Immunohistochemical analysis of adiponectin in atherosclerotic lesions of human aorta

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Original Article

Abstract

BACKGROUND: Metabolic syndrome, a cluster of interrelated disorders including abdominal obesity, insulin resistance, dyslipidemia, and hypertension (HTN) plays an important role in development of atherosclerotic lesions in arterial wall. Dysregulation of adipose tissue hormones (adipokines) production is a possible link between abdominal obesity and other manifestations of metabolic syndrome. Adiponectin is a well-known adipokine which affects metabolism and inflammatory response. However, data on its role in atherogenesis are still controversial. The aim of this study is to investigate whether adiponectin is present in atherosclerotic lesions of human aorta.

METHODS: Thirty-five autopsy segments from abdominal, thoracic aortas, and aortic arch of four men (mean age: 57 years) were fixed and stained for lipids [Oil Red O (ORO)], cells [hematoxylin-eosin (H&E)], and adiponectin [indirect immunoperoxidase assay (IPA) method]. Samples of both stable and unstable plaques were selected for analysis. Human adipose tissue, THP-1 monocytes/macrophages, and human endothelial hybrid cell line (EA.hy926) were chosen for detection of adiponectin messenger ribonucleic acid (mRNA) using reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: Adiponectin accumulations were found inside endothelial cells covering both stable and unstable atherosclerotic plaques. Focal depositions of adiponectin were also found in fibrous caps of stable lesions and atheromatous core of both stable and unstable plaques and also in adventitia. RT-PCR revealed mRNA expression of adiponectin gene in adipose tissue, but not in mononuclears and endothelial cells.

CONCLUSION: Adiponectin is present in aortic plaques of humans, but is not synthesized in endothelial cells and mononuclears, at least in culture conditions. Detection of adiponectin in atherosclerotic lesions can serve as indirect evidence of possible participation of this adipokine in atherogenesis.

Keywords: Adiponectin, Atherosclerosis, Aorta, Endothelium

Introduction

Atherosclerosis is a complex chronic disease of arterial wall, which leads to development of major cardiovascular events including myocardial infarction (MI) and stroke. In spite of intensive research in this field, mechanisms of initiation and progression of atherosclerosis are still not completely understood. In particular, the molecular triggers of focal infiltration of apolipoprotein B-containing lipoproteins in atherosclerosis-prone areas of large arteries still need to be determined. Subsequent events in arterial wall depend on interaction between lipoproteins, extracellular matrix, and intimal cells: endothelial cells, mononuclears, and migrated smooth muscle cells (SMCs).

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Specifics of this interplay will lead either to formation of stable plaque with thick fibrous cap or development of unstable plaque, containing small number of SMCs, substantial necrotic core, and thin fibrous cap confining the active inflammatory process.[1]

One of the important risk factors of atherosclerosis is metabolic syndrome, a cluster of interrelated disorders, including abdominal obesity, insulin resistance, dyslipidemia, and hypertension (HTN).[2,3] These disorders are accompanied by dysregulation of production of adipose tissue-derived hormones and cytokines, named "adipokines", mostly due to expansion of abdominal adipose mass.[4] Adiponectin is a unique adipokine, which production in obesity, opposed to most other adipokines, is decreasing. Adiponectin has insulin sensitizing and lipid metabolism modulating effects, mostly implying its action on liver and muscles, and has some local effects on vascular wall as well.[4,5] Particularly, adiponectin modulates inflammatory response in endothelial cells and macrophages,[6-8] decreases mononuclears adhesion, enhances nitric oxide (NO) production by endotheliocytes,[9,10] inhibits foam cell formation,[8,11] and migration and proliferation of vascular SMC.[12,13] These effects are mediated by adiponectin receptors (AdipoRs), mostly by AdipoR1.[9,10] Recently, expression of T-cadherin in vascular wall was shown, a receptor that specifically binds to high-molecular-weight (HMW) adiponectin and mediates its cardio- and atheroprotective effects in mice.[14-16]

Nevertheless, the data regarding adiponectin influence on development of atherosclerosis are controversial. Adiponectin retarded atherosclerosis development or had no effects on this disease in mice,[17,18] and clinical studies found both negative and positive correlations of plasma adiponectin levels with frequency of atherosclerosis and its outcomes.[19,20] Hypothetically, such controversies could be explained by differences in vascular wall adiponectin accumulation, where this adipokine could act on atherogenesis directly. Previously, adiponectin was found in mouse but not in human aorta.[15,21] The aim of this study was to estimate the adiponectin presence and localization in aortic atherosclerotic lesions of human subjects.

Materials and Methods

For determination of adiponectin protein in aortic lesions, autopsy segments of aortic arch as well as thoracic and abdominal regions of aortas (in total

n = 35) have been retrieved from four men (mean age of 57 ± 6 years, range: 50-62 years) who died of acute MI or sudden cardiac death. The exclusion criterion was the history of chronic autoimmune and inflammatory diseases. The study was approved by Ethical Committee of the Institute of Experimental Medicine, Saint Petersburg, Russia.

