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Increased sensitivity of Apolipoprotein E knockout mice to swainsonine dependent immunomodulation

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Abstract

The mechanisms that mediate accelerated atherosclerosis in autoimmune diseases remain unclear. One common mechanism that has been documented in autoimmune diseases and atherosclerosis is formation of hypoglycosylated N-glycans on the cell surface. In this study we tested the effects of swainsonine, a class II α -mannosidase inhibitor which results in formation of hypoglycosylated N-glycans, on atherogenesis and immune cell dynamics in the atheroprone and hypercholesterolemic ApoE $-/-$ mouse. Wild type or ApoE $-/-$ mice (8 weeks of age) were fed a normal chow diet and administered swainsonine via the drinking water for 8 weeks at which time, atherosclerosis, and systemic markers of markers of inflammation were evaluated. Interestingly, no change in the rate of atherosclerosis development was observed in ApoE $-/-$ mice treated with swainsonine. However, swainsonine significantly increased the number of peripheral blood leukocytes in ApoE $-/-$ mice, with trends toward similar increases in swainsonine treated wild type mice noted. Assessment of leukocyte subsets using specific markers of all major blood lineages indicated that the increase in circulating leukocytes was due to the elevated number of progenitor cells. Consistent with swainsonine having a greater effect in ApoE $-/-$ vs. wild type mice, increases in circulating inflammatory markers (IgA, IgG and chemokines) were observed in the former. Collectively, these data demonstrate that predisposition of ApoE $-/-$ mice to vascular disease is associated with sensitization to the immunomodulatory effects of swainsonine and indicate that changes in N-glycans may provide a mechanism linking autoimmunity to atherogenesis.

Keywords

Atherosclerosis; Hypoglycosylation; Inflammation; Leukocytes; N-glycan

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Conflict of interest

The authors have no conflicts to declare.

Introduction

Autoimmune diseases represent one of the fastest growing categories of disease afflicting over 9% of the population (Cooper et al., 2009). A common sequelae of these diseases is accelerated cardiovascular disease including atherosclerosis (Skaggs et al., 2012; Gerli et al., 2007; Karmon et al., 2012). While the genetic and environmental risk factors underlying development and onset of autoimmune diseases vary, most are characterized by changes in protein N-glycosylation with enrichment in mannose rich structures (van Kooyk and Rabinovich, 2008; Green et al., 2007; Chui et al., 2001), and recently it has been demonstrated that these same alterations on endothelial cells are associated with atherosclerosis development (Scott et al., 2012, 2013).

N-glycosylation is an enzyme driven post-translational modification of proteins whereby carbohydrates are added onto the amide residue of asparagines in an N-X-S/T sequon. N-glycans mature from a high-mannose to hybrid to complex state as they transit through the endoplasmic reticulum and Golgi complex. Critical to this maturation process is removal of mannose residues by a family of alpha-mannosidase enzymes that allows for addition of N-acetylglucosamine branches which support further monosaccharide additions. Under normal physiologic conditions the overwhelming majority of N-glycans are thought to be complex. During autoimmune disease however, these N-glycans fail to develop into the fully complex state with their maturation halted at the high mannose and hybrid state (hereafter referred to as hypoglycosylated N-glycans) with these epitopes now being recognized by the hosts innate immune system as “non-self” danger signals which promote systemic immune activation (Rabinovich et al., 2012; Rachmilewitz and Glycosylation, 2010).

