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Aged garlic extract inhibits homocysteine-induced scavenger receptor CD36 expression and oxidized low-density lipoprotein cholesterol uptake in human macrophages in vitro

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ABSTRACT

Aim of the study: Expression of CD36 scavenger receptors on macrophages is involved in oxidized low-density lipoprotein (OxLDL) uptake and foam cell formation during atherosclerotic lesion development. It has been shown previously in vitro and in vivo that the atherosclerotic risk factor homocysteine (Hcy) stimulates macrophage CD36 expression and OxLDL uptake. We now examined the effects of aged garlic extract (AGE), a garlic preparation enriched in water-soluble organic sulfur-containing compounds, on Hcy-induced CD36 expression and foam cell formation in human monocytes/macrophages.
Results: Incubation with Hcy (200 μM for 72 h) in THP-1-derived macrophages and primary human macrophages caused a 37.8 ± 5.2% and 60.7 ± 4.2% increase in CD36 expression compared to control, respectively. Coincubation with AGE (5 mg/ml) significantly suppressed CD36 expression in THP-1 derived macrophages by 48.6 ± 9.0% compared to Hcy-incubated cells only. AGE (1–5 mg/ml) dose dependently inhibited Hcy-induced CD36 expression in primary human macrophages, and decreased binding of nuclear proteins to a PPARγ response element. Preincubation with AGE significantly inhibited DiI-labeled OxLDL uptake.
Conclusions: These data indicate that AGE inhibits CD36 expression and OxLDL uptake in human macrophages by modulating the PPARγ pathway, and suggest that the extract could be useful for the prevention of atherosclerotic lesions.

1. Introduction

Mild hyperhomocysteinemia is an independent risk factor for atherosclerotic vascular disease and atherothrombosis (Weiss et al., 2002; Weiss, 2005). The mechanisms by which homocyteine (Hcy) exerts its proatherogenic effects are still not completely understood but include induction of endothelial activation and dysfunction (Weiss et al., 2001), proliferation of vascular smooth muscle cells (Tsai et al., 1994), modification of low-density lipoprotein (LDL) and scavenger receptor uptake (Griffiths et al., 2006), platelet activation (Durand et al., 1997), and monocyte adhesion to endothelial cells (Postea et al., 2006). All these events are considered to contribute to the initiation and progression of atherosclerotic lesions and thrombus formation.

During formation of early atherosclerotic lesions, macrophage differentiation and increase in cholesterol scavenging receptor expression play important roles in the uptake of oxidized LDL (OxLDL) and foam cell formation (Schaffner et al., 1980; Takahashi et al., 1992; Endemann et al., 1993). CD36, a class B scavenger receptor, is an 88 kDa glycoprotein expressed on platelets, monocytes, macrophages, capillary endothelial cells and adipocytes (Greenwalt et al., 1992). CD36 is upregulated by OxLDL and other stimuli via the peroxisome proliferator-activated receptor γ (PPARγ) pathway (Nagy et al., 1998) and via protein kinase C (PKC) (Feng et al., 2000). These findings suggest that CD36 is crucially involved in foam cell formation in atherosclerotic lesions. Previously we have shown that incubation of human monocyte-derived macrophages with Hcy increases CD36 expression (Ide et al., 2006). Furthermore, Hcy-fed apolipoprotein E knockout mice showed increased immuno-positive staining for CD36 in atherosclerotic lesions together with increased fatty streak lesions compared to mice fed a normal chow (Thampi et al., 2008).

Garlic (*Allium sativum* L.) and its constituents have been used in traditional medicine for centuries. Among the various garlic preparations available, aged garlic extract (AGE) has a unique manufacturing process leading to an enrichment in water-soluble

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organic sulfur-containing compounds such as S-allylcysteine and S-allylmercaptocysteine but in a reduction of harsh compounds from typical garlic odor (Amagase et al., 2001). Several pharmacological effects of AGE had been identified (Morihara et al., 2002, 2006). AGE and its constituents have been reported to possess beneficial properties such as the prevention of some malignancies (Amagase and Milner, 1993; Tanaka et al., 2004) and of cardiovascular diseases (Budoff et al., 2004). The cardiovascular effects of AGE are thought to be at least partly due to its antioxidant and thiol modifying properties (Ide and Lau, 1999, 2001; Morihara et al., 2005). Most importantly, AGE has recently been shown to inhibit the progression of coronary calcifications in patients with coronary artery disease already under treatment with statins (Budoff et al., 2004; Budoff, 2006).

In the present study, we determined the effects of AGE on Hcy-induced CD36 expression and foam cell formation in human monocytes/macrophages (THP-1 cells and primary human monocytes) using flow cytometry (FACS) and explored potential molecular mechanisms. We here report that AGE inhibits Hcy-induced CD36 expression by modulating the PPARγ pathway and inhibits OxLDL uptake into human macrophages. Our data suggest that AGE could modulate the formation of early atherosclerotic lesions and therefore may be useful for the prevention of atherosclerosis.

