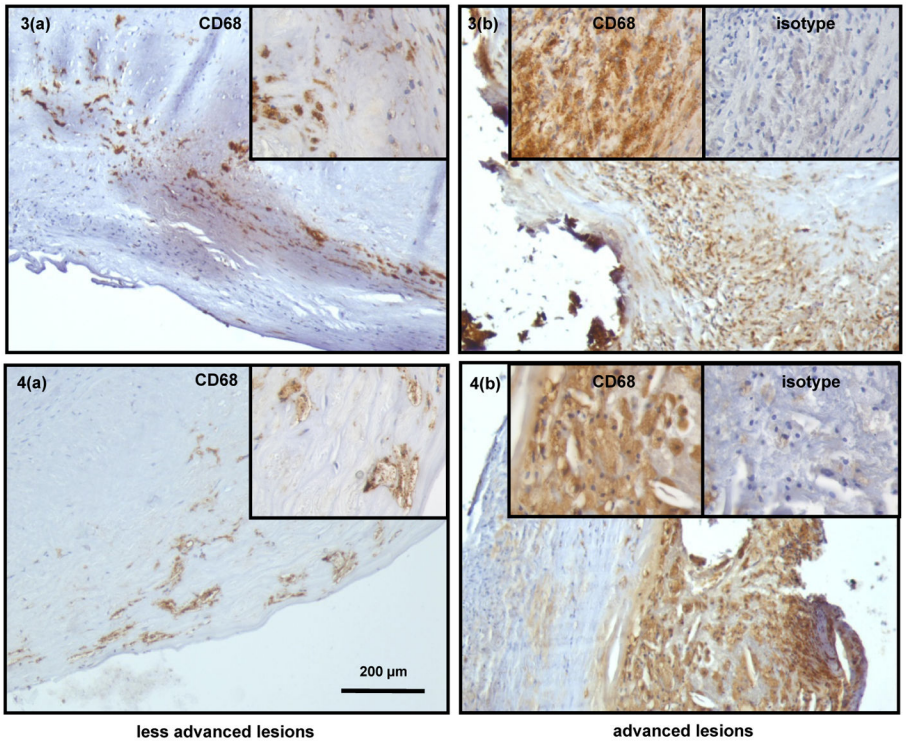

Fig. 3.

Immuno.histochemistry for CD68 in less advanced atherosclerotic lesions compared to advanced lesions. Carotid artery samples were divided into tissue fragments that contained advanced lesions and areas where the wall of the artery looked less affected, i.e. less advanced lesions. Representative IHC staining for CD68 (a macrophage marker) on tissue sections from less advanced lesions [3(a) and 4(a)] exhibit less extensive staining for CD68 compared to tissue sections from more advanced lesions [3(b) and 4(b)]. Inserts within each image show higher power magnification of the tissue stained for CD68 isotype control. IHC staining for CD68, combined with the data presented in Fig. 2, suggests that the presence of Wnt5a transcripts in tissues from advanced lesions correlates with regions containing a high number of macrophages/foam cells. Bar = 200 μm.

