Recent Advances on the Nutritional Effects Associated with the Use of Garlic as a Supplement

Molecular Basis by Which Garlic Suppresses Atherosclerosis

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ABSTRACT The aim of this study was to determine the mechanism by which the aged garlic extract “Kyolic” has a protective effect against atherosclerosis. Plasma cholesterol of rabbits fed a 1% cholesterol-enriched diet for 6 wk was not reduced by supplementation with 800 μL Kyolic/(kg body · d). In spite of this, Kyolic reduced by 64% (P < 0.05) the surface area of the thoracic aorta covered by fatty streaks and significantly reduced aortic arch cholesterol. Kyolic also significantly inhibited by ~50% the development of thickened, lipid-filled lesions in preformed neointimas produced by Fogarty 2F balloon catheter injury of the right carotid artery in cholesterol-fed rabbits. In vitro studies found that Kyolic completely prevented vascular smooth muscle phenotypic change from the contractile, high volume fraction of filament (Vvmyo) state, and inhibited proliferation of smooth muscle cells in the synthetic state with a 50% effective dose (ED50) of 0.2%. Kyolic also slightly inhibited the accumulation of lipid in cultured macrophages but not smooth muscle, and had no effect on the expression of adhesion molecules on the surface of the endothelium or the adherence of leukocytes. It is concluded that Kyolic exerts antiatherogenic effects through inhibition of smooth muscle phenotypic change and proliferation, and by another (unclarified) effect on lipid accumulation in the artery wall. J. Nutr. 131: 1006S–1009S, 2001.

KEY WORDS: atherosclerosis • garlic • rabbits • smooth muscle • cholesterol

Extracts of garlic are known to reduce serum cholesterol levels in humans, inhibit cholesterol biosynthesis, suppress LDL oxidation, lower plasma fibrinogen and increase fibrinolytic activity (Bordia et al. 1977, Harenberg et al. 1988), and to possess antiatherosclerotic properties (Lau et al. 1987, Phelps and Harris 1993). However, atherosclerosis is a complex disease, which can be described as an excessive inflammatory, fibro-fatty, proliferative response to damage of the artery wall involving several cell types, particularly smooth muscle cells (SMC), monocyte-derived macrophages, T-lymphocytes and platelets (Schwartz et al. 1993). Many people believe it can be induced from simple dysfunction of the endothelial lining as occurs with hyperlipidemia, hypertension or cigarette smoke, causing imbalance of angiotensin II and nitric oxide production in the artery wall (Dusting et al. 1998, Luscher and Noll 1995). This initiates a cascade of events, which includes expression of adhesion molecules on the surface of the endothelium, the oxidation of lipoproteins, monocyte invasion of the vessel wall, foam cell formation, smooth muscle phenotypic change, and proliferation and platelet deposition (Ross 1993).

In this study, we determined the effect of the aged garlic extract (AGE) Kyolic (Wakunaga Company, Mission Viejo, CA) on the development of atherosclerosis in a rabbit model of the disease as an extension of our earlier report (Efendy et al. 1997), and examined how it might function at a cellular and molecular level using cell culture techniques.

MATERIALS AND METHODS

In vivo

At time 0, New Zealand White rabbits (female) had their right carotid artery de-endothelialized with a 2F Fogarty balloon catheter to induce the formation of a myointimal thickening (Efendy et al. 1997). Two weeks after surgery the rabbits were divided randomly into four groups (n = 6/group) as follows: Group 1, standard diet (Norco High Fiber pellets mixed 1:1 with oaten and lucerne chaff); Group 2, standard diet + 800 μL Kyolic/(kg body · d); Group 3: 1% cholesterol-enriched diet; Group 4: 1% cholesterol-enriched diet + 800 μL Kyolic/(kg body · d). The 1% cholesterol diet was prepared by dissolving 200 g cholesterol (Sigma Chemical, St. Louis, MO) in 1 L diethyl ether, which was mixed with 10-kg standard pellets. After overnight evaporation of the solvent, the 2% cholesterol-impregnated pellets were mixed 1:1 with chaff to obtain a 1% cholesterol diet. Rabbits in Groups 2 and 4 were administered Kyolic via a 1-ml syringe inserted between their front incisors and back molars each morning at the time of feeding. After a further 6 wk, the rabbits were
killed by a lethal dose of sodium pentobarbitone and the vessels excised and prepared for morphometric, histologic and biochemical examination.

At time 0, 2, 4, 6 and 8 wk (termination), all rabbits were bled (2–2.5 mL) via their central ear vein for plasma cholesterol analysis using a Cobas-Bio centrifugal autoanalyzer (Roche, Basel, Switzerland) utilizing the commercial diagnostic kit Monotest Cholesterol (Boehringer Mannheim, Mannheim, Germany). Cholesterol concentration (mg/g tissue) of the washed and blotted aortic arch was determined after lipid extraction and purification using a spectrophotometric assay (Zlatkis et al. 1953).