In support, transgenic mice that fail to generate complex N-glycans develop autoimmune associated inflammation (Green et al., 2007; Chui et al., 2001; Lee et al., 2007; Malhotra et al., 1995) and display elevated T-cell activation (Demetriou et al., 2001; Bowlin et al., 1989) and proliferation (Mkhikian et al., 2011). Pharmacological inhibition of N-glycan maturation *in vivo* is possible through inhibition of alpha-mannosidase with compounds such as swainsonine, a class II alpha-mannosidases inhibitor which restricts N-glycan maturation at the hybrid state. Prolonged administration of swainsonine is known to induce autoimmune-like phenotypes including lupus like renal disease (Huxtable and Dorling, 1983) and exposure of mice or cells to swainsonine leads to elevated secretion of certain glycoproteins and inflammatory cytokines including interferon- γ (Bowlin et al., 1989; Morgan et al., 2004; Yeo et al., 1985). Additionally, swainsonine is a known immunomodulator that induces progenitor cell proliferation and release into the circulation in rodents and has been considered to boost immune cell function in cancer patients (Oredipe et al., 2003; White et al., 1991). Mice deficient in the MAN2A gene, one of the protein targets of swainsonine, are susceptible to development of autoimmunity characterized by increased T-cell activation, increased levels of circulating immunoglobulins and immune complex mediated glomerular nephritis (Chui et al., 2001). Indeed, several studies have now shown that loss of N-glycan branching in T-cells is associated with hyper-activation and increased proliferation (Lee et al., 2007; Mkhikian et al., 2011).

Because of this apparent association of hypoglycosylation with both atherosclerosis and autoimmune diseases, and due to the correlation of the diseases states with each other, in the current work we sought to examine if atherosclerosis prone ApoE $-/-$ mice, which have also been used in models of autoimmunity and atherosclerosis (Aprahamian et al., 2004; Richez et al., 2013), would be more susceptible to the immunomodulatory effects of swainsonine than wild type mice. Herein we show that swainsonine induces increased immunomodulatory effects on ApoE $-/-$ mice compared to wild type as measured by increased levels of circulating leukocytes, increases in serum IgG and IgA, and increased levels of circulating cytokines.

Results

Phenotypic effects of swainsonine ingestion

ApoE $-/-$ mice are intrinsically hypercholesterolemic and therefore eventually develop atherosclerosis even when fed a standard chow diet. In contrast, many studies utilize atherogenic diets (high fat/high cholesterol diets or diets supplemented with cholate) in order to accelerate the disease. In the current study we opted for a standard chow diet regimen as opposed to a atherogenic diet to test the effects of swainsonine in the presence of a milder hypercholesterolemic/inflammatory background. Fig. 1 shows that increased cholesterol (Fig. 1A) and triglyceride (Fig. 1B) levels in ApoE $-/-$ mice compared to WT mice was not affected by swainsonine treatment. As mentioned above, previous reports indicate that swainsonine induces progenitor cell expansion in normocholesterolemic mice and increases total circulating leukocyte counts so we next determined if a similar effect would be observed in ApoE $-/-$ mice. As seen in Fig. 1C, a trend toward increased levels of circulating leukocytes in wild type mice treated with swainsonine was observed (not significant by one-way ANOVA, but $p = 0.01$ by t -test between wt and wt + SW). This effect was however amplified and significant in ApoE $-/-$ where swainsonine increased the levels of leukocytes significantly above all other groups including the treated wild type animals. These data show that swainsonine administration does not alter the hypercholesterolemic phenotype of ApoE $-/-$ mice, but that these mice display an increased sensitivity to swainsonine dependent expansion of leukocyte populations.

Swainsonine alters leukocyte profiles in ApoE $-/-$ mice

To better understand the specific effects swainsonine ingestion was having on circulating cells, leukocytes were collected and stained with ConA to determine the effects of swainsonine on leukocyte N-glycan status. Additionally, leukocytes were stained with antibodies against CD19 (B-cells), CD11b (myeloid cells), and CD4 and CD8 (all major circulating T-cells). As seen in Fig. 2A, swainsonine administration induced a significant increase in circulating ConA positive cells indicating that the treatment was inhibiting N-glycan maturation as expected. Additionally, swainsonine significantly decreased the proportion of CD11b positive cells in both WT and ApoE $-/-$ mice relative to respective untreated controls, and significantly decreased the proportion of CD19, CD4, and CD8 positive cells only in ApoE $-/-$ mice.

