Gain and Loss of Function for Glutathione Synthesis: Impact on Advanced Atherosclerosis in Apo E Deficient Mice

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Abstract

Glutamate-cysteine ligase (GCL) is the rate-limiting step in glutathione synthesis. The enzyme is a hetero-dimer composed of a catalytic subunit GCLC and a modifier subunit GCLM.

Objective—We generated apo E–/– mice deficient in GCLM (*apoE–/–/Gclm–/–*) and transgenic mice that over-express GCLC specifically in macrophages (*apoE–/–/Gclc-Tg*) to test the hypothesis that significantly altering the availability of glutathione has a measurable impact on both the initiation and progression of atherosclerosis.

Methods and Results—Atherosclerotic plaque size and composition were measured in the innominate artery in chow-fed male and female mice at 20, 30, 40 and 50 weeks of age and in the aortic sinus at 40 or 50 weeks of age. The *apoE–/–/Gclm–/–* mice more rapidly developed complex lesions while the *apoE–/–/Gclc-Tg* mice had reduced lesion development as compared to the littermate *apo E–/–* control mice. Transplant of bone marrow from the *apoE–/–/Gclm–/–* and *apoE–/–/Gclc-Tg* mice into *apo E–/–* mice with established lesions also stimulated or inhibited further lesion development at 30 weeks post-transplant.

Conclusions—Gain and loss of function in the capacity to synthesize glutathione especially in macrophages has reciprocal effects on the initiation and progression of atherosclerosis at multiple sites in apo E–/– mice.

Keywords

Atherosclerosis; macrophages; glutathione; *apo E–/–*

Introduction

Reduced glutathione (GSH) is a tripeptide thiol (glutamate-cysteine-glycine) that is made by most mammalian cells at up to mM concentrations. It is a major endogenous antioxidant and enzyme cofactor[1] and participates in diverse cellular processes[2]. The enzyme glutamate-

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cysteine ligase (GCL) is the rate-limiting step in the GSH synthetic pathway. GCL is a hetero-dimer composed of a heavy catalytic subunit (GCLC, 72.8 kDa) and a lighter modifier subunit (GCLM, 30.8 kDa)[2]. The two subunits are products of separate genes in both mice and humans[3–5]. The catalytic efficiency is dramatically increased when the two subunits interact[6] although GCLC has catalytic activity in the absence of GCLM[6, 7]. Knock out of the GCLC gene is embryonic lethal, while GCLC heterozygote mice exhibit a 20% reduction in GSH levels[8]. Reduced GCLC expression and activity has been associated with diabetes mellitus, inflammatory lung diseases, AIDS, and aging[9–11].

The promoter/enhancer region of both the GCLC and GCLM genes contain a consensus antioxidant response element (ARE) and in response to changes in the cellular redox status, GCLC and GCLM expression are increased by the activation and binding of the transcription factor Nrf-2 to the ARE[12, 13]. We have previously shown that mouse macrophages have increased expression of both GCLC and GCLM when treated with oxidized LDL or homocysteine *in vitro* and that this increase in expression is mediated by binding of Nrf-2 to the ARE in the promoter for both genes[13, 14]. Increased GCLC and GCLM expression have also been reported in a variety of different cell types following treatment with other electrophiles[15, 16].

Depletion of GSH may play a pivotal role in cardiovascular disease. For example, polymorphisms in the 5' flanking region of the GCLC and GCLM genes are associated with an increased risk of myocardial infarction and endothelial dysfunction[17, 18]. Pharmacological manipulation of GSH levels modestly alters early atherosclerotic lesion development in hyperlipidemic mice[19]. The GSH content of the aorta is reduced prior to and during lesion development in mice[20] and GSH dependent antioxidant enzyme expression is reduced as lesions progress[21]. However, the effects of genetically altering GSH levels on advanced lesion development and composition have not been previously reported. Thus, in this study we have evaluated the effects of GCLM deficiency that causes a dramatic systemic reduction in GSH availability[22] and increased GCLC expression specifically in macrophages to test the hypothesis that alterations in glutathione availability especially in macrophages will have an impact on the development and composition of advanced atherosclerotic lesions in the innominate artery and aortic sinus in chow-fed apo E−/− mice between 20 and 50 weeks of age.

