Intestinal Immunity and Gut Microbiota as Therapeutic Targets for Preventing Atherosclerotic Cardiovascular Diseases

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Atherosclerosis is considered a chronic inflammatory disease and an intervention targeting the inflammatory process could be a new therapeutic strategy for preventing atherosclerotic cardiovascular diseases (CVD). We hypothesized that the intestine, which is considered the biggest immune organ in the human body, could be a therapeutic target for preventing CVD. We demonstrated that oral administration of anti-CD3 antibody or an active form of vitamin D3 reduced atherosclerosis in mice via induction of regulatory T cells and tolerogenic dendritic cells in the gut-associated lymphoid tissues. Similar to regulatory immune responses achieved by oral tolerance, our method had systemic effects that ultimately contributed towards atherosclerosis reduction. Recently, we have been interested in the gut microbiota, which have been reported as highly associated with intestinal immunity and systemic metabolic disorders, including obesity and diabetes. Notably, the guts of obese individuals are predominantly colonized by Firmicutes over Bacteroidetes. The association between atherosclerosis and microbiota has been attracting increased attention, and gut microbiota have been shown to participate in the metabolism of a proatherogenic compound called trimethylamine-N-oxide (TMAO) and aggravate CVD. Our investigation of the relationship between susceptibility to CVD and the gut microbiota revealed a characteristic flora type. Here, we discuss the evidence for the relationship between the gut microbiota and cardiometabolic diseases, and consider the gut microbiota as new potential therapeutic targets for treating CVD.

Key Words: Atherosclerosis; Gut microbiota; Intestinal immunity; Regulatory T cell; Tolerogenic dendritic cell

Atherosclerosis and the resulting cardiovascular diseases (CVD) are the leading causes of mortality in many developed and developing countries. Clinical studies and animal experiments have demonstrated that elevated plasma cholesterol, mainly transported by low-density lipoprotein (LDL), promotes atherosclerotic CVD and on the basis of this finding, statin-based lipid-lowering therapy has been shown to reduce CVD and cardiovascular events. However, several clinical trials revealed that at least 50% of residual cardiovascular risk remains despite aggressive reduction of LDL-cholesterol to target levels. Unfortunately, innovative progress in the treatment of CVD beyond lipid-lowering agents has not yet been made. Given this unfulfilled need for effective treatment, there is augmenting interest in targeting novel pathways that underlie CVD pathology.

Atherosclerosis is considered not only a disorder of lipid accumulation in the arterial wall but also a chronic inflammatory disease in which both innate and acquired immunity are involved. Inflammation of the vessel walls is an important feature of atherosclerosis, and contributes to both instability of plaques and thrombotic occlusion of arteries, resulting in CV events such as acute coronary syndrome and stroke. Chronic inflammation is associated not only with atherosclerosis but also metabolic syndrome (MetS), including abdominal obesity, dyslipidemia, and type 2 diabetes mellitus (T2DM), with adipose tissue being considered the main source of pro-inflammatory cytokines. As a next-generation treatment, many researchers, including us, have been interested in anti-inflammation therapy for CVD. We subsequently thought the intestine could be a therapeutic target for preventing and treating CVD and have focused on intestinal mucosal immunity.

The gut mucosa is the largest immunologically active organ in the human body and protects the host from invading microorganisms, while it also harbors several hundred trillions of bacteria that are collectively referred to as the “gut microbiota”. Fortunately, most of these microbes are not harmful to the host and provide beneficial effects through symbiotic relationships. In the past decade, the expanded use of mice lacking gut microbiota, known as “germ-free (GF) mice”, and the
tions of the gut microbiota and metabolic diseases: obesity, MetS, and CVD. In this review, we will describe how specific gut microbial changes could affect host metabolism, and how these findings lead to novel therapeutic targets for CVD as well as MetS.

Proinflammatory and Antiinflammatory Immune Responses in Atherogenesis

Atherosclerosis is considered a chronic inflammatory disease involving many types of immune cells.4,7 Several immune responses are critical in the initiation and progression of atherosclerosis (Figure 1). The first step preceding the development of various “omics” technologies, including genomics, proteomics, and metabolomics, have enriched our understanding of the ecological system of commensal bacteria in the intestine. Now, this understanding has prompted large-scale projects to comprehend microbe-host interactions, such as the United States’ Human Microbiome Project (HMP)15 and the European Metagenomics of the Human Intestinal Tract (MetaHIT)16 Consortium.