As there were significantly elevated levels of total leukocytes in the mice ingesting swainsonine (Fig. 1C), we next wanted to determine the absolute numbers of circulating leukocyte subsets. These were calculated from the total leukocyte counts per μ l blood with the percent positive cells for each marker tested (Fig. 2B–E). In terms of specific markers for the principal hematopoietic lineages in blood, there was no change in the levels of any cell subsets except for CD8 positive T-cells which were significantly decreased in ApoE $-/-$ + SW compared to wt and wt + SW. These data show that swainsonine was effective at reducing N-glycan complexity in both wt and ApoE $-/-$ and did so without major changes in circulating levels of B cells, T cells, or myeloid cells with the exception of decreasing circulating CD8+ T cells in ApoE $-/-$ mice. In the above analysis there were no major changes in absolute levels of leukocyte subsets (B cell, T cells, myeloid cells) despite a significant increase in the total number of circulating cells. Fig. 2E plots these other lineage negative cells, which we ascribe to as progenitor cells. Interestingly, the increase in these lineage negative progenitor cells was highest in ApoE $-/-$ mice treated with swainsonine. Collectively, these data demonstrate that the normal circulating leukocyte profile has been altered in mice ingesting swainsonine with significantly increased levels of progenitor cells and that these effects were more pronounced in ApoE $-/-$ vs. WT mice.

Swainsonine ingestion elevates serum immunoglobulins and cytokines in swainsonine treated ApoE $-/-$ mice

Treatment of cells with swainsonine is known to sensitize T-cells to activation (van Kemenade et al., 1994; Wall et al., 1988) and activate resident macrophages (Das et al., 1995). Transgenic mice lacking MAN2A display increased levels of circulating immunoglobulins, suggesting an increased level of leukocyte activation when N-glycan maturation is inhibited. To test if swainsonine affected circulating immunoglobulins, and if so, was this more sensitive in ApoE $-/-$ mice, we next assessed serum levels of IgG, IgA, and cytokines. As seen in Fig. 3A, swainsonine increased serum IgG levels in ApoE $-/-$ mice compared to all other groups tested. There was a modest increase in IgG between wt and wt + SW, but this did not reach significance by one-way ANOVA ($p = 0.06$ by t -test). As seen in Fig. 3B, swainsonine treated ApoE $-/-$ mice displayed significantly increased IgA compared to all other groups. While there was a difference between ApoE $-/-$ and ApoE + sw this did not reach significance by one-way ANOVA, but was highly significant by t -test analysis ($p = 0.009$).

We next determined the effect of swainsonine injection on circulating cytokine levels. Serum was pooled from the four mice in each group and analyzed using a mouse cytokine profiler array containing antibodies against 40 common murine cytokines. Supplementary Fig. S1 shows that ApoE $-/-$ had elevated levels of several cytokines compared wt control. Surprisingly, wt mice administered swainsonine had increased levels of some cytokines (CCL1, MCP5, MIG, SDF1), and ApoE $-/-$ + SW mice had significant increases in nearly all cytokines measured. Combined, these data suggest that swainsonine's immunomodulatory effects are potentiated in ApoE $-/-$ mice.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2014.02.011>.

Swainsonine ingestion does not alter early atherosclerosis development in ApoE $-/-$ mice

ApoE $-/-$ mice are characterized by development of atherosclerotic lesions which are completely absent in wt mice otherwise expressing the *ApoE* gene, even if fed high fat diets (Plump et al., 1992). While many studies utilizes modified high fat and cholesterol diets to accelerate development of the disease, atherosclerotic lesions will form in ApoE $-/-$ mice fed standard rodent chow with the earliest development occurring in the aortic root. In order to test if swainsonine administration alters atherosclerotic development, lesion formation in the aortic root was assessed by Oil-red-O staining of aortic cryosections. As can be seen in Fig. 4, atherosclerosis development was clearly visible in both ApoE $-/-$ and ApoE $-/-$ + SW mice which when quantified (Fig. 4B) was at similar levels between the two groups. These data show that while swainsonine demonstrates potent immunomodulatory effects in ApoE $-/-$ it does not alter early atherosclerosis development.