Materials and methods

Animals

Mice doubly deficient in *apo E* and *Gclm* (*apoE*−/−/*Gclm*−/−) were generated by breeding *Gclm*−/− mice on a C57Bl/6 background[22] with *apoE*−/− mice on a C57Bl/6 background and backcrossing for more than 6 generations. Genotypes were confirmed by PCR and littermate *apoE*−/− mice were used as wild-type controls. Mice that over-express GCLC specifically in macrophages were generated using a construct that contained a 342 bp fragment of the CD68 gene promoter in addition to the CD68 first intron, the 1920 bp full length coding sequence for GCLC and the bovine growth hormone polyadenylation signal. Clones were screened for correct orientation and then sequenced. CBA/B6 eggs were microinjected with purified DNA and then transferred into pseudo-pregnant foster mothers using standard techniques. Transgene expression was measured using real-time quantitative PCR and founder lines were mated with C57Bl/6 mice. The *Gclc-Tg* mice were crossed with *apoE*−/− mice on a C57Bl/6 background (but derived from a separate breeding colony of *apo E*−/− mice than those that were crossed with the *Gclm*−/− mice) to generate *apoE*−/−/*Gclc-Tg* mice. Genotypes were confirmed by PCR and littermate *apoE*−/− mice were used as controls. Western blotting was also used to verify over-expression of GCLC in thioglycollate elicited peritoneal macrophages isolated from the transgenic and littermate

control mice (figure 1, on-line data supplement). All of the mice included in this study were fed standard mouse chow. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Genotyping mice by PCR

DNA was isolated from mouse tail snips using the DNeasy kit (Quiagen). The mutant and the wild-type allele for GCLM and the GCLC transgene were amplified by PCR. The targeted allele for GCLM was identified by the following primer: forward 5'-CAGTTTGAGGGGACGACGACA-3'. The wild type allele was identified by the forward primer 5'-GCCCGCTCGCCATCTCTC-3'. Both alleles shared the reverse primer 5'-GTTGAGCAGGTTCCCGGCT-3'. Analysis of the GCLC transgene used the following primers: forward 5'- TTCTCGGCTCTGTGAATGACA -3'; reverse 5'-CAGCCCTCTCTTGGAAAGGAGG-3'.

Bone marrow transplantation

To determine whether a reduced or increased GSH content in macrophages would influence the progression of established atherosclerotic lesions, we performed bone marrow transplantion (BMT) with bone marrow from 8 week old male *apoE*–/–, *apoE*–/–/*Gclm*–/–, or *apoE*–/–/*Gclc-Tg* mice transplanted into irradiated female *apoE*–/– mice (the recipient mice were all obtained from the same breeding colony as the apo E–/– mice used to generate the apo E–/– GCLCTg mice). One hundred and twenty, 20-week-old *apoE*–/– females received a dose of 950 rad whole-body x-ray irradiation. The irradiated recipients were divided into three groups that were injected with 1×10^7 bone marrow cells from the *apoE*–/–, *apoE*–/–/*Gclm*–/– or *apoE*–/–/*Gclc-Tg* male mice by tail vein injection. Atherosclerosis development and composition were analyzed at 20 and 30 weeks after BMT.

Mouse perfusion and dissection

At 20, 30, 40 and 50 weeks of age (40 or 50 weeks in the BMT studies), blood was collected via the retro-orbital sinus and the animals were euthanized by pharmacological overdose of a xylazine and ketamine mix (IP). The mice were first perfused with 20 ml of PBS and then with 20 ml of fixative (4% paraformaldehyde) at physiological pressure via the left ventricle. The heart with the aortic root and the thoracic aorta and its branching vessels were dissected out intact. The ascending aorta and carotid arteries were separated from the heart and the heart was further fixed in 1% paraformaldehyde and then paraffin embedded. The innominate arteries were dissected, processed and paraffin embedded. Five micron thick sections from each of the paraffin blocks were generated and every fifth section was stained with a modified Movat's pentachrome stain[23, 24].

Plaque size and composition analysis

The cross sectional lesion area was determined in each Movat's stained section using computer assisted morphometric analysis (Image Pro, Media Cybernetics, Silver Spring, MD). We also tabulated the frequency of features of plaque composition in each Movat's stained section. These included the following: thin fibrous cap (defined as < 3 cell layers), large necrotic core (defined as occupying >50% of the volume of the plaque), intra-plaque hemorrhage (defined as the presence of red blood cells), medial enlargement/erosion (defined as the replacement of the normal media by plaque components), calcification, presence of foam cells, presence of chondrocyte-like cells, and lateral xanthomas (defined as the presence of aggregates of macrophage-derived foam cells situated on the lateral margins of the plaques). These parameters were recorded as binary outcomes and the frequency per lesion for each animal was determined. The total number of cells in the lesions was determined by counting nuclei in each section and the number was then normalized to the

area of the lesion. All data collection was done with the operator blinded to the treatment groups.

Immunohistochemistry

Paraffin-embedded sections of the innominate artery were de-paraffinized and rehydrated. The endogenous peroxidase activity was blocked by incubation with Peroxoblock (Invitrogen). Smooth muscle cells (SMCs) and macrophages were detected using mouse anti-smooth muscle actin (Dako), and rat anti-Mac2 (Pharmingen) according to the manufacturer's protocols. Negative controls included an irrelevant antibody and omission of the secondary antibody.

