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Review Article

Impact of Gut Microbiota Extracellular Vesicles on Inflammatory and Metabolic Disease

Tae-Seop Shin#, Eun Kyoung Kim#, Andrea Mc Dowell# and Yoon-Keun Kim*

MD Healthcare Inc., Seoul, Republic of Korea

#These authors contributed equally to this work

***Address for Correspondence:** Yoon-Keun Kim, MD Healthcare Inc., Seoul, Republic of Korea, E-mail: ykkim@mdhc.kr

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Introduction

The microbiome is the collection of genetic material associated with the microbial community of bacteria, archaea and eukaryotes that coinhabit a given environment. The number of symbiotic microbes in the human body has been conventionally estimated to be 100 trillion, 10 times more than human cells, and contribute over 100 times more genetic material than the human genome [1]. The intestinal microflora is the largest component of the human microbiome and is known to play an important role in human physiology. For example, the intestinal microbiota metabolizes substances that humans cannot process through digestion, contributes to immune system maturation, and has been associated withdevelopment of metabolic diseases such as obesity and diabetes, anxiety, depression, dementia and other mental health disorders [2].

Extracellular vesicles (EVs) were long thought to be passivecellular secretions, but recent studies have shown that cells release EVs through a targeted mechanism. While only eukaryotic or Gram-negative bacteria were initially known to secrete EVs, recently it has been revealed that Gram-positive bacteria also release EVs despite the thick peptidoglycan layers composing their cell walls. (Elizabeth Work, et al., Ann NY Acad Sci. 1966, Lee EY, et al, Proteomics, 2009). Bacterial extracellular vesicles are spherical lipid bilayers with a diameter of 20-200 nm that contain various proteins, DNA, and RNA [3–5]. The method of EV isolation is performed using ultracentrifugation and EV characterization includes transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and protein pattern analysis. Metabolomics and proteomics can also be used to validate the materials composing the EVs isolated from a given sample.

Recently, functional studies of extracellular vesicles secreted from various microbial strains and their impact on pathogenesis have been conducted through assessment of pathogen associated molecular patterns (PAMP). Aside from investigation of the pathogenic potential of bacterial EVs, the anti-inflammatory properties of EVs secreted by probiotic strains such as lactic acid bacteria (LAB) and use as vaccine candidates are also under investigation. However, the exact mechanism through which microbial EVs affect inflammatory responses and various diseases such as inflammatory bowel disease (IBD) and type 2 diabetes (T2D) mellitus remains uncertain.It is generally hypothesized that as the external EV envelope contains membrane components of EV-derived bacteria, EVs are predicted to affect disease through similar mechanisms as the parent bacterial cells the EVs are derived from such as TLR2, TLR4, and PAMP pathways. Further studyis necessary to validate the precise mechanisms and components through which EVs affect host immune modulation and disease.

Human Microbiome

Although the number of microorganisms living in the human body is known to be greater than 100 trillion, it is not easy to study the intestinal microbiome because only 20-40% of intestinal microorganisms can be cultured. All bacteria possess 16S ribosomal RNA (rRNA) composed of conserved and hypervariable regions unique to each species. Isolation of the bacterial 16S rRNA genes in a given environmental sample enables identification to the species levelregardless of the ability to culture a given species. Furthermore, recent development of high throughput pyrosequencing methods to analyze all genes in a sample has spurred the rapid expansion of microbiome research capabilities [6]. This metagenomic approach has been widely adopted for microbiome analysis for its ability to simultaneously analyze the target 16S rRNA nucleotide sequence of all microorganisms in a given environment, particularly those previously unknown due to limitations of current bacterial culture methodology.

The microbial genome can be defined as the entire genome of the microflora present in an environment. Since 2007, the National Institute of Health has collected samples from 15-18 human regions (skin, nasal cavity, mouth, intestine, genitourinary system, etc.) of 242 humans, and conducted theHuman Microbiome Project (HMP) from the collected samples. Following this process, in 2010 the HMP published the microbial meta-genome sequences of 178 samples [7]. In 2012, the Human Microbiome Project consortium classified 5,177 microorganisms and analyzed 800 microbial genome sequences in the intestinal, skin, and vaginal microbiota and found that even healthy individuals can have vastly differing microbiota compositions [8]. In addition, comparison of microbial composition between families revealed that twins possess the highest similarity, followed by mothers, offspring, and strangers who share less than 50% of the total microbiota [9]. Our understanding of the general universality of the microbiome has been further confirmed through comparison ofthe total microbial composition among different ethnic groups including Koreans, Japanese, Chinese, and Americans.Mounting evidence that host genes and dietary habits influence the composition of intestinal microbiota also suggests that the human microbiome is a complex ecosystem influenced by a variety of environmental factors [10].