2. Materials and methods

2.1. Chemicals

AGE was kindly provided by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan). Bovine serum albumin (BSA), dexamethasone, dimethyl sulfoxide (DMSO), Ficoll-Hypaque, ethylendiaminetetraacetic acid (EDTA), Hcy, paraformaldehyde, phorbol 12-myristate 13-acetate (PMA), 2-chloro-5-nitro-N-phenylbenzamide (GW9662), troglitazone and Kodak BioMax Light film were purchased from Sigma–Aldrich Co. (Taufkirchen, Germany). Accutase and RPMI 1640 medium were obtained from PAA laboratories (Coelbe, Germany). Novex 6% DNA retardation gel, penicillin–streptomycin solution, fetal bovine serum (FBS) and 1,1′-dioctadecyl-3,3,3′3′-tetra-methylindocyanide percholorate (DiI) were from Invitrogen (Karlsruhe, Germany). Econo-Pac 10 DG column and DC Protein Assay Kit were purchased from BioRad Laboratories (Munich, Germany). Millex-GP sterilizing filter units (0.22 μm) were obtained from Millipore (Bedford, MA, USA). Cupric sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) was from Merck (Darmstadt, Germany). Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD36 were obtained from Immunotech (Marseille, France). FITC-conjugated mouse IgG1 isotypic control was purchased from Beckman Coulter, Inc. (Miami, FL, USA). Transfer membrane Biodyne B was from Pall Life Sciences (Dreieich, Germany). PPARγ antibody was obtained from Affinity BioReagents (Golden, USA). Nuclear extraction kits and EMSA gel-shift kits were purchased from Panomics (Fremont, CA, USA).

2.2. Aged garlic extract (AGE)

AGE is manufactured as follows: garlic gloves (*Allium sativum* L.) grown under strictly controlled organic conditions without the use of chemical fertilizers, herbicides or pesticides are sliced and soaked in an aqueous ethanol solution and extracted/aged up to 20 months at room temperatures. Chromatographic experiments including a LC/MS method identified that AGE as used in this study contained S-allylcysteine as a major sulfur-containing compound in the range of 1.6–2.4 mg/g (dry weight). Other constituents such as S-allylmercaptocysteine, fructosyl-arginine and tetrahydro-β-carboline derivates have been identified. S-allylcysteine concentration is used to standardize the extract according to the US Pharmacopeia/Natural Formula (USP/NF) garlic fluid extract monograph (United States Pharmacopeial Convention I, 2005).

AGE was obtained as a 300 mg/ml stock solution and diluted in cell culture medium to the final concentrations used (1–5 mg/ml). This concentration range had been used in previous in vitro studies and mimics the situation in human trials, where daily doses of 1–5 g are supplemented (Budoff et al., 2004; Budoff, 2006).

2.3. Cell culture

Human THP-1 cells (Tsuchiya et al., 1980) were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 200 units/ml penicillin and 0.2 mg/ml streptomycin. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 3–4 days before being used for experiments. Cells were differentiated into macrophages by incubation with PMA (10 nM).

Human primary mononuclear cells were isolated from healthy volunteers by density-gradient centrifugation using Ficoll-Hypaque. Freshly isolated monocyte-derived macrophages have been reported to express CD36 after 8–12 days of culture (Devaraj et al., 2001). Therefore isolated cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 200 units/ml penicillin and 0.2 mg/ml streptomycin for 7 days and medium was exchanged every 2–3 days before experiments.

Viability of cells throughout the experiments was always >95% as determined by trypan blue exclusion. None of the incubation modalities had any effect of cell viability.

2.4. Isolation and modification of low density lipoprotein

Low density lipoprotein (LDL; 1.019–1.063 g/ml) was isolated from EDTA–plasma of fasting healthy volunteers by sequential ultracentrifugation, desalted through a column (Econo-Pac 10DG) and sterilized by filtration through a 0.22 μm pore size filter. Protein concentration was determined using the DC Protein Assay Kit. OxLDL was prepared by incubation of LDL (0.5 mg protein/ml) with 10 μM $CuSO_4$ overnight at 37 °C. The extent of oxidative modification was evaluated by examining relative electrophoretic mobility of LDL in an agarose gel and by measuring conjugated dienes at 234 nm as described previously (Jialal et al., 1995).

OxLDL was labeled using the fluorescent probe DiI according to the method described by Innerarity et al. (1986). Briefly, OxLDL was incubated overnight at 37 °C with 50 μl of DiI in DMSO (3 mg/ml) per mg of LDL protein. Labeled OxLDL was then reisolated by ultracentrifugation.

2.5. Flow cytometry analysis for CD36 expression

THP-1 cells in the presence of PMA (10 nM) or primary human monocytes after culture for seven days (1×10^6 cells in 6-well plates) were incubated with Hcy (200 μM) and/or AGE (1, 2.5 and 5 mg/ml) at 37 °C for 72 h. Incubation medium was exchanged every 24 h. Coincubation with dexamethasone (10 nmol/l), an inhibitor of macrophage differentiation, was used as a negative control. After incubation, cells were washed with PBS and detached from the plates using accutase at 37 °C for 10 min. Floating cells were collected and centrifuged at 2000 rpm for 5 min at 4 °C, washed with ice cold PBS, and centrifuged again. The pellet was resuspended in PBS containing 1% BSA, incubated with FITC-conjugated antibodies against CD36 or an isotypic control antibody on ice for 50 min. Cells were then washed with ice cold PBS to remove unspecific bound