Recent studies have demonstrated that gut microbe-derived factors may actually lead to many chronic diseases, such as ulcerative colitis and Crohn’s disease.17 In addition, massive data from both animal models and human studies are expanding our understanding of the associations between the functions of the gut microbiota and metabolic diseases: obesity, MetS, and CVD. In this review, we will describe how specific gut microbial changes could affect host metabolism, and how these findings lead to novel therapeutic targets for CVD as well as MetS.

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Intestinal immunity and gut flora play a crucial role in atherogenesis, the process by which atherosclerotic plaques develop. These plaques are characterized by lipid accumulation, inflammation, and fibrous tissue formation, often leading to endothelial dysfunction and life-threatening coronary thrombosis. The classic risk factors for atherosclerosis, such as dyslipidemia, hypertension, diabetes, and smoking, contribute to plaque formation by promoting endothelial dysfunction and LDL cholesterol deposition in the arterial wall. Oxidized LDL (OxLDL) accumulation is a key event in the progression of atherosclerosis, leading to the recruitment of monocytes and other leukocytes that can induce inflammation and foam cell formation.

The intestine is a major site for the delivery of antigens and is critical in shaping the immune response. The mucosal immune system discriminates between potentially harmful foreign antigens and harmless ones, allowing for the development of a tolerant immune environment. Tolerogenic dendritic cells (DCs) and regulatory T cells (Tregs) are key players in the induction and maintenance of oral tolerance. CTLA-4, a protein that inhibits T-cell activation, plays a critical role in the regulation of immune responses in the gut.

Intestinal immunity and gut flora have also been implicated in the prevention and treatment of atherosclerosis. Therapeutic interventions targeting the inflammatory process in atherosclerosis may lead to the establishment of definitive treatments for atherosclerotic cardiovascular disease (CVD). Oral tolerance, achieved through anergy/apoptosis of CD4+ effector T cells or induction of Tregs, is a promising strategy for preventing atherosclerosis.

**Intestine as a Therapeutic Target for Preventing Atherosclerosis**

The mucosal and intestinal immune system discriminates between potentially harmful foreign antigens and harmless ones. The gut acts to tolerate harmless antigens, but remains able to eliminate harmful pathogens. To accommodate the exposure to harmless antigens, including food components and commensal gut bacteria, the gut has evolved an anti-inflammatory environment. Tolerogenic DCs in the gut present food antigens to T cells as tolerogens and induce antigen-specific immune suppression. Recent research has revealed that tolerogenic DCs in the gut present food antigens to T cells as tolerogens and induce antigen-specific immune suppression. The development of such therapies could provide a novel approach for preventing atherosclerotic CVD.
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Retinoic acid promotes the conversion of naïve CD4+ T cells into Foxp3+ peripherally inducible Tregs (pTreg) with the help of TGF-β in the gut, suggesting significant roles for intestinal DCs that produce retinoic acid. Oral tolerance is locally induced in an antigen-specific manner, but its effects are not constrained to the local immune response within the gut. The anti-inflammatory immune responses could be seen in other tissues or organs, including distal non-lymphoid organs such as skin. Taken together, we hypothesized that modulation of intestinal immunity or induction of oral tolerance affects the systemic immune responses and might prevent atherosclerosis.

Recent studies have shown that oral anti-CD3 antibody is biologically active and induces TGF-β-producing CD4+ LAP+ Tregs, namely Th3, that suppress experimental autoimmune encephalitis, autoimmune DM in a TGF-β-dependent fashion. Autoimmune diseases are shown to be suppressed by only low doses of oral anti-CD3 antibody in association with
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an increase in LAP⁺ Tregs, but not by high doses, although there is no evidence of antigen specificity with oral anti-CD3 antibody. As observed in mucosal tolerance induction, only low doses of oral anti-CD3 antibody administration may result in the induction of Th3 by delivering a weak signal to T cells, although further elucidation of the cellular and molecular mechanisms underlying induction of various Tregs is needed.