Discussion

Protein N-glycosylation is a primary signal controlling protein transport to the cell surface and excretion from the cell. Seminal studies have shown that dysfunction in N-glycan processing leads to various diseases that are autoimmune in nature (Green et al., 2007; Chui et al., 2001; Lee et al., 2007; Demetriou et al., 2001; Huxtable and Dorling, 1983). Moreover, emerging studies are demonstrating that N-glycosylation is dynamically controlled during inflammatory diseases including atherosclerosis. Specifically we have demonstrated that pro-atherogenic/inflammatory stressors inhibit endothelial α -mannosidase activity leading to hypoglycosylated structures on the cell surface that then mediate increased inflammatory cell adhesion (Scott et al., 2012, 2013; Chacko et al., 2011). Given that autoimmune diseases are risk factors for atherosclerosis, our objectives in this study were to determine the effect of pharmacological inhibition of α -mannosidases on atherosclerosis *in vivo*.

Surprisingly, swainsonine did not affect atherosclerosis development in ApoE $-/-$ mice. However significant increases in circulating leukocyte numbers were observed which were not associated with any changes in B cells, myeloid cells, or CD4⁺ T cells. In fact, a decrease in CD8⁺ T cells was noted. The increased blood leukocytes were negative for all major hematopoietic lineage markers tested (CD11b, CD19, CD4, and CD8), and therefore most likely reflect a progenitor cell population. Interestingly however, this effect of swainsonine was more pronounced in ApoE $-/-$ mice compared to WT mice, suggesting that the inherent hypercholesterolemia and/or chronic inflammation in the former animals sensitizes them to the effects of α -mannosidase inhibition. One possible mechanism explaining this is that α -mannosidase activity is already lower in the ApoE $-/-$ mice.

We note that it is not possible to firmly conclude that swainsonine treatment does not affect atherosclerosis. It is possible that when combined with a disease promoting high fat-diet, or if administered at higher levels or for longer periods of time, swainsonine may accelerate the atherogenic process. Also, it is important to remember that despite a robust increase in circulating blood cells, there was no increase in the specific leukocyte populations known to participate in atherosclerosis development, and precisely how (pro- or anti-inflammatory) progenitor cells would influence the atherogenic process is unclear. The data presented

suggest that additional work examining if the autoimmune like effects brought on by swainsonine ingestion (nephritic disease) are worsened in ApoE $-/-$ mice are also warranted. It will also be interesting to see if the increase in progenitor cells occurs in other animal models of chronic inflammation. These data also suggest that any further development of swainsonine as an anti-tumor drug need to factor underlying chronic inflammation in altering the dose-dependent effects of the drug.

The relationship between multiple disease states is important to understand as persistent hypoglycosylation during inflammation could act as a sterile danger signal which activates the innate immune system. While the synergistic interrelationship between autoimmune disease and atherosclerosis is well appreciated, a fundamental understanding of the underlying mechanisms at the molecular and cellular level remains incompletely understood. As it becomes increasingly apparent that changes in glycosylation occur in both diseases, a better understanding of the role of these sugar structures becomes paramount as they may provide key therapeutic targets for disease prevention and control.

Methods

Eight-week-old C57/bl6 (wt) and Apolipoprotein E $-/-$ (ApoE $-/-$) mice were obtained from Jackson Laboratories. Over the next 8 weeks, mice were provided *ad libitum* access to standard rodent chow and water with some mice being supplemented with 17.5 μ M swainsonine in their drinking water. All experiments were carried out under approval of the University of Alabama at Birmingham Animal Care and Use Committee. There were four mice in each group.

