Water-Soluble Hexasulfobutyl[60]fullerene Inhibit Low-Density Lipoprotein Oxidation in Aqueous and Lipophilic Phases (44517)

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Abstract. **Oxidative modification of low-density lipoprotein (LDL) plays a pivotal role in the pathogenesis of atherosclerosis. Increasing the resistance of LDL to oxidation may therefore mitigate, or even prevent, atherosclerosis. A new water-soluble C_{60} derivative, hexasulfobutyl[60]fullerene [C_{60} - $(CH_2CH_2CH_2CH_2\text{-}SO_3Na)_6$; FC_4S], consisting of 6 sulfobutyl moieties covalently bound onto the C_{60} cage is a potent free radical scavenger. This study explored the antioxidative effect of sulfobutylated fullerene derivatives (FC_4S) on LDL oxidation. FC_4S was found to be effective in protecting LDL against oxidation induced by either Cu^{2+} or azo peroxyl radicals generated initially in the aqueous or lipophilic phase, respectively. Levels of the oxidative products, conjugated diene and thiobarbituric acid-reactive substances, and the relative electrophoresis mobility of the LDL were decreased. The addition of 20 μ*M* FC_4S at the early stage of oxidation increased the kinetic lag time from 69 ± 11 to 14 ± 10 min ($P < 0.05$) and decreased the propagation rate from 17.1 ± 2.6 to 6.3 ± 1.0 mOD/min ($P < 0.005$). Persistent suppression of peroxidation reaction was observed upon further addition of FC_4S after full consumption of all endogenous antioxidants during the propagation period. Intravenous injection of hypercholesterolemic rabbits with FC_4S (1 mg/kg/day) efficiently decreased atheroma formation. Data substantiate the use of FC_4S as an excellent hydrophilic antioxidant in protecting atheroma formation, *via* removing free radicals, in either aqueous or lipophilic phase.**

[P.S.E.B.M. 2000, Vol 224:69–75]

The oxidative modification of low-density lipoprotein (LDL) is apparently a contributing factor to the pathogenesis of atherosclerosis. It is known that oxidatively modified LDL is internalized *via* a scavenging receptor on macrophages, leading to lipid accumulation and foam cell formation (1–3). Thus, increasing the resistance of LDL to oxidation should mitigate or even prevent atherosclerosis. Several studies have been performed on the ability of natural and synthetic antioxidants to attenuate atherosclerosis in animal (4–6) and human models (7, 8). Owing to its high solubility in lipid and association with the LDL particle, vitamin E is generally regarded as one of the most important antioxidants for inhibiting lipid peroxidation of LDL (9, 10). This antioxidative role of vitamin E has undergone re-evaluation. Bowry and Stocker (11) suggested that α-tocopherol facilitates the transfer of radical reactions from the aqueous phase into LDL and mediates radical chain reactions within the lipoprotein particle. Much effort is currently underway in searching for new effective antioxidants lacking such prooxidant activity.

Since the discovery and large-scale synthesis of fullerene (C_{60}), its physical and chemical properties have been studied extensively (12, 13). Despite interest in potential activities in biological systems, information remains limited primarily due to the poor solubility of the compound in aqueous environments. The recent synthesis of water-soluble derivatives of fullerenes offers more promise toward the preparation of new biologically active compounds. Such

This study was supported by grants 87-2314-B002-351 and 86-2316-B002-015-M29 from the National Science Council of the Republic of China.

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Received October 4, 1999. [P.S.E.B.M. 2000, Vol 224]
Accepted January 14, 2000.

0037-9727/00/2242-0069$15.00/0

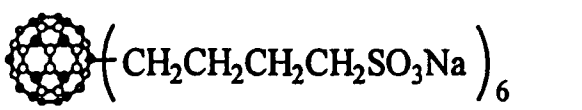
derivatives can affect the activity of certain enzymes (14). The water-soluble polyhydroxylated fullerene derivatives (fullerenols) and C_{60}-dimalonic acid are both highly effective in eliminating superoxide radicals (15, 16). Since free radicals and redox active transition metals are assumed to be responsible for LDL oxidation (17), it is conceivable that water-soluble fullerene derivatives might be protecting LDL from oxidation.