We applied this method for the treatment of atherosclerosis in apolipoprotein E-deficient (apoE⁻/⁻) mice and demonstrated that oral anti-CD3 antibody treatment induced Th3 and Foxp3⁺ Tregs, which suppressed pathogenic immune processes pivotal for atherogenesis through a TGF-β-dependent mechanism and consequently inhibited atherosclerotic plaque formation (Figure 3). Furthermore, we examined the effect of oral anti-CD3 antibody treatment on the phenotypes of DCs in the mesenteric lymph nodes in mice and confirmed that CD80 and CD86 expressions in DCs were reduced in anti-CD3 antibody-treated mice compared with controls.

Active vitamin D₃ (calcitriol) has been shown to induce immature DCs and Tregs. We tried to examine the effects of orally administered calcitriol on atherosclerosis in animal models and first demonstrated that it decreases atherosclerosis in apoE⁻/⁻ mice by promoting induction of tolerogenic DCs and Foxp3⁺ Tregs. A cell-based therapy strategy using tolerogenic DCs revealed that apolipoprotein B100 (apoB100)-pulsed tolerogenic DCs inhibit the proliferative and proinflammatory T-cell response to apoB100, promoted Treg induction, and reduced atherosclerotic lesion formation. Oral administration of Hsp60 or Hsp65 might also induce Tregs in the gastrointestinal tract and affect atherogenesis (Figure 3).

Taken together, modulation of intestinal immunity, including the function and quantity of Tregs and tolerogenic DCs, could be a novel strategy for preventing atherosclerotic CVD.

Gut Microbiota and Their Regulatory Effects on the Intestinal Immunity

Colonization of the gastrointestinal tract begins after birth, and continues throughout life. The profile of the predominant

Figure 4. Human gut commensal microbiota and their classification. The most dominant Gram-positive phylum in the human gut flora is Firmicutes and the most dominant Gram-negative phylum is Bacteroidetes. The 4 major phyla of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria account for more than 98% of all human gut microbiota. The total number of bacteria in the human intestine is more than one hundred trillion. Those bacteria are classified into several hundreds of species.
phylum in the gut changes during childhood and youth, and almost stabilizes by adulthood. Most bacterial species in the adult human and mouse gut belong to the phyla *Firmicutes* and *Bacteroidetes*, with less abundant bacterial phyla such as *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* (Figure 4). The major 4 phyla of *Firmicutes*, *Bacteroides*, *Actinobacteria*, and *Proteobacteria* occupy more than 98% of all human gut microbiota. A lot of gut bacteria cannot be cultivated in vitro, making their categorization and identification difficult. Although there is no clear definition of a normal “healthy” gut microbiome in humans, recent metagenomics has allowed 3 major clusters of gut bacteria named “enterotypes” to be distinguished in humans, based on the predominant bacterial genera in fecal specimens: type I is characterized by high levels of *Bacteroides*, type II has few *Bacteroides* but *Prevotella* are common, and type III has high levels of *Ruminococcus* (Figure 4). The composition of the gut microbiota is remarkably diverse and dynamic over short periods of time because dietary exposure significantly affects the microbial community, but its composition appears to remain remarkably stable over time within individuals and their family members.

It has been recently demonstrated that specific bacterial species are associated with differentiation of specific subsets of T cells in the intestine. Both human and mouse *Clostridium* clusters IV and XIVA, a spore-forming component of indigenous intestinal microbiota, have been implicated in the induction of FOXP3+ Tregs in the colon of mice (Figure 3). Additionally, *Prevotella* and *Atopobium* group, *Eubacterium* were enriched in healthy controls. Individuals with a lower number of gut bacterial genes are characterized by enhanced dyslipidemia, overall adiposity and insulin resistance, which may lead to CVD.