Isolation and stimulation of mouse peritoneal macrophages

Macrophages were collected from the peritoneum of the *apo E–/–/gclm–/–*, *apoE–/–/Gclc-Tg,* and *apo E–/–* mice four days after i.p. injection of 2 ml of 4% thioglycollate. For details of the culturing of the peritoneal macrophages, see the on-line data supplement.

Caspase 9 activity assay

Thioglycollate elicted peritoneal macrophages from the *apo E–/–/gclm–/–*, *apoE–/–/Gclc-Tg,* and *apo E–/–* mice were grown in 6 well plates at a density of 6×10^4 cells/cm^2 and treated with 10mM acrolein for 6h. Cells were also treated with 0.5mM staurosporine as a positive control for caspase activation. Caspase 9 activity was measured in 3 repeated experiments with the Caspase-9 LEHD-AFC fluorimetric assay kit (Biovision, Mountainview, CA) according to the manufacturer's protocol.

Measurement of cellular and tissue GSH content

The total glutathione content (nmoles/mg protein of GSH+GSSG) in the peritoneal macrophages, liver, kidney, spleen and lung was determined in a 96-well fluorescent microtiter plate assay as described previously[25, 26]. For details of this assay, see the on-line data supplement.

Measurement of plasma cholesterol and triglycerides levels

Total plasma cholesterol and triglyceride levels were measured using commercially available kits (Sigma). Lipoprotein cholesterol profiles were generated by FPLC as previously described[27] (figures 4 and 5 on-line data supplement).

Statistical analysis

All data were expressed as mean ± SE. Significant differences between means in serum cholesterol and triglycerides and lesion size were determined by using the Student's two-tailed t test and by ANOVA for multiple comparisons. Frequency measures and non-normally distributed data were analyzed using the Mann-Whitney test. Values of $p < 0.05$ were considered statistically significant.

Results

Tissue Content of Total GSH

The levels of total GSH were measured in liver, spleen, kidney, lung, and in thioglycollate-elicited peritoneal macrophages from *apoE–/–/Gclm–/–*, *apoE–/–/Gclc-Tg* and control *apo E –/–* mice (table 1). There were radically reduced levels of GSH (>80%) in the liver, kidney, spleen, lung, and macrophages from the *apoE–/–/Gclm–/–* as compared with the control *apoE–/–* mice. The level of GSH in macrophages of the *apoE–/–/Gclc-Tg* mice was about 3

fold higher as compared with macrophages from the control apoE–/– mice. There were no significant differences in the GSH content of the liver, kidney, spleen, and lung from the transgenic and control apo E–/– mice.

Initiation and Progression of Atherosclerotic Plaques

The average cross sectional area of atherosclerotic plaque in the innominate artery was evaluated in both female and male mice at 20, 30, 40 and 50 weeks of age. In comparison to the sex- and age-matched littermate *apo E*–/– controls, the *apoE*–/–/*Gclm*–/– mice more rapidly developed larger and more complex lesions. In the females the difference was already significant at 20 weeks of age, while in the males the difference between groups was significant starting at 30 weeks of age (fig 1 a, c). In contrast, the *apoE*–/–/*Gclc-Tg* mice developed smaller lesions than the litter-mate controls (fig 1 b, d). The decrease of lesion area in the males was significant at 30, 40 and 50 weeks of age (fig 1b). The *apoE*–/–/*Gclc-Tg* female mice had a significant reduction in lesion area only at 50 weeks of age (fig 1d). To make sure that the effects of the GCLM deficiency or GCLC transgene expression was not site specific, we also measured the average lesion area in the aortic sinus of *apoE*–/–/*Gclm*–/– female mice at 40 weeks of age and *apoE*–/–/*Gclc-Tg* male mice at 50 weeks of age. Again, there were significantly larger lesions in the sinus of the *apoE*–/–/*Gclm*–/– mice than the corresponding littermate control mice (fig 1e) and significantly smaller lesions in the *apoE*–/–/*Gclc-Tg* mice than the corresponding control mice (fig 1f).

Effects of Bone Marrow Transplantation on the Progression of Established Atherosclerotic Plaques

In order to determine whether the reduced or increased availability of GSH in macrophages contributes to the acceleration or the reduction in the progression of established lesions, we conducted bone marrow transplantation studies. There was about a 60% increase in lesion area in the *apoE*–/– mice transplanted with bone marrow from the a*poE*–/–/*Gclm*–/– mice as compared to *apoE*–/– mice transplanted with *apoE*–/– bone marrow at 30 weeks post-transplant (fig 2a). The rate of progression of the lesions was reduced by 35% at 30 weeks post-transplant when bone marrow from a*poE*–/–/*Gclc-Tg* mice was transplanted into the *apoE*–/– recipients (figure 2b).