A new class of water-soluble C_{60} derivatives, namely, hexasulfobutyl[60]fullerene (FC_4S) [C_{60}-($CH_2CH_2CH_2$-$CH_2SO_3Na)_6$] (Fig. 1), composed of six sulfobutyl functional groups covalently bound on a C_{60} cage, was synthesized (18) and used in this study for evaluation of its efficacy as an antioxidant in preventing LDL oxidation. The radical initiation systems used for the induction of LDL oxidation include Cu^{2+} ions and azo initiators 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), yielding water-soluble and lipid-soluble peroxyl radicals, respectively, at known and reproducible rates after thermolysis (19).

Materials and Methods

Chemicals. The compound FC_4S was synthesized in a yield of 80%–85% by the treatment of fullerene molecules in a mixture of toluene and tetrahydrofuran or dimethoxyethane with sodium naphthalide at ambient temperatures, followed by reacting the resulting hexaanionic fullerene intermediates with an excess of 1,4-butane sultone (18). AAPH, AMVN, and diethylenetriaminepentaacetic acid (DTPA) were obtained from Waco Pure Chemical (Osaka, Japan). The $CuSO_4$, NaCl, Na_2HPO_4, KH_2PO_4, KCl, thiobarbituric acid, *n*-butanol ethylenedi-aminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) were purchased from Merck (Darmstadt, Germany), and bovine serum albumin and 1,1,3,3-tetraethoxypropane were obtained from Sigma (St. Louis, MO).

Isolation of LDL. Plasma was prepared from healthy, normolipidemic volunteers after overnight fast from blood collected in EDTA (3.4 m*M*). LDL was isolated by sequential ultracentrifugation in sodium bromide solution at a density between 1.021 g/ml and 1.063 g/ml (20). The lipoproteins were dispersed in phosphate-buffered saline (PBS) consisting of NaCl (140 m*M*), KCl (2.7 m*M*), Na_2HPO_4 (8.13 m*M*), KH_2PO_4 (1.47 m*M*) pH 7.4, were flushed with N_2. Samples were stored at 4 ° C and used within 1 week. For oxidation, lipoprotein preparations were dialyzed against 3 × 1000 volumes of vacuum degassed PBS at 4°C for 24 hr. The concentration of LDL is reported as mg of protein as measured by the modified Lowry method (21) using bovine serum albumin as a standard.



Hexa(sulfobutyl)[60]fullerene (FC_4S)

Figure 1. The structure of water-soluble hexasulfobutyl[60]fullerene [C_{60}—($CH_2CH_2CH_2CH_2$-$SO_3Na)_6$; FC_4S]

Oxidation of LDL. Unless otherwise stated, all reagents were suspended (LDL) or prepared in PBS. Samples of LDL (1 mg/ml) were diluted to 100 μg/ml and incubated at 37°C. Oxidation reactions were initiated by the addition of 2 μl of 1 m*M* $CuSO_4$–1000 μl of LDL at a final concentration of 2 μ*M* (17) or by adding 5 μl of 400 m*M* AAPH and 10 m*M* DTPA (final concentrations of 2 m*M* and 50 μ*M*, respectively) to 1000 μl of the LDL preparation (22). Oxidation reactions were also carried out using 2 m*M* AMVN by adding 5 μl of 400 m*M* AMVN dissolved in ethanol to 1000 μl of LDL aqueous suspension with the final ethanol concentration less than 1% (v/v) (23). The experiment was performed in the absence or presence of FC_4S at a final concentration of 10–100 μ*M*, which was added either at the beginning of the oxidation reaction or at various times thereafter. Each sample had its own blank containing all components except LDL. A sample of LDL in the absence of initiating agents was used to assess spontaneous autoxidation, a value that was excluded from all experimental readings. Unless otherwise indicated, incubation was continued until the steady state of the maximum lipoprotein oxidation was achieved. The reaction was terminated by addition of 3 m*M* EDTA and 100 μ*M* BHT and transfer to 4°C.

Monitoring Oxidative Modification. *Conjugated diene formation.* Kinetics of the conjugated diene formation was followed by continuously monitoring the change in the absorbance at 234 nm using a Beckman DU 70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA), thermostatted at 37°C and equipped with an automatic sample change permitting the simultaneous monitoring of up to 12 samples. The duration of the lag time was calculated by extrapolating the propagation phase. The propagation, rate expressed as the change in absorbance/min, is the mean reaction rate of the propagation phase in the kinetic curve. The peak time is defined as the time when the maximum absorbance was reached (24).

