Microbial Colonization of Germ-Free Mice Restores Neointimal Hyperplasia Development After Arterial Injury

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***Background*—**The potential role of the gut microbiome in cardiovascular diseases is increasingly evident. Arterial restenosis attributable to neointimal hyperplasia after cardiovascular procedures such as balloon angioplasty, stenting, and bypass surgery is a common cause of treatment failure, yet whether gut microbiota participate in the development of neointimal hyperplasia remains largely unknown.

***Methods and Results*—**We performed fecal microbial transplantation from conventionally raised male C57BL/6 mice to age-, sex-, and strain-matched germ-free mice. Five weeks after inoculation, all mice underwent unilateral carotid ligation. Neointimal hyperplasia development was quantified after 4 weeks. Conventionally raised and germ-free cohorts served as comparison groups.

***Conclusions*—**Germ-free mice have significantly attenuated neointimal hyperplasia development compared with conventionally raised mice. The arterial remodeling response is restored by fecal transplantation. Our results describe a causative role of gut microbiota in contributing to the pathogenesis of neointimal hyperplasia. **(*J Am Heart Assoc*. 2020;9:e013496. DOI: 10.1161/JAHA.119.013496.)**

Key Words: microbiome • neointimal hyperplasia • restenosis

Neointimal hyperplasia is a prevalent cause of restenosis after bypass surgery, balloon angioplasty, and stenting. However, the complex direct causal effects and interactions of genetic and environmental influences in this process are not well understood.[1] Specifically, the gut microbiome may be an important environmental factor influencing susceptibility to neointimal hyperplasia development after arterial injury despite the lack of direct contact between gut microbes and the peripheral vasculature. We previously observed that germ-free (GF) mice develop significantly less neointimal hyperplasia 4 weeks after unilateral carotid ligation compared with an age- and sex-matched conventionally raised (CONV-R) cohort.[2] In addition, GF mice had an altered systemic and local arterial inflammatory response to carotid ligation, corroborating data by others that microbiota regulate acute inflammatory responses.[3]

The goal of this study was to further elucidate the causative role of gut microbiota in the arterial remodeling process by recolonizing GF mice using fecal transplantation (GF-FT). We hypothesized that microbial colonization would restore the neointimal hyperplasia phenotype after arterial injury.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Male C57BL/6 mice in the CONV-R cohort were housed in conventional conditions at Northwestern University with standard irradiated chow and autoclaved drinking water provided ad libitum. Fresh donor fecal samples were collected from the CONV-R cohort, snap frozen at −80°C immediately after collection, and stored frozen until use. Left carotid ligation in 18- to 22-week-old mice was performed as previously described.[2] Age- and sex-matched GF C57BL/6 mice were bred and maintained in flexible film isolators at the University of Chicago Gnotobiotic Research Animal Facility. On the day of fecal transplantation, a subcohort of GF mice (GF-FT) were transferred in sterile transport caging to the

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quarantine facility for fecal transplantation. Donor fecal samples were thawed, pooled, and homogenized (100 mg/mL) in sterile PBS. An aliquot of the fecal slurry was stored at −80°C for DNA extraction. Mice in the GF-FT cohort each received 150 μL of the same fecal slurry by oral gavage. Mice were subsequently housed in sterile conditions in semi-rigid isolators under positive pressure. Five weeks after inoculation, GF-FT mice underwent carotid ligation. GF mice that remained sterile and underwent carotid ligation under sterile conditions served as the comparison cohort. Four weeks after carotid ligation, mice were euthanized and bilateral carotid arteries were harvested and processed as described previously.[2] All animal procedures were approved and conducted in accordance with the Northwestern University and University of Chicago Institutional Animal Care and Use Committees. All animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Neointimal hyperplasia, defined as intima area, intima+media area, intima/(intima+media), and intima/media, was quantified on arterial sections at evenly spaced 350 micron intervals across the common carotid artery (≈3.5 mm long) and an average value was calculated. Fecal samples from all mice were collected weekly for genomic DNA isolation. Fecal bacterial 16S rRNA copy number in CONV-R and GF-FT mice was determined using quantitative real-time polymerase chain reaction.[4] Unweighted unifrac beta diversity was used to assess the community level differences between microbiomes of experimental groups without weighting by microbe abundance.[5] A statistical framework called analysis of composition of microbiomes (ANCOM) was used to search for differentially abundant microbes between groups.[6]