Plaque Composition

We have previously described the age-related changes in lesion characteristics in the advanced lesions in the innominate arteries of chow-fed apo E–/– mice[28]. In order to determine the effects of GCLM deficiency and of GCLC transgene expression in macrophages on the same developmental changes in plaque composition, the frequency of these features were tabulated in the Movat's stained sections from each mouse. At 20 weeks of age there were no differences in lesion composition between the *apoE*–/–/*Gclm*–/– and *apoE*–/– male mice. However, by 30 weeks the *apoE*–/–/*Gclm*–/– male mice had a higher frequency of thin fibrous caps, large necrotic cores, cholesterol clefts, lateral xanthomas and foam cells, intra-plaque hemorrhage and chondrocytes-like cells (table 1 on-line data supplement). In the *apoE*–/–/*Gclm*–/– female mice an increased frequency of most of these features was already evident by 20 weeks of age and there continued to be higher frequencies of a large necrotic core and cholesterol clefts at both 30 and 40 weeks of age and intra-plaque hemorrhage at 30 weeks of age in the *apoE*–/–/*Gclm*–/– female mice. There were no differences in the frequency of plaque calcification in either the male or female mice at any time points (table 1, on-line data supplement). The transplanted mice were also analyzed for features of plaque composition. At 20 weeks post-transplant the mice transplanted with the a*poE*–/–/*Gclm*–/– bone marrow had a higher frequency of thin fibrous caps and chondrocyte like cells compared to the controls. At 30 weeks post-transplant there

was a higher frequency of cholesterol clefts, lateral xanthoma and calcification in the experimental group compared to the control (table 2, on-line data supplement).

There were no consistent differences at any time point or in either gender for any of the features of plaque composition between the *apoE–/–/Gclc-Tg* and *apoE–/–* mice. For example, at 20 weeks of age there were no differences while by 30 weeks both the male and female *apoE–/–/Gclc-Tg* mice had a lower frequency of thin fibrous caps. At 30 and 50 weeks of age, the *apoE–/–/Gclc-Tg* males had a higher frequency of plaques containing foam cells, while there was no significant difference in the females. In the female *apoE–/–/Gclc-Tg* mice at 40 and 50 weeks of age there was an increased frequency of extra-cellular cholesterol clefts. The lesions from the mice transplanted with bone marrow from the *apoE–/–/Gclc-Tg* mice were also analyzed for changes in plaque composition. There were few consistent differences for both time points (table 2, on-line data supplement). At 20 weeks post transplant, the lesions in the *apoE–/–* recipients had a higher frequency of foam cells, and a reduced frequency of chondrocyte-like cells and calcification as compared to the control mice. At 30 weeks post transplant there was also an increased frequency of foam cells and a reduced frequency of large necrotic cores and chondrocyte-like cells (table 2, on-line data supplement).

Immunohistochemistry

The presence of macrophages and smooth muscle cells in the plaques was confirmed by immunoperoxidase staining for the macrophage-specific marker Mac2 and for smooth muscle actin. There were fewer macrophages in the lesions of the a*poE–/–/Gclm–/–* male mice as compared to age matched littermate controls at 30 weeks of age. (fig 3a). The number of cells staining positive for smooth muscle actin increased with formation of the fibrous cap in the a*poE–/–/Gclm–/–* male mice at 20 weeks of age (fig 2 on-line data supplement). The lesions in the a*poE–/–/Gclc-Tg* mice had more macrophage staining as compared to age matched controls at 30 weeks of age in both male and female mice (fig 3b,d). The smooth muscle actin content of the lesions in the *apoE–/–/Gclc-Tg* was higher in the males at 20 weeks of age (fig 2 on-line data supplement).

Reduced Lesion Cellularity and Activation of Caspase 9

There was a reduced overall cellularity of the lesions (total # nuclei/1000 μm^2) in both the male and female *apoE–/–/Gclm–/–* mice as compared to the littermate controls at all time points (fig 4a,c). There were no differences in the total cellularity of the lesions in both the male and female *apoE–/–/Gclc-Tg* mice as compared to the littermate controls at any time point (fig 4b,d). To help explain the reduced macrophage content and lesion cellularity in the a*poE–/–/Gclm–/–* mice, we investigated whether peritoneal macrophages from the a*poE–/–/Gclm–/–* mice were more susceptible to apoptosis induced by the pro-oxidant acrolein and whether the macrophages from the *apoE–/–/Gclc-Tg* mice were less susceptible than macrophages from *apoE–/–* mice. There was an increase in caspase 9 activity (1.8 fold) in the macrophages of the a*poE–/–/Gclm–/–* mice following treatment with acrolein. The cells were also more susceptible to staurosporine (2.2 fold increase) and when cultured in standard media they showed a higher baseline caspase 9 activity (~ 1.8 fold increase) than the macrophages from the control apo E–/– mice (fig 4e). There was no difference in caspase 9 activity in the peritoneal macrophages from the a*poE–/–/Gclc-Tg mice* after treatment with acrolein as compared to macrophages from the *apoE–/–* mice (figure 4f).