Diet-based Gut Enterotypes

The human intestinal microflora is divided into four core phyla: Gram-negative Bacteroidetes and Proteobacteria and Gram-positive Firmicutes and Actinobacteria [11]. Among these core phyla, Bacteroidetes and Firmicutes are known to make up the majority of intestinal microorganisms while Proteobacteria and Actinobacteria account for less than 10%. Thehuman bacteriological ecosystem can be generally classified into three main enterotypes based on the dominance of either *Bacteroides*, *Prevotella*, or *Rumminococcus* [12].

A study comparing the intestinal microflora of children in rural Europe and Africa found that *Prevotella* and *Xylanibacter*, which have genes that hydrolyze fiber and polysaccharides in African children who eat mainly fruits or grains is more abundant than European children who primarily eat meatsand processed flour. Further, feces of African children have been found to be richer in biodiversity, have higher concentrations of short-chain fatty acids and butyric acid, which are effective in preventing colon cancer, and less *E. coli*, *Shigella* and *Salmonella* than European children [13]. Based on these findings, Western diets high in protein, fat and simple sugars maylead to homogenization of various intestinal microflora. In addition, a study analyzing the intestinal microflora of Japanese people confirmed that microorganisms rich in enzymes that degrade porphyran contained in seaweeds were distributed in Japanese people who consumed algae [14]. While the precise distribution of intestinal microflora in response to differing dietary habits is not yet known, generally it has been shown that those with high intakes of protein and animal fats are more likely to have a gut enterotypes predominantly composed of *Bacteroides*. Furthermore, those who consume high amounts of sugary carbohydrates have been reported to commonly have a *Prevotella* enterotype while those who consume primarily high-fiber carbohydrates are associated with a *Ruminococcus*-dominant enterotype [12]. Altogether, these findings support the theory that dominant gut enterotypes may be associated with long term eating habits.

Relationship between gut microbiota and Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is characterized by chronic low-grade inflammation of the gastrointestinal tract resulting in potentially severe symptoms that can severely diminish patient quality of life [15]. While the precise etiology of IBD remains under investigation, disruptions in the normal flora inhabiting the intestinal lumen as well as intestinal barrier function have been attributed to IBD development [16]. Commensal gut microorganisms comprise a complex, diverse ecosystem that continuously interacts with gut epithelial cells and have been shown to play key roles in maintenance of the mucosal barrier. However, alterations in the gut microbial ecosystem due to environmental factors including antibiotics, dietary patterns, and compromised immunity can lead to dysfunctional immunomodulation and metabolic activity in the gut [17]. Such disruptions have been linked to diminished mucus barrier functionality including decreased tight junctions in the intestinal epithelial lining resulting in increased intestinal permeability. When the integrity of the mucosal barrier is significantly diminished, the passage of inflammatory agents such as microbial components from the intestinal lumen can trigger long-term, low-grade mucosal inflammation associated with IBD [18].

Intestinal tissues of patients with IBD have been found to have decreased Firmicutes and Bacteroidetes *spp*., increased Actinobacteria and Proteobacteria *spp*., in addition to reduction of overall intestinal microbial diversity [19]. Another study assessing tissuesprocured from patients with IBD showed increased Bacteroidetes and decreased Firmicutes and microbial diversity compared to healthy controls. Furthermore, comparison ofinflamed and non-inflamedintestinal tissue samples of IBD patients showed significant differences in the mucosal microbiota composition with high inter-individual variation [20]. In addition, five bacterial species were identified through denaturing gradient gel electrophoresis (DGGE) profiling of fecal samples from patients with Crohn's disease and normal subjects [21]. In patients with Crohn's disease, *Ruminococcus gnavus* was shown to be increased while *Dialister invisus*, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, and an uncharacterized species of Clostridium cluster XIVa was decreased. Based on these findings, the intestinal microbiota plays an important role in inflammatory growth disease, and fecal microbiota transplantation (FMT) was performed in patients with Crohn's disease. In addition, probiotics have been shown to be effective in the prevention of pouchitis in patients with ulcerative colitis.