Lipid peroxide measurement. The content of thiobarbituric acid-reactive substances (TBARS) was used as a measure of lipid peroxide levels (25). TBARS were expressed as malondialdehyde equivalents, using freshly diluted 1,1,3,3-tetramethoxypropane as a standard, and measured by fluorimetric assay (26). Briefly, native or oxidized lipoprotein was added to thiobarbituric acid and incubated at 95°C for 1 hr to produce the adducts. After cooling to room temperature, *n*-butanol was added to extract the product, and fluorescence was measured with excitation and emission at 515 and 552 nm, respectively.

Electrophoresis. The relative level of negative surface charges on lipoproteins was measured by electrophoresis on 0.5% agarose gels in 0.05 *M* barbital buffer, pH 8.6, at 150 V for 40 min. The gel was stained with Sudan Black B. The relative electrophoretic mobility was defined as the

ratio of the migration distance of oxidized to native LDL (17). If FC_4S was used during oxidation, the same concentration of FC_4S was added to the native LDL before electrophoresis.

Delineation of Atherosclerotic Lesions in Rabbits. *Animals and diets.* Male New Zealand white rabbits weighing 2.5–3 kg were divided randomly into three groups of five. The reference rabbits were fed a regular laboratory rabbit chow (Purina 5321, St. Louis, MO). The other two groups were both fed a high-fat and high-cholesterol diet to induce atheroma formation as in our previous study (27). One group of these rabbits was injected with FC_4S at a dose of 1 mg/kg/day iv. All rabbits were housed in individual cages with raised screen bottoms in a room maintained at a constant temperature and kept on a 12:12-hr light:dark cycle. Food and water were given daily. The study conformed to the guidelines for the care and use of animals approved by the National Taiwan University Medical Center.

Tissue harvest for the delineation of atherosclerotic lesions. At the end of 6 weeks of feeding test diets, rabbits were anesthetized and sacrificed for delineation of atherosclerotic lesions (27). A midline thoracotomy was performed, and the ascending aorta was quickly removed from the aortic valve cups to the arifice of the left subclavian artery. The excised aorta was fixed in neutral 10% formation for 24 hr, rinsed in 70% ethanol, and immersed in Herxeheimer's solution (5% Sudan IV in ethanol and acetate) at room temperature 15 min. The tissues were transferred to 80% ethanol for 20 min and washed in running water for 1 hr. The percentage of aortic intima infiltrated by lipids was delineated by planimetry from the distribution of sudanophilia.

Statistical Analysis. Unless otherwise indicated, the data are presented as the mean ± standard deviation of triplicate determinations. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by a Scheffe test. A value of $P < 0.05$ was considered significant.

Results

Conjugated Diene Formation. Nonoxidized LDL had a low TBARS content (0.04 ± 0.02 nmole/100 μg protein). Incubation of LDL at 37°C in PBS with 2 μ*M* Cu^{2+}, 2 m*M* AAPH/50 μ*M* DTPA, or 2 m*M* AMVN resulted in oxidative modification of LDL as shown by increased levels of conjugated diene (Fig. 2). Addition of FC_4S at concentrations as low as 10 μ*M* inhibited both Cu^{2+}-induced and AAPH-induced LDL oxidation. Interestingly, the water-soluble FC_4S also inhibited LDL oxidation induced by the lipid-soluble peroxyl radicals resulting from the thermolysis of AMVN. As shown in Table I, FC_4S modified the kinetics of conjugated diene production, increasing the lag time from 69 ± 11 min in the control to 118 ± 12 min and 142 ± 10 min in the presence of 10 μ*M* and 20 μ*M* FC_4S, respectively (both $P < 0.05$). Peak time was similarly affected in the presence of FC_4S (from 163 ± 18 min for control to 261 ± 20 min and 300 ± 28 min for 10 μ*M* and 20 μ*M* FC_4S,