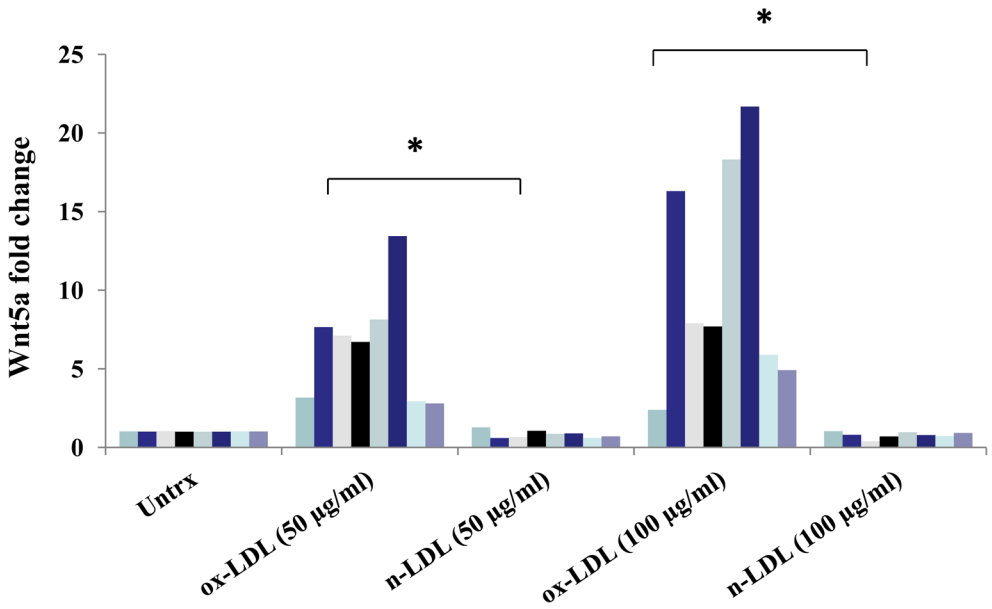

Fig. 4.

Ox-LDL induces Wnt5a transcription in human monocyte derived macrophages. Human monocyte derived macrophages from 8 independent donors were treated with ox-LDL or n-LDL both at 50 and 100 μg/ml for 4 hours. Wnt5a expression was measured and normalized to HPRT 1 mRNA levels by quantitative RT-PCR. *, $P \leq 0.02$, significant difference between groups. For a given treatment condition, each bar represents a separate donor.

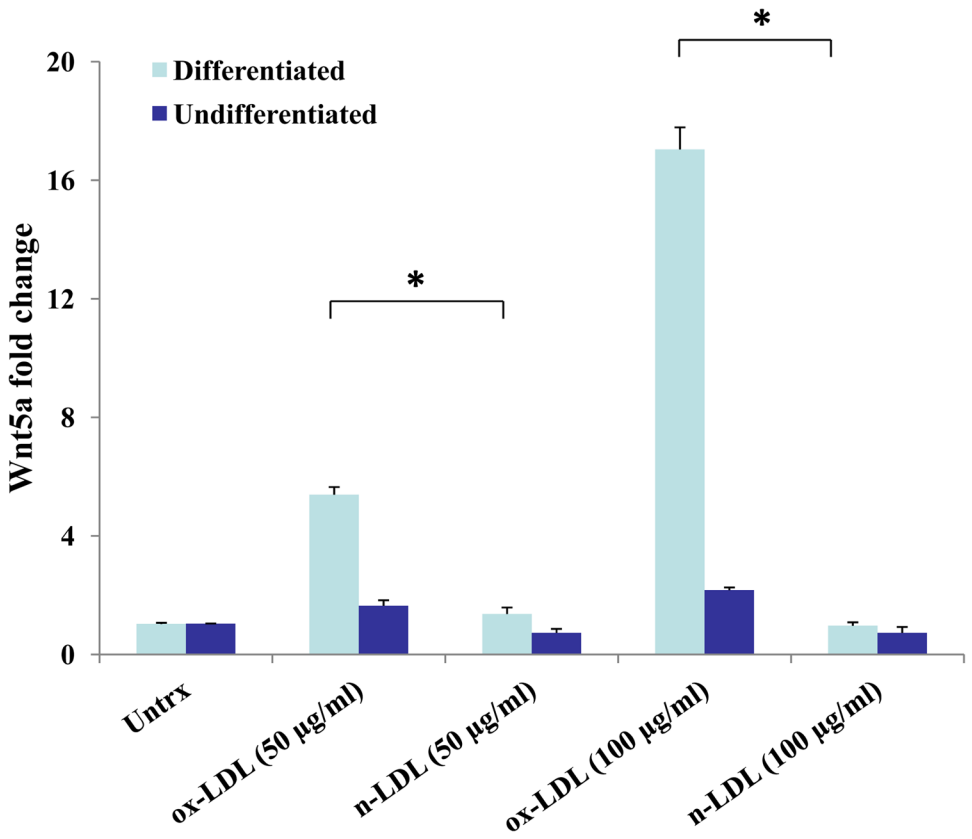

Fig. 5.

Ox-LDL induces Wnt5a transcription in differentiated and undifferentiated THP-1 cells. THP-1 monocytes were incubated with/without PMA (50 ng/ml) and then stimulated with ox-LDL or n-LDL both at 50 and 100 µg/ml for 4 hours. Wnt5a expression was measured and normalized to HPRT 1 mRNA levels by quantitative RT-PCR. Experiments were performed in triplicates; mean ± SD of two independent experiments are depicted. * $P \leq 0.04$.