Plasma Cholesterol and Triglycerides Levels

There were no differences in the total plasma cholesterol levels between the age matched *apoE–/–/Gclm–/–* and *apoE–/– or* the *apoE–/–/Gclc-Tg* and control *apo E–/–* mice (table 2). There were also no differences in total plasma triglycerides. However, the analysis of the

lipoprotein cholesterol profiles by FPLC showed a significant difference between the *apoE–/–/Gclm–/–* and *apoE–/–* mice at 20 weeks of age with the majority of the cholesterol in the VLDL fraction in *apoE–/–/Gclm–/–* mice with corresponding reductions in LDL and HDL cholesterol. These differences disappeared in the *apoE–/–/Gclm–/–* mice at 50 weeks of age (fig 4, on-line data supplement). In contrast, there were no differences in the lipoprotein cholesterol profiles in the *apo E–/–* mice 20 weeks following transplant with bone marrow from the *apoE–/–/Gclm–/–* mice (fig 5, on-line data supplement) suggesting that the absence of GCLM in macrophages did not account for the altered profile seen in the *apoE–/–/Gclm–/–* mice at 20 weeks of age.

Discussion

An association between glutathione metabolism and cardiovascular disease has been previously established[17, 18, 29–33] and experimental studies have provided further support for this association[20, 21, 34–36]. For example, Rosenblat et al., previously demonstrated that manipulation of GSH levels in very young apo E–/– mice by pharmacological interventions modestly increased or protected against early fatty streak development in the aortic sinus[19]. Our current data is consistent with these previous observations and with previous *in vitro* and *in vivo* studies that show that increased GSH levels have anti-atherosclerotic effects[14, 15, 37, 38]. Importantly, this study is the first to show that increasing or decreasing endogenous antioxidant levels in macrophages is sufficient to have a measurable impact on established atherosclerosis.

The *apoE–/–/Gclm–/–* and *apoE–/–/Gclc-Tg* colonies were generated several years apart and were originally derived from separate apo E–/– breeding stocks. Thus, with the exception of the bone marrow transplant studies where the recipient mice were all from the same colony, all comparisons were made with the separate littermate control *apo E–/–* mice. However, there were a few consistencies between the groups. For example, there were more dramatic effects of the reduced availability of GSH in the females from the *apoE–/–/Gclm–/–* mice and more modest effects of increased GSH in the females from the *apoE–/–/Gclc-Tg* mice. It is currently unclear why there were gender dependent differences although it is well known that female mice on a C57Bl/6 background develop larger lesions[39, 40]. Estrogen also plays a role in repressing the expression of ARE dependent genes[41]. These observations reinforce the point that it is important to evaluate lesions in both sexes and at multiple sites[42].

The up to 8 fold increase in lesion area in the innominate artery of the *apoE–/–/Gclm–/–* mice is a much larger increase than observed with most other genetic or pharmacological interventions reported so far for apo E–/– mice. This may reflect the fundamental role of GSH in many cellular processes. The reduced availability of GSH in the *apoE–/–/Gclm–/–* mice had the most dramatic effect at 20 and 30 weeks of age, a point at which the plaques were rapidly progressing[43]. Initiation and progression of atherosclerotic lesions in mice is largely dependent on cellular influx and proliferation coupled with lipid uptake and connective tissue deposition. This suggests that GSH may play an essential role in regulating these processes[37, 44–47]. It is surprising however that the larger lesions in the *apoE–/–/Gclm–/–* mice at 20 and 30 weeks of age also exhibited characteristics of more advanced lesions normally seen in the apo E–/– mice at 40 or more weeks of age[43]. Thus, the currently unknown processes that lead to formation of a thin fibrous cap, a large central necrotic core and intra-plaque hemorrhage in mice are also all accelerated with the reduction of GSH.

The more rapid formation of the necrotic core for example, may involve an increase in foam cell death due to increased oxidative stress[48]. To address this possibility, we originally attempted to measure cell death in the plaques in all of the mice. Unfortunately, in keeping

with previous reports, we could not get consistent data using the TUNEL assay or staining for activated caspases with formalin fixed and paraffin embedded tissue[49, 50]. Thus, we compared the temporal changes in the overall cellularity of the plaques and in the macrophage and SMC content. There were fewer macrophages in the male mice at 30 weeks of age and a reduced number of total cells in the lesions from all of the *apoE–/–/Gclm–/–* mice and more macrophages in the lesions from some of the *apoE–/–/Gclc-Tg* mice suggesting that the differences in the GSH levels may have contributed to controlling the influx of monocytes/macrophages and/or the turnover of the cells. This is in keeping with previous in vitro studies that have shown that depletion of GSH makes vascular cells more susceptible to pro-oxidant induced cytotoxicity[48, 51–53]. It is also consistent with studies showing that alterations in macrophage glutathione content affects NFkB activation and expression of pro-inflammatory cytokines that stimulate adhesion molecule expression by endothelial cells and thus recruitment of monocytes/macrophages into developing lesions[54–56].