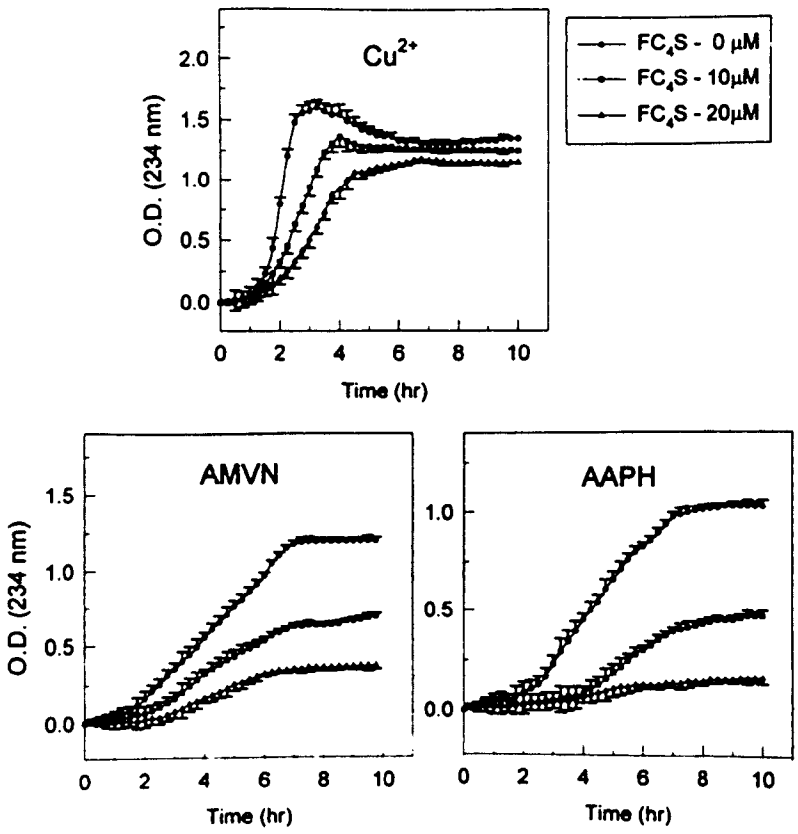


Figure 2. FC_4S protects LDL against oxidation by copper, AMVN or AAPH: conjugated diene formation. LDL (100 μg/ml) was incubated at 37°C with 2 μ*M* copper, 2 m*M* AAPH/50 μ*M* DTPA, or 2 m*M* AMVN for the indicated time period. Data are expressed as mean ± SD.

Table I. Alterations in Parameters of Cu^{2+}-Induced LDL Oxidation in the Absence or Presence of FC_4S

Parameters	FC_4S (μM)		
	0	10	20
Lag time (min)	69 ± 11	118 ± 12[a]	142 ± 10[a]
Peak time (min)	163 ± 18	261 ± 20[b]	300 ± 28[b]
Propagation rate (mOD/min)	17.1 ± 2.6	8.8 ± 1.6[a]	6.3 ± 1.0[b]
Max. OD	1.6 ± 0.3	1.3 ± 0.2	1.1 ± 0.2[a]

Note. All data are the mean ± SD.
Statistics: [a] $P < 0.05$ as compared with 0 μM FC_4S group, [b] $P < 0.005$ as compared with 0 μM FC_4S group.

respectively; both $P < 0.005$). The propagation rate also decreased by FC_4S [17.1 ± 2.6 mOD/min in the control and 8.8 ± 1.6 mOD/min ($P < 0.05$) or 6.3 ± 1.0 mOD/min ($P < 0.005$) at 10 μM or 20 μM FC_4S, respectively]. Finally, the maximal OD was decreased upon addition of 20 μM FC_4S (from 1.6 ± 0.3 to 1.1 ± 0.2, $P < 0.05$), indicating a decrease in the total amount of diene formation.