Samples of aorta were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH: 7.2-7.4). Paraffin and cryostat 3-5 μm slices were prepared for histological and immunohistochemical analysis. For evaluation of plaque stability, preparations were stained for lipids and cells, using Oil Red O (ORO) and hematoxylin-eosin (H&E), respectively. Unstable lesions were characterized by thin, disorganized, or damaged fibrous caps infiltrated with lipids and mononuclears (Figure 1).[22]
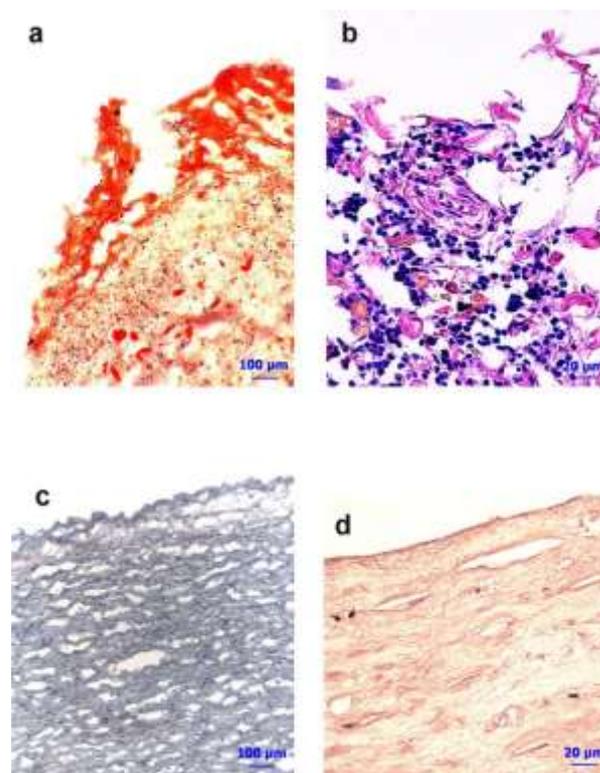
Figure 1. Morphological characteristics of stable and unstable atherosclerotic lesions of human aorta
(a) Huge depositions of lipids in surface layer of destructed fibrous cap of unstable lesion; (b) Mononuclear infiltration in damaged area of unstable lesion's cap; (c) Absence of lipids in the fibrous cap of stable plaque; (d) Absence of mononuclear infiltration in the fibrous cap of stable plaque; a and c: Oil Red O (ORO) staining; b and d: hematoxylin-eosin (H&E) staining

Detection of adiponectin in aortic slices was performed using indirect immunoperoxidase assay (IPA). Endogenous peroxidase was quenched by

treatment with peroxidase blocking reagent (cell and tissue staining kit, R&D Systems). The primary antibodies for adiponectin were taken from human adiponectin enzyme-linked immunosorbent assay (ELISA) kit (conjugate solution, BioVendor); goat anti-Rabbit IgG secondary antibody horseradish peroxidase (HRP)-conjugates were obtained from Abcam (ab97051, 50 μg/ml). The product has been developed after adding 3.3'-diaminobenzidine (brown staining). The nuclei were stained by methyl green (Dako). Negative control without primary antibodies was also assessed (Figure 2c). Microscopy was performed using Leica DM2500, and microphotographs were obtained by Leica DFC420 photocamera and Leica Application Suite software (version 3.4.0).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay: For evaluation of possible adiponectin synthesis in vascular wall, we performed adiponectin qRT-PCR analysis in cell cultures of human macrophages and endothelial cells. For doing this, THP-1, a human monocytic cell line, and EA.hy926, an immortalized endothelial hybrid cell line, were applied in the study. THP-1 and EA.hy926 cells were cultivated, respectively, in RPMI-1640 and Dulbecco's Modified Eagle Medium (DMEM) media, supplemented with 10% fetal bovine serum and antibiotics as described earlier.[23,24] THP-1 mononuclears were differentiated into macrophages by treatment of these cells with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, 24 h).[23] Total ribonucleic acid (RNA) was isolated from the cells, using TRI reagent (Ambion) according to manufacturer's protocol. 500 ng of RNA was reverse-transcribed and SYBR Green PCR was performed as in Mogilenko et al.[23] Primers were designed using Primer3 software (http://primer3.sourceforge.net/): adiponectin transcript 1, forward 5'-TCTGATTCCATACCAGAGGAGAC-3' and reverse 5'- GCCCTGATGTCAGGAGTTTC-3', adiponectin transcript 2, forward 5'-GATTCCATACCAGAGGGGCT-3' and reverse 5'-ATGACCGGGCAGAGCTAATA-3', CD31, forward 5'- GGCTTGGAGTCCTGCTGA-3' and reverse 5'- AAGCACTGCAGGGTCAGG-3'. CD31 or platelet/endothelial cell adhesion molecule-1 (PECAM-1) was used as a marker of endothelial and mononuclear cells.[25] The specificity of PCR was determined evaluating melting curves and agarose gel electrophoresis of amplicons. The expression of genes of interest was normalized by geometric mean of relative content of three references: β-actin, cyclophilin A (CyPA), and ribosomal protein lateral stalk subunit P0 (RPLP0).[26]

