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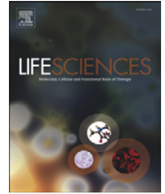

Aqueous or lipid components of atherosclerotic lesion increase macrophage oxidation and lipid accumulation

Niroz Abu-Saleh [a], Michael Aviram [a,*], Tony Hayek [a,b]

[a] The Lipid Research Laboratory, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel
[b] Department of Internal Medicine E, Rambam Health Care Campus, Haifa, Israel

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ABSTRACT

Introduction and objective: Understanding the interactions among atherosclerotic plaque components and arterial macrophages, is essential for elucidating the mechanisms involved in the development of atherosclerosis. We assessed the effects of lesion extracts on macrophages.
Methods: Mouse peritoneal macrophages from atherosclerotic normoglycemic or hyperglycemic apoE$^{-/-}$ mice were incubated with aortic aqueous or with aortic lipidic extracts (mAAE or mALE) derived from these mice. In parallel, J774A.1 cultured macrophages were incubated with increasing concentrations of extracts prepared from human carotid lesions: polar lesion aqueous extract (hLAE), nonpolar lesion lipid extract (hLLE), or with their combination. In all the above systems we performed analyses of macrophage oxidative status, cholesterol, and triglyceride metabolism.
Results: Aqueous or lipid extracts from either mice aorta or from human carotid lesions significantly increased macrophage oxidative stress as determined by reactive oxygen species (ROS) analysis. In parallel, a compensatory increase in the cellular antioxidant paraoxonase2 (PON2) activity and in macrophage glutathione content were observed following incubation with all extracts. Macrophage triglyceride mass and triglyceride biosynthesis rate were both significantly increased following treatment with the lipid extracts, secondary to upregulation of DGAT1. All extracts decreased cholesterol biosynthesis rate, through downregulation of HMGCR, the rate limiting enzyme in cholesterol biosynthesis. The combination of the human lesion extracts had the most significant effects.
Conclusion: The present study demonstrates that atherosclerotic plaque constituents enhance macrophage cellular oxidative stress, and accumulation of cholesterol and triglycerides, as shown in both in vivo and in vitro model systems.

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1. Introduction

Atherosclerosis is a multifactorial, progressive, and an inflammatory disease [1,2]. The atherosclerotic process initiates following dysfunction of arterial endothelial cells including, increment in adhesive molecules and increased permeabilization, combined with changes in the composition of the extracellular matrix. These effects promote macrophage foam cell formation [3–5]. Most important, an increase in cellular oxidative stress contribute to the development of the atherosclerotic lesion which involve increment in arterial wall cells cholesterol and oxysterol accumulation in the arterial wall [6–8].

Macrophages play a major role during early atherogenesis since they accumulate oxidized lipids, cholesterol and triglycerides, leading to foam cells formation [9]. Foam cell accumulation and matrix-degradation are associated with weakening of the fibrous cap leading to plaque rupture and thrombosis [6,10–13]. Macrophage cholesterol accumulation can result from an increased uptake of low-density lipoprotein (LDL) [14–18], decreased rate of high-density lipoprotein (HDL) -mediated cholesterol efflux from the cells [19–22], and/or from increased cholesterol biosynthesis rate [14,18,23]. Macrophage triglyceride accumulation can result from increased triglyceride biosynthesis rate, and/or from decreased triglyceride hydrolysis rate [24–26].

The interaction between the atherosclerotic plaque components and the blood elements, mainly monocyte derived macrophages, is fundamental for understanding atherosclerotic development. Previous studies from our lab evaluated the effects of different extracts from human atherosclerotic plaques on

Abbreviations: 4-HNE, α,β-unsaturated hydroxyl-alkenal (4-Hydroxynonenal); DCFH, 2′,7′-dichlorofluorescin diacetate; DGAT, acyl-coenzyme A: diacylglycerol acyltransferase; FITC, fluorescein–isothiocyanate; HDL, high-density lipoprotein; hLAE, human Lesion Aqueous Extract; hLLE, human Lesion Lipid Extract; HMGCR, HMG-CoA reductase; LDL, low-density lipoprotein; mAAE, mice Aortic Aqueous Extract; mALE, mice Aortic Lipid Extract; MPM, mouse peritoneal macrophages; PON1, paraoxonase 1; PON2, paraoxonase 2; ROS, reactive oxygen species.

* Corresponding author at: The Lipid Research Laboratory, Technion Rappaport Faculty of Medicine, Bat – Galim, Haifa 31096, Israel.
E-mail address: aviram@tx.technion.ac.il (M. Aviram).
URL: http://www.aviramlipids.com (M. Aviram).

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plasma and on cultured macrophages. Tavori et.al [27,28] and Cohen et.al [29–31] analyzed the biochemical composition of human carotid plaque extracts and their influence on the potent antioxidant paraoxonase1 (PON1) activity. Additionally, the effects of the non-polar hydrophobic lesion lipid extract (hLLE) on macrophages [mouse peritoneal macrophages (MPM), and J774A.1 murine macrophage cell line] was accessed. hLLE changed the macrophage morphology to a foam cell-like appearance, extensively increased macrophage oxidative stress, and decreased HDL ability to induce cholesterol efflux [28].

In the above studies, macrophages were cultured in high glucose concentration (25 mM) which is considered high when compared to the normoglycemic blood concentrations (5 mM). High serum glucose concentration in diabetes increases oxidative stress [32–34], alters lipids metabolism [35,36] and is a major risk factor for cardiovascular diseases [37–40], suggesting that the high medium glucose level may further contribute to the effects of the atherosclerotic lesion extracts on macrophages. However, the effects of the different lesion extracts on macrophages have not been examined previously under high glucose levels versus normal glucose level.