Lipid Peroxide Levels and Relative Electrophoretic Mobility. The lipid peroxide content (as assessed by TBARS), of LDL incubated with Cu^{2+} increased with time up to 8 hr, followed by a decrease in the value due to oxidation entering the decomposition phase (Fig. 3). Addition of FC_4S (10–100 μM) resulted in a dose-dependent decrease in Cu^{2+}-induced TBARS levels with complete suppression of peroxidation observed at 100 μM. After 8 hr, the levels of TBARS increased or remained unchanged in samples containing 10 μM and 20 μM FC_4S whereas a concentration of 100 μM FC_4S continued to block TBARS formation. These data showed the necessity of comparing TBARS levels for the test groups before the decomposition phase. Thus, an incubation period of 6 hr was chosen for the measurement of TBARS levels during Cu^{2+}-induced oxidation in subsequent experiments. In the case of AAPH- or AMVN- induced LDL oxidation, TBARS levels increased up to 12 hr (Fig. 3) at a rate inversely proportional to the concentration of the peroxidants. Results of the relative electrophoretic mobility were compatible with those obtained above, being lower in the presence of FC_4S than in the control group [3.2 ± 0.5 for the control and 2.2 ± 0.3 ($P < 0.05$) and 1.6 ± 0.3 ($P < 0.01$) when 10 μM and 20 μM FC_4S, respectively, were present].

Effect of FC_4S on Oxidation During Propagation Phase. To determine whether FC_4S remains active in inhibiting LDL oxidations after the oxidative reaction enters the propagation phase, FC_4S was added 2 hr after the beginning of Cu^{2+}-induced oxidation and 4 hr after initiation with AAPH and AMVN. The propagation phase has been shown to begin after endogenous antioxidants are depleted (17). As shown in Figure 4, reduced levels of conjugated dienes were detected in both the ion-dependent (Cu^{2+}) and ion-independent (AAPH or AMVN) processes upon the addition of 20 μM FC_4S. To assess the effect on lipid peroxide levels, FC_4S was added either at the beginning or at various time intervals after initiation of oxidation. Incubation was continued for a period of 6 hr for Cu^{2+}-induced oxidation or 12 hr for AMVN or AAPH (the difference in incubation time was to avoid reaching the decomposition phase and thus underestimating lipid peroxide levels, as shown in Fig-

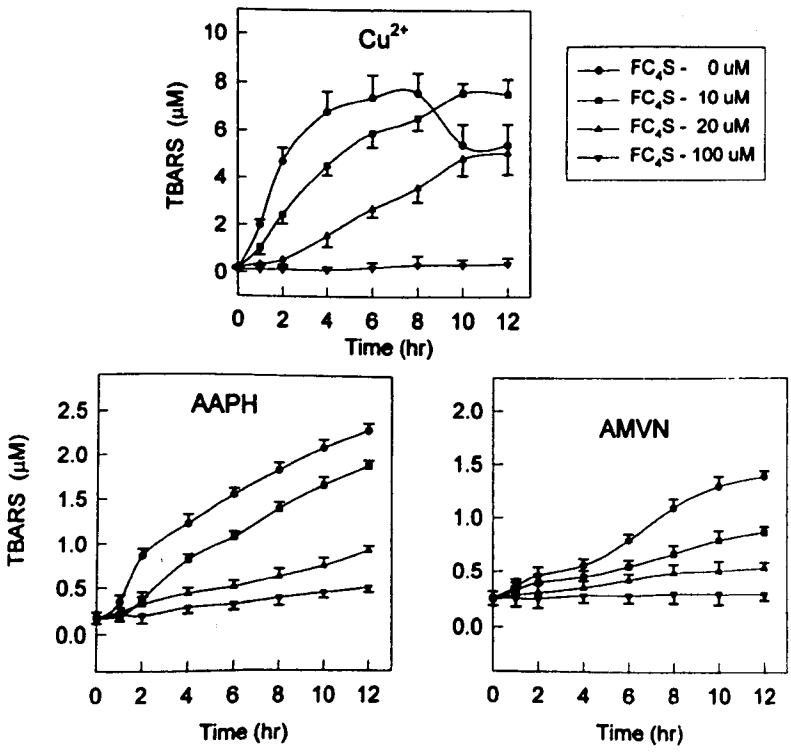


Figure 3. FC_4S protects LDL against copper-, AMVN- or AAPH-induced lipid peroxidation. LDL (100 μg/ml) was incubated at 37°C with 2 μM copper, 2 mM AAPH/50 μM DTPA, or 2 mM AMVN in the absence(●) or presence of 10 μM (■), 20 μM (▲) or 100 μM (▼) FC_4S. The lipid peroxide level was measured as TBARS. Data are expressed as mean ± SD.