As a positive control for adiponectin gene expression, fragments (3-5 g) of femoral adipose tissue were obtained from one man after liposuction and put into TRI reagent for subsequent RNA extraction and qRT-PCR. Informed consent was obtained from this patient.

Results

Adiponectin detection in atheromas of human aorta:
Immunohistochemical analysis of atherosclerotic lesions revealed localizations of adiponectin in different layers of aorta. Local intracellular depositions of adiponectin were visualized in endothelial layer covering both stable and unstable atherosclerotic plaques (Figures 2a and 2b). In addition, adiponectin was found in superficial and deep areas of fibrous caps (Figures 3a and 3b), probably due to migration of this adipokine into depth of vascular wall.
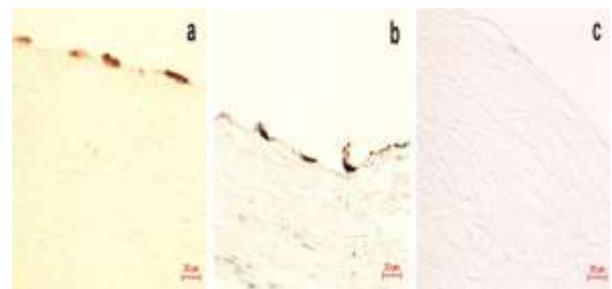

Figure 2. Adiponectin in endothelium of stable and unstable atherosclerotic plaques of human aorta
Focal adiponectin depositions in cytoplasm of endothelial cells covering the stable (a) and unstable (b) atherosclerotic plaques; disorganized fibrous cap and damaged endothelium in unstable plaque are seen in (b); (c) Negative reaction for adiponectin in a preparation of stable plaque, elaborated without primary antibodies [immunoperoxidase assay (IPA)]

Adiponectin has also been identified in atheromatous core of stable atherosclerotic plaques, mainly around lipid deposits (Figure 3d) and in adventitia underneath the stable plaque (Figure 3c). Adiponectin was also detected in deep areas of unstable lesions (Figure 3e).

Adiponectin gene expression in THP-1 and EA.hy926 cell lines:
qRT-PCR analysis revealed adiponectin mRNA in adipose tissue but not in EA.hy926 endothelial cells and human THP-1 monocytes/macrophages, while endothelial and mononuclear marker CD31 was expressed in all cell types studied (Figure 4).
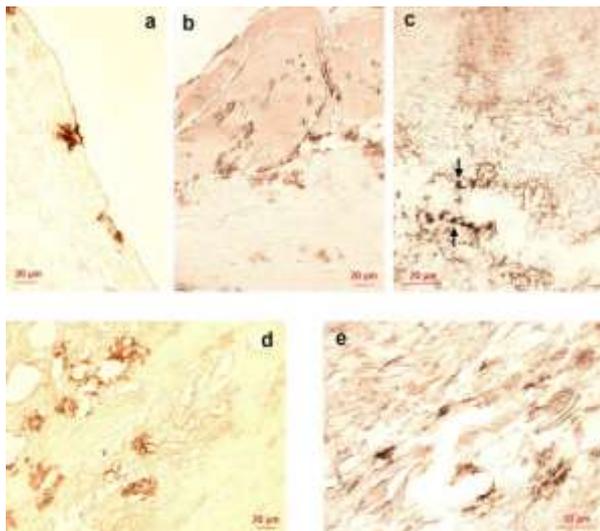
Figure 3. Adiponectin depositions in different parts of atherosclerotic plaque of human aorta

(a) Focal depositions of adiponectin in a surface layer of stable atherosclerotic plaque; (b) Adiponectin in deep areas of thick fibrous cap of stable plaque; (c) The sites of adiponectin depositions (arrows) in adventitia under the stable plaque; (d) Adiponectin depositions at the atheromatous core of stable plaque; (e) Intracellular adiponectin depositions in intima of ruptured plaque [immunoperoxidase assay (IPA)]