Whole lengths of thoracic aorta (3–4 cm) were cut longitudinally, rinsed, fixed in 10% buffered formal saline and stained for 40 min in a saturated solution of Oil-Red-O (Sigma Chemical) in isopropyl alcohol. The percentage of luminal area of the thoracic aorta covered by fatty streaks was then measured using the Mocha 227 Image analysis system (Jandel Scientific, San Raphael, CA). For morphometric analysis of the atherosclerotic lesions formed in the de-endothelialized right carotid artery, the vessel from each rabbit was fixed in 10% buffered formal saline, embedded in paraffin, and 7-μm cross sections were stained with toluidine blue and photographed under light microscopy. The area of intima as a percentage of wall area (intima plus media) was measured using the Mocha 227 Image analysis system.

**In vitro**

**Culture procedure.** SMC from the aortic media of rabbits of mixed strains (Central Animal Breeding House, University of Queensland, Queensland, Australia) were dispersed into single cells and plated at $2 \times 10^6$ cells/90-mm dish or $4 \times 10^4$ cells/well in 24-well plates in Medium 199 (M199) + 5% fetal calf serum (FCS) (Campbell and Campbell 1993). For subculture, cells were passaged 3 times, then seeded at $4 \times 10^4$ cells/well in 24-well plates.

The macrophage cell line J774 (American Type Culture Collection, from BALB/C mice) was seeded at $3 \times 10^4$ cells/well in Dulbecco’s modified Eagles medium (DMEM) + 10% FCS. Endothelial cells and SMC were harvested from the rat thoracic aorta according to standard procedure (Campbell and Campbell 1993), plated in primary culture and passaged 5 times before seeding at $3 \times 10^4$ cells/well on glass coverslips in RPMI + 10% FCS + 100 μg/mL heparin (Sigma Chemical) + 20 μg/mL endothelial cell growth supplement (Sigma Chemical) (endothelial cells) or DMEM + 10% FCS (smooth muscle).

**Antibody staining.** Rat endothelial and SMC on coverslips were incubated in 0, 0.1, 1 and 5% Kyolic for 24 h, fixed in methanol at −20°C for 10 min, and blocked in 1% bovine serum albumin in PBS for 20 min at room temperature. Antibody to the adhesive molecule ICAM-1 (Sekagaku, Tokyo, Japan) was added for 2 h; the cells were then washed and incubated with the fluoresceinated secondary antibody fluorescein isothiocyanate (FITC) (Silenus, Victoria, Australia) for 1 h, washed and mounted. Frozen sections of carotid artery were similarly stained with antibodies to smooth muscle α-actin (HHF-35, Dako Laboratories, San Diego, CA).

**Lipid uptake.** Passaged rabbit aortic SMC and J774 macrophages were incubated for 24 h with DMEM + 0.5% FCS to maximize lipoprotein receptor number. Medium was replaced with DMEM + 5% hyperlipidemic serum (which had been exposed to J774 mac-

**FIGURE 1** ICAM-1 immunofluorescence on cultures of rat endothelial cells (A) without and (B) in the presence of 1% Kyolic. Oil-Red-O staining of J774 macrophages after exposure to minimally oxidized 5% hyperlipidemic serum (C) without and (D) in the presence of 1% Kyolic.
enzyme activity.

RESULTS AND DISCUSSION

Due to the general unpalatability of the diet, the rabbits fed cholesterol-enriched pellets weighed 20% less than those provided the standard pellets over the 6-wk period, although this was not significant. In addition, with both the standard and cholesterol-enriched diet, the weight gain over 6 wk in the Kyolic-treated groups (Groups 2 and 4) vs. control (Groups 1 and 3, respectively) was slightly less (−16%), but again this was not significant.

Plasma cholesterol in the Group 3 cholesterol-fed rabbits rose significantly compared with the standard diet Group 1 rabbits (6.0 ± 0.6 vs. 1.3 ± 0.2 μmol/L, with no decrease induced by Kyolic administration in either case (Group 4: 6.2 ± 0.7 and Group 2: 1.3 ± 0.1 μmol/L). There was also no change in lipoprotein profile (VLDL, LDL and HDL) with Kyolic administration.