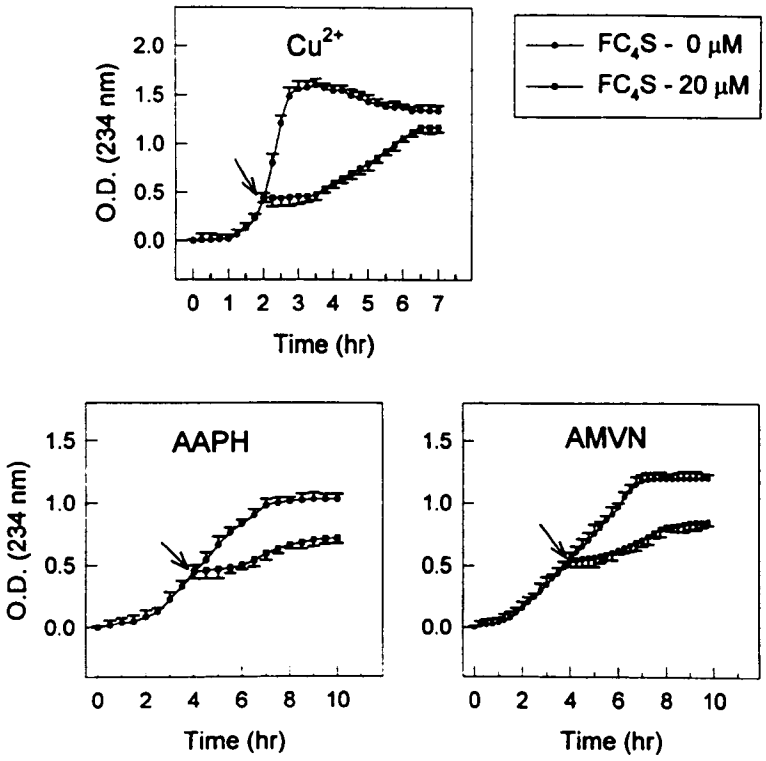


Figure 4. Effect of FC_4S on conjugated diene formation in oxidized LDL entering the propagation phase. LDL (100 μg/ml) was incubated at 37°C with 2 μ*M* copper, 2 m*M* AAPH/50 μ*M* DTPA, or 2 m*M* AMVN in the absence (●) or presence of 20 μ*M* (■) FC_4S. FC_4S was added at 2 hr after initiation of oxidation by Cu^{2+} or 4 hr after initiation by AAPH or AMVN. Data are expressed as mean ± SD.

ure 2). After incubation for the indicated period, the TBARS levels were measured. The percentage of inhibition by FC_4S was calculated by subtracting the control value (without FC_4S) from the experimental data collected at various concentrations (with FC_4S), followed by dividing the difference by the control value. Table II shows that the degree of inhibition was related to the time of addition of FC_4S to mixtures with Cu^{2+}. Furthermore, FC_4S remained active in inhibiting further lipid peroxide production even when the propagation phase of the oxidation process was ongoing. It should be noted that the delayed addition of FC_4S attenuates in apparently less inhibition. The same trend of inhibitory effects was seen using AMVN and, although less marked, AAPH (Table III). Therefore, FC_4S inhibited LDL oxidation irrespective of the administration sequence. Moreover, there was no promotional effect observed.

Table II. Inhibitory Effect of FC_4S on TBARS Formation in Cu^{2+}-Induced LDL Oxidation when FC_4S Added at the Beginning or at Various Time Intervals After Initiating Oxidation

Addition time (min)	% inhibition		
	FC_4S (m*M*)		
	10	20	100
0	16.1 ± 3.2	59.7 ± 10.2	95.1 ± 10.6
30	11.5 ± 4.2	56.1 ± 13.2	84.4 ± 11.6
60	9.3 ± 3.6	52.6 ± 11.5	77.9 ± 10.8
90	6.5 ± 3.7[a]	53.1 ± 12.5	73.9 ± 11.2
120	6.4 ± 2.8[a]	47.8 ± 10.9	63.2 ± 9.8[a]
150	6.2 ± 3.1	45.0 ± 9.0	64.9 ± 10.6[a]
180	6.4 ± 2.6[a]	44.1 ± 9.2	48.4 ± 9.6[b]
240	6.2 ± 3.2[a]	27.0 ± 8.0[a]	46.0 ± 13.5[b]
300	3.3 ± 1.2[b]	26.5 ± 9.4[a]	29.8 ± 9.3[c]
360	2.2 ± 1.0[b]	22.0 ± 7.2[a]	30.5 ± 10.2[c]

Note. All data are the mean ± SD.
Statistics: [a] $P < 0.05$, [b] $P < 0.005$, [c] $P < 0.001$ as compared with respective 0 min group.