In addition, the frequent use of antibiotics in the first year of life has been reported to increase the risk of developing Crohn's disease [22], suggesting that the interaction between the host and the microflora during infancy plays an important role in maintaining the intestinal immune homeostasis. The above findings suggest that the intestinal microflora plays an important role in the development of IBD, but it is still unclear whether the intestinal microflora is the cause or result of inflammation. In addition, many researchers agree that intestinal microorganisms are important in the pathogenesis of inflammatory diseases, but the exact substance and mechanism responsible for inflammation is still unknown.

Importance of Microbial EVs in IBD

In order to further elucidate the dynamic interaction between

the gut microbiota and IBD, we performed metagenomic analysis of microbial EVs derived fromdextran-induced colitis mouse stool. Through this study, it was confirmed that extracellular vesicles derived from TM7 bacteria were significantly increased in the stool of IBD mice, while the extracellular vesicles derived from the absolute anaerobic bacteria *Akkermansia muciniphila* and *Bacteroides acidifaciens* were decreased. To date, the uncultivated candidate TM7 phylum has often been associated with human inflammatory disease without a clear understanding of the underlying mechanism. The increased TM7 EVs observed in this study in addition to reduced *A. muciniphila* EVs offers a new insight into the influence of dextran-induced intestinal inflammation on gut microbiota activity. These results indicate that although it is difficult to confirm the directeffect of many microorganisms on colon homeostasis, absolute anaerobic bacteria such as *A. muciniphila* appear to play an important role in IBD. In addition, as EVs secreted by absolute anaerobic bacteria such as *A. muciniphila* have a very important effect on IBD, further study should be conducted to determine the exact mechanism through which anaerobic microbial EVs impact colon homeostasis [23]. Furthermore, a recent study reported that application of *Lactobacillus*-derived extracellular vesicles exerted a therapeutic effect on IBD [24]. It was also confirmed that inflammatory cytokines including IL-8 were reduced when Caco-2 cells were treated with EVs obtained from *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, and *Lactobacillus kefirgranum*, which are isolated from a fermented milk product called kefir. Moreover, when *Lactobacillus*-derived EVs were administered to 2,4,6-trinitrobenzenesulfonic acid-induced mice with IBD, rectal bleeding was reduced and stool consistency was improved.

Relationship between Gut Microbiota and Metabolic Disease

The commensal bacteria residing in our GI tract play an invaluable role in nutrient absorption and fermentation of dietary fibers otherwise indigestible by human metabolic mechanisms. Our gut microbiota provide a particularly critical function in the production of short-chain fatty acids (SCFAs) including acetate, butyrate, and propionate necessary as energy sources as well as epigenetic regulation via histone deacetylase (HDAC) inhibition [25]. Gut microbiota dysbiosis resulting in irregular SCFA production can lead to dysregulated host metabolic functionality. For example, a group in China conducted a metagenome-wide association study and determined that T2D patients were characterized by decreased abundance of certain universal butyrate-producing bacteria such as *Clostridiales sp. SS3/4*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and *Roseburia intestinalis* [26]. Butyrate is known to activate intestinal gluconeogenesis via the gut-brain axis, exerting a positive influence on metabolic regulation of body weight and glucose [27]. Therefore, the finding that the T2D microbiome tends to have diminished butyrate-producing bacterial populations supports a critical relationship between the microbiome and metabolic disease.

The first evidence that obesity, a representative metabolic disease, is associated with changes in the intestinal microflora was when Gordon et al. reported increased Firmicutes and decreased Bacteroidetes in the intestinal microflora of Leptin deficient ob/ob mice in 2005 [28]. Similar studies have shown that consumption of high-fat diets in wild-type mice results in microflora with increased Firmicutes and reduced Bacteroidetes abundance [29].