However, protection from oxidant induced cytotoxicity may not have contributed significantly to the modestly reduced atherosclerosis in the *apoE–/–/Gclc-Tg* mice as there were no differences in the cellularity of the plaques or in the caspase 9 activity in the peritoneal macrophages. This suggests that there may be minimal oxidative stress in chow-fed apo E–/– mice. It is conceivable that there would have been more dramatic protective effects had we fed the mice a high fat diet and/or induced diabetes or uremia to elevate the levels of oxidative stress. The athero-protective effect of increasing GSH in macrophages may also be due to the role that GSH plays in maintaining redox dependent signal transduction[11, 57] and protein glutathionylation[58, 59], or to indirect effects mediated by other antioxidants such as alpha lipoic acid[60].

The current results showing that increasing the capacity of macrophages to make GSH provides some protection from lesion progression even after lesions have been established is in contrast to our recent study showing no effect on the progression of established lesions in the apo E–/– mice following dietary treatment with antioxidants[61]. As antioxidant supplementation in humans has also not been very effective at reducing clinical events related to cardiovascular disease[62], our data suggest that increasing endogenous antioxidant production rather than supplementing with dietary antioxidants may be an effective alternative therapeutic approach for controlling the progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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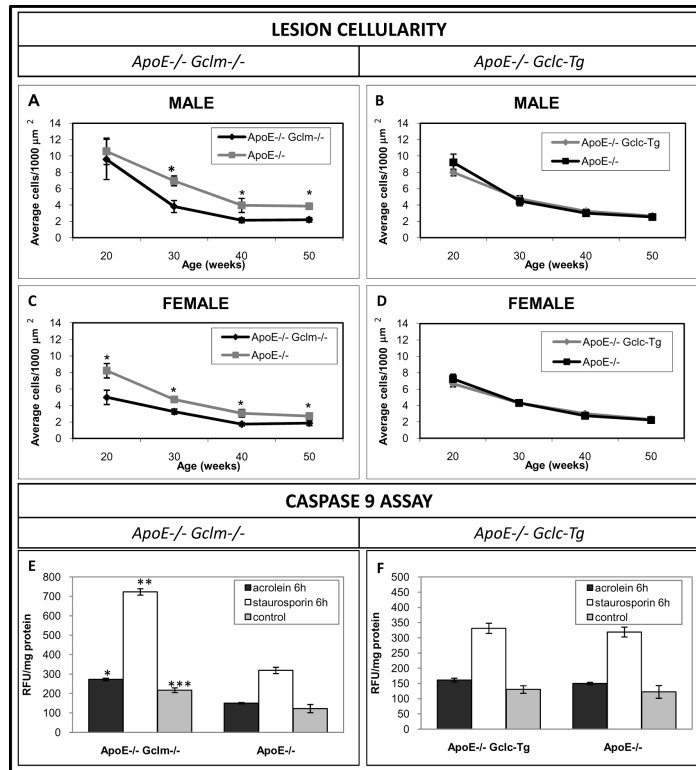
Figure 1. Average Lesion Area in the Innominate Artery and Aortic Sinus
***ApoE–/–/Gclm–/–* Mice** (A) Male mice at 20 weeks (*apoE–/–/Gclm–/–* n=5, *apoE–/–/ Gclm+/+* n=17), 30 weeks (*apoE–/–/Gclm–/–* n=8, *apoE–/–/Gclm+/+* n=13), 40 weeks (*apoE–/–/Gclm–/–* n=7, *apoE–/–/Gclm+/+* n=10) and 50 weeks (*apoE–/–/Gclm–/–* n=9, *apoE–/–/Gclm+/+* n=4); (C) female mice at 20 weeks (*apoE–/–/Gclm–/–* n=5, *apoE–/–/ Gclm+/+* n=24), 30 weeks (*apoE–/–/Gclm–/–* n=8, *apoE–/–/Gclm+/+* n=14), 40 weeks (*apoE–/–/Gclm–/–* n=6, *apoE–/–/Gclm+/+* n=10) and 50 weeks (*apoE–/–/Gclm–/–* n=5, *apoE–/–/Gclm+/+* n=20). (E) Aortic sinus of female mice at 40 weeks of age (*apoE–/–/ Gclm–/–* n=5, *apoE–/–/Gclm+/+* n= 5)
***ApoE–/–/Gclc-Tg* Mice.