Effect of FC_4S on Atherosclerotic Lesions. Figure 5 shows that rats fed the high-fat and cholesterol diet had marked atheroma formation compared with the reference group. FC_4S injection attenuated this effect, but the atheroma area in these rabbits was still higher than in the references.

Discussion

As expected, LDL oxidation was induced efficiently by either transition metal ions or peroxyl radicals. Water-soluble AAPH generates peroxyl radicals in the aqueous phase, whereas the lipid-soluble AMVN generates peroxyl radicals within the lipophilic lipoprotein compartment (19). It is not clear exactly where copper generates free radicals, although it has been suggested that the copper ion binds to lipoprotein and causes decomposition of lipid hydroperoxides *via* a Haber-Weiss type reaction pathway producing peroxyl and alkoxyl radicals at, or near, the lipoprotein surface (28, 29). Recently, a novel model of lipoprotein oxidation referred to as tocopherol-mediated peroxidation has been proposed. The "pro-oxidant" activity of α-tocopherol is believed to be due to the ability of the vitamin to facilitate the transfer of both radicals from the aqueous phase and transition metal ions into the lipoprotein particle resulting in a radical chain reaction (11, 30). Similarly, it has been re-

Table III. Inhibitory Effect of FC_4S on TBARS Formation in AAPH- or AMVN-Induced LDL Oxidation when Added at the Beginning or at Various Times After Initiating Oxidation

Addition time (hr)	% inhibition					
	AAPH			AMVN		
	FC_4S concentration (μM)			FC_4S concentration (μM)		
	10	20	100	10	20	100
0	24.5 ± 6.3	49.9 ± 4.9	80.1 ± 10.2	25.3 ± 7.8	78.1 ± 10.2	94.0 ± 12.7
1	23.2 ± 5.2	46.6 ± 5.6	78.9 ± 9.8	27.0 ± 6.9	61.4 ± 9.7	89.9 ± 15.2
2	22.7 ± 5.8	39.5 ± 8.2	78.1 ± 10.7	22.6 ± 7.3	51.7 ± 9.6[a]	77.5 ± 9.7
4	22.6 ± 4.2	40.6 ± 7.9	67.3 ± 8.9	20.4 ± 5.6	40.1 ± 8.3[a]	65.5 ± 10.6[a]
6	21.0 ± 3.0	37.1 ± 9.8	63.1 ± 10.2	18.7 ± 7.9	38.9 ± 9.7[a]	62.0 ± 8.9[a]
8	22.6 ± 3.6	37.1 ± 9.6	52.5 ± 7.6[a]	16.2 ± 5.2	35.8 ± 9.1[a]	42.6 ± 7.8[b]
10	21.4 ± 4.7	36.6 ± 9.7	52.6 ± 9.7[a]	15.6 ± 4.0	30.9 ± 5.6[b]	43.9 ± 6.9[b]

Note. All data are the mean ± SD.
Statistics: [a]$P < 0.05$, [b]$P < 0.005$ as compared with respective 0 hr group.

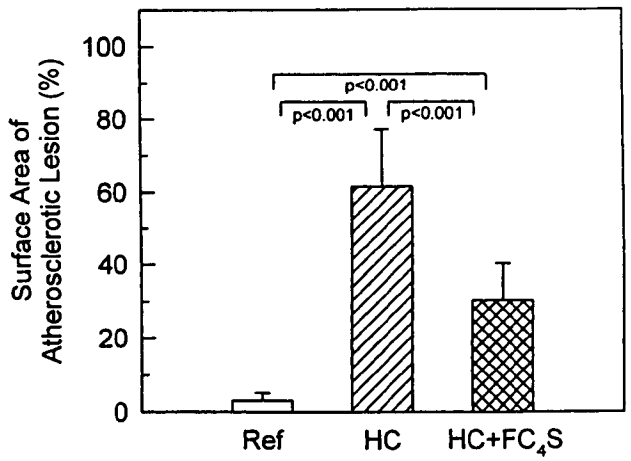


Figure 5. Effect of FC_4S on atherosclerotic lesions in the ascending aorta of rabbits. Rabbits were fed a regular laboratory chow (Ref) or a high fat and cholesterol diet with (HC + FC_4S) or without (HC) intravenous injection of FC_4S for 6 weeks. The ascending aorta was removed, and surface area of atherosclerotic lesion delineated. Data are expressed as mean ± SD ($n = 5$ in each group).