The importance of intestinal microflora as a causal factor in obesity has been thoroughly demonstrated in several studies. Germ-free mice inoculated with fecal microbiota from obese andlean mice under identical dietary conditions showed thatthe mice given obese gut microbiota transplants experienced weight gain [30]. This finding demonstrates the differential metabolic capacity of intestinal microflora associated with obesity and the significant contribution gut bacteria have on metabolic function. Furthermore, an increase in the ratio of Firmicutes/Bacteroidetes was observed in the intestinal microflora of obese people and has been correlated extensively with obesity. However, the proportion of Bacteroidetes was able to be increased through consumption of low-carb or low-fat meals, suggesting that while the intestinal flora influences metabolic function in the gut, diet can also modify the composition of our gut microbiota [31]. Furthermore, twin studies showed that obesity phenotypes show decreased intestinal microbial diversity, decreased Bacteroidetes, and enrichment of microorganisms with genes involved in carbohydrate and fat metabolism [32]. However, contrary to the above findings, there has also been a report that the ratio of Firmicutes/Bacteroidetes in obese people was reduced [33] and that there was no difference in the level of intestinal microorganisms when compared with the intestinal microflora of obese and lean people [34]. Therefore, further study of larger sample groups accounting for a wider variety of potentially confounding factors should be rigorously pursued to better understand the relationship between gut microbiota and obesity.

Intestinal microorganisms have also been reported to play an important role in the pathogenesis of T2D mellitus, which is characterized by insulin resistance. Feeding mice with high-fat meals increases intestinal permeability which in turn increases the concentration of the microbial outer membrane lipopolysaccharide (LPS) in the blood.Increasedblood LPS levels are known to cause insulin resistance associated with diabetes mellitus, obesity, and low-level inflammation [35]. In addition, it has been shown that in the absence of LPS-recognizing toll-like receptor (TLR)-4,dietary-induced insulin resistance can be prevented [36]. These results suggest that intestinal microorganisms interact with the innate immune system through the host's TLRs and are involved in the development of insulin resistance and T2D.

In conclusion, it is accepted that intestinal microorganisms play an important role in the energy-metabolism process and development of obesity and T2D. While extensive investigation of the exact mechanism through which the gut microbiota interacts with metabolic disease is ongoing, final consensus from the scientific community has yet to be gained.

Significance of Gut Microbiota EVs as a Factor in Type II Diabetes

The intestinal mucosa is a mesh-like structure that allows only nano-sized particles to pass through. Therefore, while bacteria cannot freely pass the intestinal barrier, the nano-sized EVs they release are able tofreely transverse the intestinal mucosa lining and enter circulation. For this reason, the role intestinal microbial EVs play in metabolic health is a critical area of study (Figure 1).

We determined that EVs derived from intestinal bacteria play an important role in the pathogenesis of high-fat diet induced T2D through experimental animal models [37]. This *in vivo* study revealed