Several researchers have reported that amounts of bacteria belonging to the phylum *Bacteroidetes* were reduced and those of the phylum *Firmicutes* were increased in obese individuals. These findings suggest that the gut microbiota is altered in obesity; however, it is unclear whether the change in microbial composition is a cause or a result of obesity. If the altered microbiota contributes to the pathogenesis of obesity, modulating the microbial component could be a new therapeutic option for extreme obesity. In this regard, it was demonstrated that the obesity phenotype is transmissible by fecal microbiota transplantation (FMT) into lean GF mice, resulting in increased capacity for energy harvest. Accordingly, the obese phenotype can be transplanted between individuals through the microbiota.

A potential mechanism for how gut microbiota contribute to the pathophysiology of obesity has been intensively studied in many laboratories. The microbiome of obese subjects has increased fermentation capability, resulting in increased levels of SCFAs in the feces. It was recently revealed that SCFAs such as acetate and propionate regulate appetite via gut endocrine hormone and a central homeostatic mechanism. Accordingly, these findings suggest that the gut microbiota directly contributes to obesity via increased appetite and energy harvest from the diet.

Two recent landmark papers demonstrated the diagnostic and clinical value of fecal microbiota composition in T2DM. In both studies, diabetic subjects were characterized by a reduction of *Clostridiales* species, including butyrate-producing *Roseburia* species and *Fecalibacterium prausnitzii*. Whereas Karlsson et al reported an enrichment of *Lactobacillus gasseri* and *Streptococcus mutans* in diabetic patients, Qin et al found that increased number of *Proteobacteria* could be a predictor of T2DM. In fecal samples of Japanese patients with T2DM, it became obvious that the *Clostridium cocoides* group, *Atopobium* cluster, and *Prevotella* were significantly fewer, while the total *Lactobacillus* count was higher than in those of control subjects, using a sensitive quantitative reverse transcription PCR method. In particular, the counts of the *Lactobacillus reuteri* and *Lactobacillus plantarum* subgroups were significantly higher in Japanese T2DM patients.

### Gut Microbial Alternations Associated With Atherosclerosis

Recent studies in both mice and humans show that obesity is also associated with changes in the gut microbiota compared with lean subjects. It has been indicated that the gut microbiota may function as an environmental factor that contributes to obesity and T2DM. GF mice that consume 27% more food than conventional mice have significantly less body fat, suggesting the role of the microbiota in metabolism and energy balance. In humans, a study including twins concordant for obesity and T2DM showed that, although plasma cholesterol levels were significantly higher in GF/ apoE−/− mice than in conventional ap apoE−/− mice, the effect of gut microbiota on atherosclerosis was ambiguous. Lipidomic analysis of GF and conventionally raised mice proposes that the gut microbiota affects host lipid metabolism. A detailed mechanism for how commensal bacteria may contribute to host lipid and cholesterol metabolism could potentially be explained by microbial regulation of bile acid synthesis and metabolism, but further work is required.

A sequencing study comparing the gut microbiome from patients who had stenotic and symptomatic atherosclerotic plaques in the carotid artery and from healthy controls showed that the microbiome was more proinflammatory in the people with atherosclerotic plaques. The shotgun sequencing of the gut metagenome demonstrated that the genus *Collinsella* was increased in patients with symptomatic atherosclerosis, whereas *Roseburia* and *Eubacterium* were enriched in healthy controls. The study also demonstrated that patients with symptomatic atherosclerosis were underrepresented in enterotype I, and overrepresented in enterotype III.

Our research group also has been investigating the association between the fecal microbiota and coronary artery disease (CAD). We will report the results in the near future.

### CVD and the Chemical TMAO Connection

Recently, Hazen et al have splendidly reported that gut microbial-
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Microbiota using broad-spectrum antibiotics cancelled the pro-atherosclerotic effect of dietary choline, which was associated with the reduction of plasma TMAO levels in antibiotic-treated mice. The study also investigated the relationship between fasting plasma levels of TMAO and incident major adverse cardiovascular events (death, myocardial infarction, or stroke) during 3 years of follow-up in 4,007 patients undergoing elective diagnostic cardiac catheterization. Increased plasma TMAO levels were associated with an increased risk of a major adverse cardiovascular event (Figure 5). Even after adjustment for traditional risk factors, an elevated TMAO level could predict an increased risk of major adverse cardiovascular events. Collectively, the findings suggest that pathways that are dependent on the gut microbiota may contribute to the pathophysiology of atherosclerotic CAD and suggest potential therapeutic targets.