ported that ascorbic acid may act as a pro-oxidant in the presence of transition metal ions (31). The data presented here show that FC_4S is effective as an antioxidant against ion-dependent or ion-independent LDL oxidation. Interestingly, our finding of high efficiency of water-soluble FC_4S to protect LDL against oxidation induced by the lipophilic peroxyl radical is in marked contrast to the generally accepted concept that water-soluble antioxidants scavenge free radicals only in the aqueous phase. For example, vitamin C inhibits AAPH-induced lipoprotein oxidation, but not AMVN-induced oxidation (32). The primary mechanisms by which water-soluble antioxidants protect lipoprotein from oxidation involve scavenging of aqueous free radicals prior to their attack on the lipoprotein lipids and/or sparing and regenerating lipoprotein-associated antioxidants (33). The lipid-soluble antioxidants associated with lipoprotein act as chain-breaking agents to suppress lipid peroxidation by reducing chain-carrying lipid peroxy radicals or by forming covalent adducts with them. In contrast to water-soluble preventive antioxidants, chain-breaking antioxidants cannot prevent the initiation of lipid peroxidation or preserve endogenous antioxidants in lipoprotein (34, 35). With respect to these two properties, our data reveal a hitherto unrecognized antioxidative activity of water-soluble antioxidants with FC_4S acting as both a preventive and chain-breaking antioxidant, as shown by substantial prolongation of the lag phase time and reduction of the propagation rate (Fig. 2 and Table I) and the decreased reactivity of the propagation phase (Fig. 4, Tables II and III).

Although it is not known how FC_4S protects LDL against metal ion–dependent oxidation and peroxyl radical, a hypothetical mechanism involves the trapping of aqueous free radicals owing to its easy partition into aqueous media. The later phenomenon arises from the unique high electronegativity of fullerene derivatives. The other possibility includes specific binding of FC_4S to LDL, although the partition ratio remains unknown. However, it provides a plausible explanation for how water-soluble FC_4S compounds inhibit the lipophilic initiator-induced LDL peroxidation and markedly decrease further lipid peroxidation after even oxidation has entered the propagation phase. Other water-soluble fullerene derivatives bearing amino acid and dipeptide moieties that exhibit membranotropic properties have been reported to localize inside the artificial membrane, penetrate into the liposomes through the lipid bilayer, and perform activated transmembrane transport of bivalent metals (36). FC_4S may behave similarly. Our present data do not differentiate between the possible roles of FC_4S in enhancing activated transport of the divalent transition metal ions into lipoprotein particles versus modulating the radical chain reaction within the lipoprotein particle. Nevertheless, lipid peroxidation in the LDL did occur more slowly with the coapplication of FC_4S than was the case with Cu^{2+} alone.

In conclusion, we demonstrated that FC_4S is an effective water-soluble antioxidant that protects LDL from oxidation by removing both aqueous and lipophilic free radicals and prevents atheroma formation of hypercholesterolemic rabbit. Steinberg *et al.* (37) and Witztum and Steinberg

(38) have suggested that lipoprotein oxidation responsible for its atherogenecity occurs more within the artery wall than in the circulation. Thus lipoprotein-associated antioxidants are more effective against atherogenesis when the lipoprotein has left the circulation. In addition, water-soluble radical trapping antioxidants are suggested to be the first line of defense against radical damage under oxidative stress. From both aspects, FC_4S may represent a promising antiatherogenic agent due to its unique characteristics of scavenging both aqueous and lipophilic free radicals, a property not commonly seen in many of well-known water-soluble antioxidants.

The authors are grateful to Miss Hsiu-Tsu Yeh for technical assistance.

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