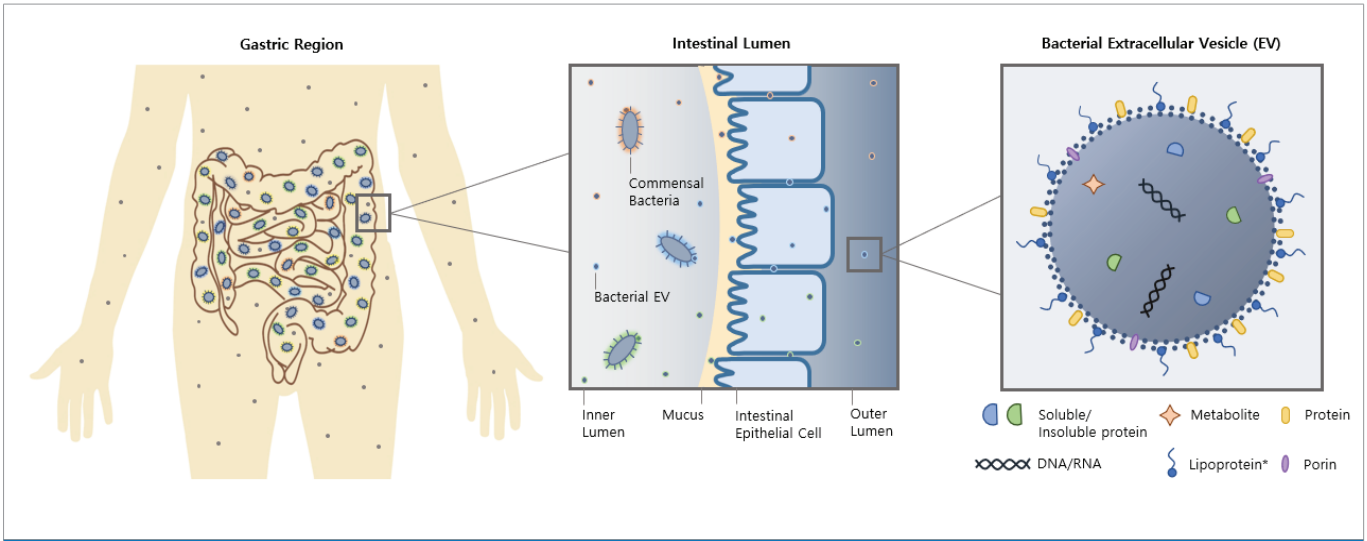

Figure 1: Systemic circulation of intestinal microbiota extracellular vesicles.
The commensal bacteria residing in the intestines release extracellular vesicles (EVs) during their proliferation and death. While the bacterial cells themselves are unable to bypass the intestinal mucosal and epithelial cell barrier, their nano-sized EV counterparts are able to transverse the intestinal barrier into the outer lumen. Thus, intestinal bacterial EVs are able to circulate the body and deliver bacterial components including both soluble and insoluble proteins, DNA, RNA, membrane proteins, lipoproteins in the case of gram-negative bacterial EVs, and potentially metabolites for distal intercellular communication.

that upon oral administration of extracellular vesicles isolated from the stool of mice fed high-fat dietsto mice fed a normal diet, insulin resistance was induced in the mice fed a normal diet at similar levels as the high-fat diet group. In addition, oral administration of extracellular vesicles isolated from *Pseudomonas panacis*, an intestinal microorganism shown to have increased extracellular vesicle activity in response to high fat diet, and to normal diet mice induced insulin resistance. The third significant finding of this study was that when *P. panacis* and its extracellular vesicles were administered orally, while the bacteria were not absorbed into the body, the extracellular vesicles were absorbed systemically after 5 minutes of oral administration. Based on these findings, the researchers determined that intestinal microbial-derived extracellular vesicles play an important role in the pathogenesis of high-fat diet induced insulin resistance associated with T2D.

In a study published in the Journal of Experimental & Molecular Medicine in 2018, we found that *Akkermansia muciniphila* EVs were more abundant in fecal samples obtained from healthy control subjects than the fecal samples of T2D patients. Furthermore, we confirmed that the group administered *A. muciniphila*-derived EVs had reduced gut permeability and improved metabolic functions through AMPK signaling in a high fat diet mouse model [38]. Obesity and T2D are known to be closely related withobesity often acting as a prognostic sign of T2D. While *A. muciniphila* has been well characterized to be associated with a lean phenotype, recently *A. muciniphila* EVs have been reported to improve symptoms of obesity. Administration of *A. muciniphila* itself or EV of *A. muciniphila* reduced weight and decreased daily food intake in a HFD model. In particular, it was confirmed that weight and food intake were more greatly reduced in the HFD group administered *A. muciniphila* EVs than the group administered the *A. muciniphila* bacteria. Furthermore, mRNA expressions of inflammatory cytokines and TLR-4 were more decreased while mRNA expression of tight junction proteins including ZO-1, OCLDN, and CLDN-1 were more increased in the group administered *A. muciniphila*-derived EVs compared with the group treated with *A. muciniphila* bacteria itself [39].

Conclusion

With the development of increasingly affordable metagenomic sequencing technology, research on the interaction between symbiotic microorganisms and host health and disease has been rapidly progressing. However, to date, no clear mechanism of communication between symbiotic microorganisms and hosts in the human body has been agreed upon. This is probably because while the microbes themselves are important, the role of soluble and insoluble microbial metabolites is often overlooked.

EVs secreted by bacteria were first observed through an electron microscope in the 1960s. However, at that time bacterial EVs were dismissed as simply wastes that bacteria excrete extracellularly and awareness of their role in human health is still lacking in the scientific community. Unlike bacteria, bacterial EVs are nano-sized, so they can travel through the intestinal barrier and circulate throughout the body and directly interact with the host systemically. Due to this ability to travel throughout the body, intestinal microbial EVs can have far-reaching influence on health and disease in other areas of the body outside the confines of the intestines. In this regard, EVs derived from intestinal microorganisms can be understood to be directly linked to the health and disease of the whole human body.

The dynamic network of meaningful interactions between the host and microbiota-derived EVs adds yet another complication to our understanding of the complex relationship between the microbiome and disease. Recently emerging methodologies such as molecular pathological epidemiology (MPE) should be explored in order to integrate molecular pathological signatures with a diseased individual's unique epidemiological factors and microbiome

signature. Further study on the complex environment-disease-host interactions between diet, gut microbiota, and their EVs is necessary to determine the precise mechanisms of interaction between our gut microbiota and disease.

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