It was also clarified that microbial processing of L-carnitine, which is abundant in red meat and contains a TMA structure similar to that of choline, elevates plasma TMAO concentrations and enhances atherosclerosis in a microbial-
dependent manner.\textsuperscript{45} It was also recently found that γ-butyrobetaine is the major proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMA and TMAO.\textsuperscript{46} One of the proatherosclerotic mechanisms of TMAO might be increasing macrophage foam cell formation and suppressing reverse cholesterol transport (RCT) in vivo. Bacterial taxa belonging to the families Clostridiaceae and Peptostreptococcaceae were positively associated with TMAO production in humans, suggesting that L-carnitine-metabolizing bacteria may belong to these families. However, the molecular mechanisms through which gut microbial formation of TMAO leads to inhibition of RCT are not entirely clear. Further, this study may in part explain why excessive red meat consumption has been associated with increased CVD and mortality risks.

**Future Perspectives**

As imbalance in the gut microbiota, termed dysbiosis, significantly contributes to various human diseases, the fecal microbiota are regarded as important players in the development of metabolic disorders and subsequent CVD. As currently available evidence is mainly based on animal and human cohort studies, additional work in the future is earnestly desired to clarify how altered gut microbiota can be used in both the diagnostic and therapeutic setting. For example, FMT from a healthy donor by duodenal tubing, colonoscopy, or enema has been shown as effective in patients with pseudomembranous enteritis infected by *Clostridium difficile.\textsuperscript{47} Transplanting microbiota from lean donors to recipients with MetS led to an improvement in insulin sensitivity, with increased levels of butyrate-producing bacteria.\textsuperscript{48} There will be many obstacles in the development of commercial products based on FMT, for example donor selection, quality assurance, risk of pathogen contamination, and patient acceptance. FMT from healthy donor to CVD patient might enable us to identify causally involved intestinal microbes in CVD and might be a new therapeutic strategy for CVD, as in the case of treating *Clostridium difficile* infection.

**Concluding Remarks**

Intestinal immunity has been attracting much attention as a novel therapeutic target for atherosclerosis. Since the discovery of Tregs and DCs, knowledge about the biology and pathophysiology of these regulatory immune cells has accumulated in the fields of atherosclerosis and autoimmune diseases. It is now clear that several types of Tregs and tolerogenic DCs are essential for the regulation of pathogenic T-cell immune responses in atherogenesis. Gut-associated immune tolerance induction by oral administration of drugs or therapeutic agents possessing immunoregulatory activities is simple, easy, and a hopeful way of regulating inflammatory diseases, though the detailed mechanisms of how intestinal immunity affects systemic immunity remain to be clarified.

In association with intestinal immunity in atherogenesis, we have been interested in the gut bacteria that might be involved in the pathogenesis of atherosclerotic CVD and the associated gut flora types. Simple classification of the gut microbiota associated with CVD, especially CAD, must be the first step. Sequencing the microbial genes by a metagenome-wide association study is the second step. However, this may be insufficient, because the presence of DNA alone neither necessarily translates into protein synthesis nor relates to function. Further studies are needed for understanding of the functional level of some specific microbial pathways and their products that contribute to maintaining our physiological homeostasis and that contribute to disease processes. We hope the novel therapeutic strategies that intervene in the gut microbiota for the prevention of atherosclerotic CVD will be developed in the near future and contribute to patients’ wellbeing.

**Disclosures**

The authors received some research funding described below. They declare no other conflict of interest.

**Acknowledgments**

This work was supported by Japan Society for the Promotion of Science KAKENHI Grant No. 24591114 (T.Y.), Suzuken Memorial Foundation (T.Y. and N.S.), Takeda Scientific Foundation (T.Y. and N.S.), Mochida Memorial Foundation (T.Y.), Senshin Medical Research Foundation (T.Y. and N.S.), Yakult Bioscience Research Foundation (T.Y.), Uehara Memorial Foundation (K.H. and N.S.), and The Japanese Circulation Society Translational Research Foundation (K.H.).

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