The current study was designed to investigate the of protein-rich or lipid-rich extracts from normoglycemic and hyperglycemic apoE$^{-/-}$ mice, widely used as atherosclerotic animal model, on MPM of these mice. We have also evaluated the effects of human carotid plaque extracts, rich in lipids or in proteins, as well as their combination on J774A.1 macrophage oxidative stress, cholesterol and triglyceride metabolism.

2. Materials and methods

2.1. Materials

Fluorescein-isothiocyanate (FITC), 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), streptozotocin, dihydrocumarin, and primary antibody against actin (cat # A3854) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, nystatin, L-glutamine, sodium pyruvate, and enzyme-linked chemiluminescence (ECL) solution were all purchased from Biological Industries (Beth Haemek, Israel). [^{3}H]-labeled acetate, and [^{3}H]-labeled oleic acid were purchased from PerkinElmer (Waltham, MA, USA). Silica gel plates (60F254) from Merck (Darmstadt, Germany). RNA purification kit (MasterPure TM) was obtained from Epicentre Biotechnologies (Madison, Wisc., USA). cDNA preparation kit and ABsolute Blue qPCR ROX mix were purchased from Thermo Scientific (Epsom, UK). Primary antibody against HMGCR was purchased from Santa Cruz Biotechnology cat# sc-271595 (Dallas, TX, USA). HRP-conjugated secondary antibodies were obtained from Jackson Immuno-Research (West Grove, PA, USA). Primary antibody against 4hydroxynonenal (4-HNE) was purchased from Alexis Biochemicals cat # 210-767-R100 (San Diego, CA, USA). PVDF membrane was purchased from Merck Millipore (Darmstadt, Germany).

2.2. Human carotid atherosclerotic lesions (plaques) extracts

Human carotid plaques were obtained from patients undergoing routine endarterectomy in the Department of Vascular Surgery at Carmel and Rambam Medical Centers (Haifa, Israel). Complete atherosclerotic plaques were removed, and were immediately placed in saline and stored at −80 °C. Lesion samples were laid on filter paper to absorb the liquid followed by their weight determination. Two different extracts were prepared: 1. Human lesion aqueous extract (hLAE) in PBS containing 0.1% (v/v) of protease inhibitor cocktail (1 mL/400 mg tissue) was incubated at 4 °C for 30 min followed by centrifugation and supernatant collection. The protein level was then assessed in the supernatant [41], and a stock of 1.5 mg of protein/ml was prepared and stored under −20 °C. 2. Nonpolar hydrophobic lesion lipid extract (hLLE) was prepared from the lesion sediment obtained after hLAE preparation by using ethyl acetate (EtAc) that was added while stirring for 30 min, followed by supernatant filtration and evaporation under nitrogen. The yellow lipid sediment was weighted and dissolved in dimethyl sulfoximine DMSO at a concentration of 50 mg of lipid weight/ml. The study protocol was approved by the hospital Helsinki Committees, with patient's informed consent.

2.3. Animal studies: apoE$^{-/-}$ mice

ApoE$^{-/-}$ mice are widely used as atherosclerotic animal models, since they develop severe hyperlipidemia on a regular chow diet [42]. ApoE$^{-/-}$ mice were provided by courtesy of Prof. Jan Breslow, Rockefeller University, NY, and they were bred and housed in pathogen-free conditions at the Animal Care Facility of the Rappaport Faculty of Medicine. The protocols were approved by the Committee for Supervision of Animal Experiments of the Technion-Israel Institute of Technology (Approval number: IL0080112). Hyperglycemia was induced with single intraperitoneal (IP) injection of 200 mg/kg streptozotocin at 2 months of age. Serum glucose levels were determined within one week and the mice with serum glucose levels in the range of 250–400 mg/dl were included in study group ($n = 6$). The control normoglycemic mice ($n = 7$) were at the same age as the hyperglycemic one. After hyperglycemia duration of 1 month peritoneal macrophages and aortas were harvested from each mouse.

2.3.1. Mice aortic extracts

Aortas from normoglycemic and hyperglycemic mice were isolated at necropsy and immediately stored at −80 °C until further use. Aortas were laid on filter paper to absorb the liquid followed by their weight determination. Two different extracts were prepared from each aorta as described in Section 2.2: 1. Mouse aorta aqueous extract (mAAE), and 2. Mouse aorta lipid extract (mALE). Aqueous or lipid pools of the normoglycemic or hyperglycemic mice aorta extracts were prepared.

2.3.2. Mouse peritoneal macrophages (MPM)

MPM were harvested from the peritoneal fluid of normoglycemic or hyperglycemic apoE$^{-/-}$ mice (weight 19–25 g) 3 days after IP injection

Table 1
Atherosclerotic mice (apoE$^{-/-}$) aortic extract composition of protein, cholesterol and triglycerides.

	Protein level (μg/mg aortic weight)	Cholesterol level (ng/mg aortic weight)	Triglyceride level (ng/mg aortic weight)
Normoglycemic mAAE (Aqueous)	12 ± 0.7	7.7 ± 1.2	90 ± 4.8
Hyperglycemic mAAE (Aqueous)	16 ± 0.6	5.7 ± 1.8	134 ± 26
Normoglycemic mALE (Lipid)	0.0023 ± 0.0002*	13 ± 5.5	350 ± 13
Hyperglycemic mALE (Lipid)	0.0023 ± 0.0003*	12 ± 0.8	382 ± 50

mAAE- Mouse aortic aqueous extract, mALE- Mouse aortic lipid extract. Lipidic vs. aqueous extract from the same group. #$p < 0.05$, Hyperglycemic vs. normoglycemic extract.
* $p < 0.05$.

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