** (B) Male mice at 20 weeks (*apoE–/–/Gclc-Tg* n=11, *apoE–/–/ Gclc-WT* n=12), 30 weeks (*apoE–/–/Gclc-Tg* n=7, *apoE–/–/Gclc-WT* n=6), 40 weeks (*apoE –/–/Gclc-Tg* n=6, *apoE–/–/Gclc-WT* n=11) and 50 weeks (*apoE–/–/Gclc-Tg* n=7 *apoE–/–/ Gclc-WT* n=13); (D) Female mice at 20 weeks (*apoE–/–/Gclc-Tg* n=14, *apoE–/–/Gclc-WT* n=15), 30 weeks (*apoE–/–/Gclc-Tg* n=15, *apoE–/–/Gclc-WT* n=12), 40 weeks (*apoE–/–/ Gclc-Tg* n=7, *apoE–/–/Gclc-WT* n=7) and 50 weeks (*apoE–/–/Gclc-Tg* n=8, *apoE–/–/Gclc-WT* n=18); (F) Aortic sinus of male mice at 50 weeks of age (*apoE–/–/Gclc-Tg* n=5, *apoE–/ –/Gclc-WT* n=5). Data are presented as means ± SE, *p<0.05 vs controls.
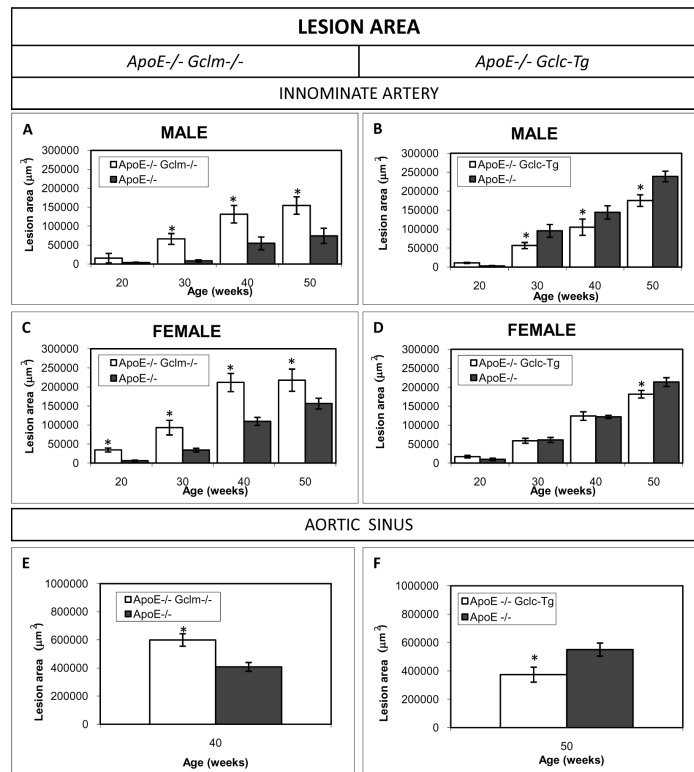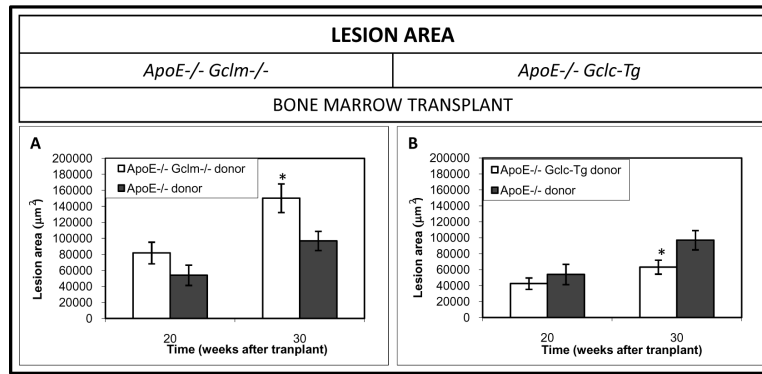
Figure 2. Average Lesion Area in the Innominate Artery Following Bone marrow Transplant
***ApoE–/–/Gclm–/–* Mice.** A) Lesion area in female *apoE–/–* mice following bone marrow transplantation at 20 weeks (*apoE–/–/Gclm–/–* donor, *apo E–/–* recipients n=15, *apoE–/–/Gclm+/+* donor, *apo E–/–* recipients n =14), and 30 weeks (*apoE–/–/Gclm–/–* donor, *apo E–/–* recipients n=13, *apoE–/–/Gclm+/+* donor, *apo E–/–* recipients n=8).
***ApoE–/–/Gclc-Tg* Mice.** B) Lesion area in female apoE–/– mice following bone marrow transplantation at 20 weeks post-transplant (*apoE–/–/Gclc-Tg* donor, *apo E–/–* recipients n=15, *apoE–/–/Gclc-WT* donor, *apo E–/–* recipients n=14), and 30 weeks post-transplant (*apoE–/–/Gclc-Tg* donor, apo E–/– recipients n=13, *apoE–/–/Gclc-WT*, apo E–/– recipients donor n=8). Data are presented as means ± SE, *p<0.05 vs controls.
All recipient *apo E–/–* mice were from the same breeding colony as the apo E–/– mice used to generate the *apo E–/–/Gclc-Tg* mice.
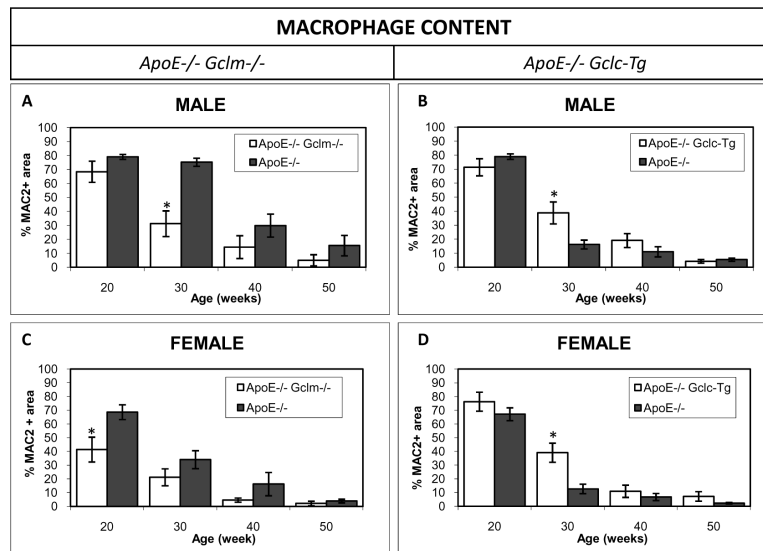
Figure 3. Macrophage Content of the Lesions in the Innominate Artery
Area of Mac 2 staining in lesions in the innominate artery as a percentage of the total area of the lesion for males (A, B), and females (C, D). Sample sizes were identical to those listed in figure 1. Data shown are the means ± SE. * p<0.05 vs littermate control.