Serum triglyceride, cholesterol, immunoglobulin and cytokine analysis

Blood was obtained by retro-orbital bleeding and serum was collected by spinning at $2000 \times g$ for 10 min after 60 min of clotting at room temperature. Total cholesterol and triglycerides were analyzed with kits from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's protocol. Serum IgG and IgA were analyzed using Ready-SET-Go! ELISA kits (eBiosciences, San Diego, CA) according to the manufacturer's protocols. Serum cytokines were measured using the Mouse Cytokine Prolifer Array (R&D biosystems, Minneapolis, MN) according to the manufacturer's protocol.

Circulating leukocyte analysis

Peripheral blood was obtained by retro-orbital bleeding and total leukocytes were measured on a Countess automated cell counter (Invitrogen) after erythrocyte lysis. Leukocyte subset frequencies were measured by flow cytometry on a BD FACS Calibur using anti-CD19 (B-cells), anti-CD11b (myeloid cells-neutrophils/monocytes), and anti-CD4/CD8 (all major T-cells) (BD Pharmingen, Franklin Lakes, NJ). For analysis of hypoglycosylated N-glycans cells were labeled with fluorescein labeled Concanavalin A (ConA) (Vector Laboratories, Burlingame, CA).

Atherosclerotic lesion analysis

At the end of the experiment, mice were euthanized with ketamine/xylazine injection, perfused with PBS, and the heart was removed and embedded in OCT. A series of five 7- μ M sections starting at the base of the aortic root and spaced 50- μ M apart were obtained and processed for atherosclerotic lesions analysis by Oil Red O staining as previously described (Baglione and Smith, 2006; Parks et al., 2005).

Statistics

Statistical analysis was calculated by *t*-test (oil red O staining) or one-way ANOVA with Tukey post-test (all other analysis unless indicated) using GraphPad 5.0. Statistical significance was assessed as $p < 0.05$ for all experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Swainsonine does not alter the hypercholesterolemia phenotype of ApoE $-/-$ mice. Mice were treated as described in Section “Methods” and serum was collected by retro orbital bleed and analyzed for total cholesterol (A), triglycerides (B), and total leukocyte counts (C). * $p < 0.05$ by one-way ANOVA versus wt and wt + SW in (A, B, and C) and # $p < 0.05$ versus ApoE $-/-$ in (C). There were four mice analyzed for each treatment condition.

Fig. 2.

Swainsonine reduces CD8⁺ T-cells in ApoE^{-/-} but does not affect other leukocyte populations. Mice were treated as described in Section “Methods” and analyzed by flow cytometry to determine the proportion of cells positive for ConA, CD11b, CD19, CD4, and CD8 (A). Total cell counts per µl for ConA (B), CD11b (C), CD19 (D), CD4 (E), and CD8 (F) were calculated by multiply percent positive cells in (A) by total leukocyte counts in Fig. 1C. In (A), **p* < 0.05 versus wt and ApoE^{-/-} and #*p* < 0.05 versus wt + SW and in (F) **p* < 0.05 versus wt and wt + SW all by one-way ANOVA with Tukey post-test. There were four mice analyzed for each treatment condition. Panel G shows the number of lineage marker negative cells. **p* < 0.05 versus wt and wt + SW all by one-way ANOVA with Tukey post-test. All values are mean ± SEM (*n* = 4).

Fig. 3.

Swainsonine induces increased serum immunoglobulins in ApoE^{-/-} mice. Mice were treated as described in Section “Methods” and serum was collected analyzed for total IgG (A) and IgG (B) levels. In (A) and (B), * $p < 0.05$ versus all other groups tested by one-way ANOVA with Tukey post test. There were four mice analyzed for each treatment condition.

Fig. 4. Swainsonine does not affect atherosclerosis development in ApoE $-/-$ mice. Mice were treated as described in Section “Methods” and aortic root atherosclerosis development was assessed by oil red O staining. Representative images of oil red O staining are shown in (A) and quantified in (B).