Discussion

Clinical,[19,20] animal,[17] and cell culture studies[6-8,10-13] strongly support involvement of adiponectin in atherogenesis. It remains unclear whether adiponectin acts locally in vascular wall.
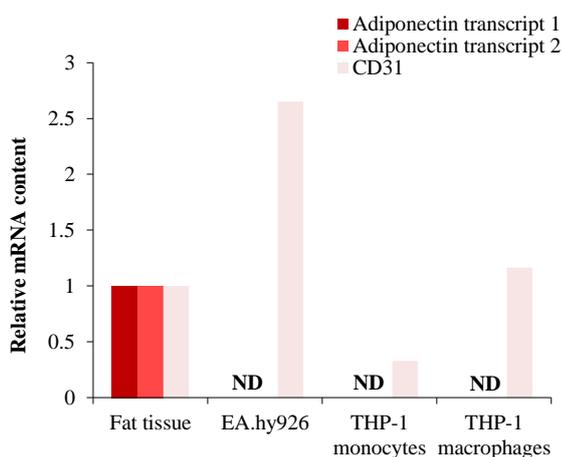
Figure 4. Relative adiponectin and CD31 gene expression in human fat tissue, endothelial hybridoma cell (EA.hy926), and THP-1 mononuclears

Gene expression levels were assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and normalized for the relative content of β-actin, cyclophilin A (CyPA), and ribosomal protein lateral stalk subunit P0 (RPLP0); ND: Not detected

We found that adiponectin was present in human aortic atherosclerotic plaques. Adiponectin was found in all layers of aorta affected by both stable and unstable lesions: endothelium, fibrotic cap, atheromatous core, and adventitia. Earlier, Kostopoulos et al.[15] did not find adiponectin protein in human aortic and coronary plaques. Only slight signal was detected in adventitia, probably due to low sensitivity of assay performed. In another research, adiponectin was found in subendothelium of injured but not intact aorta in a patient with aortic aneurysm.[27] Recent study has shown that adiponectin is present in human carotid arteries and plaques, and its content is increased in unstable lesions.[28] Similar to our observations, the authors also found adiponectin in endothelium, fibrous cap, and lipid core, presumably in areas with foam cells. Adiponectin was also detected in both atherosclerotic and normal mouse aortas, mostly on luminal surface and in intracellular vesicles of endothelial cells, and also on the surface of monocytes and SMCs in atherosclerotic lesions.[21] We examined only atherosclerosis-affected aortas, and future studies will reveal if adiponectin accumulation also occurs in normal aortic vessels of humans.

Interestingly, in contrast to above-mentioned observations,[21,27,28] we found focal adiponectin accumulation in vessels. Mechanism of its uneven deposition, particularly in endothelium and fibrous cap, needs further investigation. It could be related to focal processes of atherogenesis such as disturbed permeability of endothelium, its dysfunction, inflammatory reactions, etc. These events could lead to local synthesis, uptake from plasma, or retention of adiponectin in aorta. Adiponectin mRNA was not detected in both normal and atherosclerotic carotid arteries,[28] while it was found in murine aortas in one of the two studies.[29,30] We also have not found adiponectin mRNA in cultured human monocytes/macrophages and endothelial cells, and these data are in line with the absence of this adipokine synthesis in intimal layer of human artery, as was shown earlier.[28]

Uptake, transport, and retention of adiponectin in vessels could be mediated by AdipoRs. Indeed, AdipoRs, AdipoR1 and AdipoR2, are expressed in endothelium, SMCs, macrophages, and foam cells of human carotid artery, i.e., in the same cell types, where adiponectin was detected.[28] T-cadherin, another binding partner for adiponectin, is also expressed in endothelium and SMC of vascular wall.[15,16] Future investigations will reveal whether T-cadherin and AdipoR1/R2 are expressed and colocalized with

adiponectin deposition in human aorta.

We detected adiponectin in both stable and unstable plaques. We did not include in this investigation samples of nonlesioned aorta, so it is not known whether adiponectin specifically accumulates in atherosclerotic regions or also in unaltered areas of aorta. Nevertheless, the presence of adiponectin in aortic plaques suggests possible involvement of this adipokine in atherogenesis. The other limitation is that the data were obtained only from men with one specific age range (50-62 years). Since plasma concentration of adiponectin is gender- and age-dependent, it is not known if women and younger people have the same patterns of adiponectin accumulation as aged men. Finally, we still do not exactly know the source of adiponectin in aortic plaques. RT-PCR analysis of aortic intima/plaques will partially resolve this problem.

Conclusion

We found for the first time the presence of adiponectin in human aortic plaques. Focal depositions of adiponectin were found in endothelium, fibrous cap, atheromatous core of both stable and unstable plaques, and also in adventitia. Adiponectin RNA was not detected in THP-1 monocytes/macrophages and EA.hy926 endothelial cells, but there is possibility of local synthesis of adiponectin in aortic plaques or normal aorta. Detection of adiponectin in atherosclerotic lesions can serve as an indirect confirmation of local involvement of this adipokine in atherogenesis.

Acknowledgments

Conflict of Interests

Authors have no conflict of interests.

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