The rabbits fed a standard diet had no fatty lesions on the intimal surface of their thoracic aortae in the presence or absence of Kyolic. However, in the cholesterol-fed Group 3 rabbits, 70% of the luminal aortic surface was covered by lipid-filled lesions, with only 25% coverage in the Kyolic-treated Group 4 rabbits; this represents a 64% reduction. Similarly, in Kyolic-treated rabbits fed a cholesterol-enriched diet, the level of accumulated cholesterol in the aortic arch was significantly reduced by 26% from 1.7 ± 0.1 to 1.27 ± 0.12 mg cholesterol/g tissue [see Efendy et al. (1997)].

There was a neointima 6–7 cell layers thick in the carotid arteries of rabbits fed a standard diet in the presence and absence of Kyolic. This was greatly increased in Group 3 rabbits fed a cholesterol-enriched diet, with a concentric thickening of fibro-fatty plaque. In the Kyolic-treated, cholesterol-fed Group 4, the neointima was only half the size of the lesion in Group 3 rabbits (23.8 ± 2.3 vs. 42.6 ± 6.5%, < 0.05). There were no lipid-filled cells, and the size of the deep, smooth muscle-rich region was greatly decreased (Efendy, 1994).

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et al. 1997). This was particularly evident when fresh frozen sections were stained with FITC-labeled antibodies to smooth muscle α-actin.

We next examined the mechanism by which these in vivo effects might have occurred. Because there were fewer fatty streaks and thus fewer subendothelial lipid-laden, monocytederived macrophages, we examined the effect of 0.1, 1 and 5% Kyolic on the adhesion of leukocytes to the endothelium by scanning electron microscopy and by the expression of the adhesion molecule ICAM-1 on cultured endothelial cells. In both cases it was found that Kyolic had no effect (Fig. 1A,B). Also, the accumulation of cholesterol (both by spectrophotometry and staining with Oil-Red-O) in cultured macrophages exposed to 5% hyperlipidemic serum (minimally oxidized) for 24 h at 37°C was only slightly decreased by the addition of Kyolic at all concentrations (20 vs. 12 µg/10⁶ cells) (Fig. 1C,D), whereas there was no decreased accumulation of cholesterol in SMC under the same conditions.

However, it was found that 1% Kyolic completely inhibited the change in phenotype that is necessary before SMC can accumulate lipid, migrate, proliferate and synthesize appreciable extracellular matrix (Fig. 2). In primary cell culture, this change in phenotypic expression from a cell whose predominant function is contraction to one whose function is proliferation, synthesis of matrix or accumulation of lipid usually occurs during the first 5 d after enzyme dispersion into single cells; this is due to disruption of the heparan sulfate–rich basal lamina that surrounds each cell (Campbell et al. 1992). It is characterized by a significant decrease in the volume fraction of the smooth muscle cytoplasm occupied by myofilaments, with a concomitant increase in the volume fraction occupied by synthetic organelles such as rough endoplasmic reticulum, Golgi and free ribosomes.

The mechanism by which Kyolic exerts this effect is not known at present. However, it was confirmed by studies in which 1% Kyolic completely inhibited the uptake of [3H]thymidine in primary cultured rabbit aortic SMC over a 24-h incubation. Because the cells are maintained in the contractile phenotype, they are unresponsive to growth factors present in culture medium plus 5% FCS. Kyolic also potently inhibited the proliferation of passaged (synthetic state) SMC with 50% inhibition occurring at 0.2% Kyolic (Fig. 3), indicating a second, separate mechanism by which Kyolic may inhibit the development of a proliferative vascular lesion.

Summary and conclusion

Thus, in this rabbit model of atherosclerosis, administration of garlic in the form of the AGE Kyolic causes a decrease in aortic tissue cholesterol as determined biochemically, a decrease in fatty streak formation and a decrease in the size of atherosclerotic plaque formed in the balloon-injured carotid artery. There was no change in plasma cholesterol level or lipoprotein fractions, only a slight decrease in lipid accumulation in cultured macrophages, and no change in ICAM-1 expression or leukocyte adhesion to the endothelium. This atheroprotective effect, despite no decrease in plasma cholesterol levels, is consistent with the hypothesis of Lau et al. (1987) that Kyolic mobilizes accumulated lipid into the blood stream, where it is cleared from the body. It would also provide an explanation for the lower weight gain observed in rabbits given Kyolic.

The most significant cellular effect seen with Kyolic was the maintenance of the contractile smooth muscle cell phenotype under conditions such as injury, in which change to the proliferative, synthetic state usually occurs. In the contractile phenotype, which is the phenotype of smooth muscle in the mature, noninjured vessel wall, the cells do not migrate, synthesize appreciable matrix or accumulate lipid. They also do not respond to mitogens present in serum (Campbell et al. 1992). All of these functions are crucial to the development of atherosclerosis. Inhibition of phenotypic change to the state in which these functions can occur may be a major mechanism whereby Kyolic exerts its antiatherogenic effect.

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LITERATURE CITED