Figure 4. Total Cell Content of the Lesions in the Innominate Arteries and Caspase-9 Activity in Peritoneal Macrophages

Total # nuclei/1000 μm^2 cross sectional area in lesions in the innominate artery of both the male (A,C) and female (B,D) mice. Sample sizes were identical to those listed in figure 1. Caspase 9 activity (E,F) was evaluated in thioglycollate-elicited peritoneal macrophages after 6h treatment with acrolein or staurosporin (n= 3 mice/group). *p<0.05 vs littermate controls. Data are presented as means ± SE.

Table 1

Tissue Glutathione Content.

	ApoE–/–/Gclm–/–	ApoE–/–/Gclc-Tg	ApoE–/–[+]
GSH (nmoles/mg protein)			
Macrophages	1.10 ± 0.20[*]	21.57 ± 4.53[*]	6.67 ± 1.39
Liver	8.70 ± 0.85[*]	58.49 ± 6.33	64.82 ± 20.42
Spleen	2.72 ± 0.34[*]	12.06 ± 0.01	12.61 ± 0.78
Kidney	0.28 ± 0.02[*]	1.29 ± 0.01	1.21 ± 0.81
Lung	2.55 ± 0.54[*]	17.64 ± 2.46	15.17 ± 1.06

Data are presented as means ± SE

[*] p<0.05 vs *apoE* –/– mice.

[+] Littermate controls for *apoE–/–/Gclc-Tg* mice. n= 3 mice/group.

Table 2

Total Plasma Cholesterol and Triglycerides.

	ApoE−/−/Gclc−/−		ApoE−/−		ApoE−/−/Gclc-Tg		ApoE−/−	
	male	female	male	female	male	female	male	female
Cholesterol (mg/dl)								
20 weeks	492.4±59.1	371.3±20.9	546.6±34.2	412.4±5.9	590.4±34.4	511.9±24.9	537.2±20.5	446.5±8.0
30 weeks	563.6±63.4	377.1±41.7	535.3±29.5	433.4±23.8	655.2±36.3	608.3±25.9	585.8±14.2	499.7±19.8
40 weeks	523.9±38.4	388.5±18.2	619.9±29.4	456.3±81.4	648.2±66.7	594.7±38.2	614.0±25.0	508.5±36.3
50 weeks	605.9±56.7	457.2±44.1	640.9±49.5	496.7±23.2	634.0±29.5	593.5±26.9	631.0±25.3	558.2±22.1
Triglycerides (mg/dl)								
20 weeks	582.6±94.4	408.5±59.8	551.4±93.0	342.2±38.8	626.0±52.4	399.1±31.6	551.4±34.2	410.4±36.3
30 weeks	446.9±52.3	299.5±22.1	494.3±36.9	243.7±30.3	688.2±86.3	506.6±55.0	512.7±55.1	469.7±10.5
40 weeks	474.2±47.6	251.0±41.9	503.6±47.9	363.9±24.3	523.9±38.4	395.5±20.1	497.9±45.6	424.8±26.0
50 weeks	412.4±24.0	305.9±35.1	446.4±65.9	327.6±29.7	386.4±74.6	206.2±65.9	467.7±75.2	357.6±41.3

Data are presented as the means ± SE.

Sample sizes are identical to those listed for figure 1.