Results

As shown in Figure 1A and 1B, 4 weeks after carotid ligation, male GF mice had ≈25% less neointimal hyperplasia than the CONV-R cohort (intima: 0.005±0.002 mm^2 [GF] versus 0.021±0.004 mm^2 [CONV-R], P=0.01; intima+media: 0.029±0.003 mm^2 [GF] versus 0.055±0.005 mm^2 [CONV-R], P=0.005). Conventionalization using donor CONV-R stool attenuated this difference and restored the arterial remodeling phenotype of CONV-R mice to ex-GF mice. There were no significant differences in neointimal hyperplasia severity between GF-FT mice compared with CONV-R mice (intima [GF-FT]: 0.014±0.003 mm^2, P=0.23; intima+media [GF-FT]: 0.050±0.008 mm^2, P=0.53). Correspondingly, GF-FT mice had significantly more neointimal hyperplasia than their GF counterparts (intima, P=0.04; intima+media, P=0.02). All morphometric data are provided in Table 1. Interestingly, while there was a significant difference in intima/media between GF and CONV-R (P=0.04), there was neither a significant difference in intima/media or intima/intima+media between GF and GF-FT mice nor between CONV-R and GF-FT mice, suggesting that there is remodeling of both the intimal and media layers after injury in this model after fecal

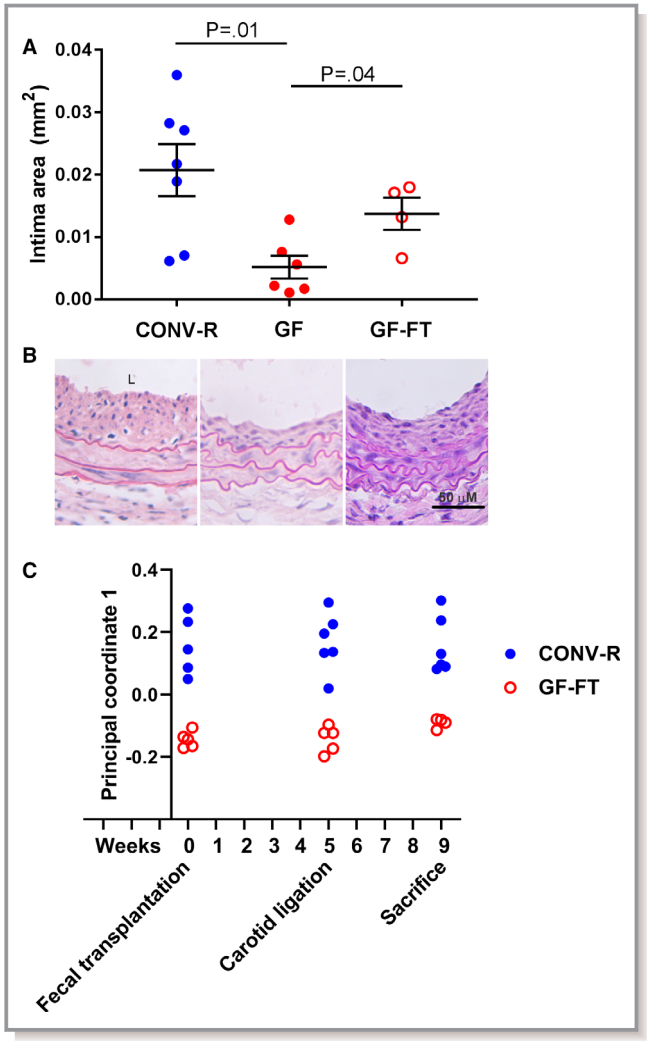

Figure. Neointimal hyperplasia after arterial injury in conventionally raised, germ-free, and germ-free after fecal transplantation mice. **A**, Mean intima area of post-ligation carotid arteries from mice in each group (conventionally raised, n=7; germ-free, n=6; germ-free after fecal transplantation, n=4). There is no significant difference between conventionally raised-and germ-free after fecal transplantation groups. **B**, Representative hematoxylin and eosin staining of post-ligation carotid arteries. Lumen (shown as L) is oriented at the top. Scale bar indicates 50 microns. Groups were considered a priori to be non-parametric and the Mann–Whitney *U* test was used to assess for differences between groups. P<0.05 was considered significant. **C**, Microbial diversity shifts in the conventionally raised and germ-free after fecal transplantation cohorts. Principal coordinate analysis of unweighted unifrac beta diversity of microbiome samples in both groups across sampling times. The first component (principal coordinate 1) is shown and explained 22% of the total variance. CONV-R indicates conventionally raised; GF, germ-free; GF-FT, GF after fecal transplantation.

Table 1. Post-Injury Vessel Parameters in Left Carotid Arteries in CONV-R, GF, and GF-FT Cohorts

	Intima Area (mm^2)	*P* Value	Media Area (mm^2)	*P* Value	Intima+Media (mm^2)	*P* Value	Intima/ Intima+Media)	*P* Value	Intima/Media	*P* Value
CONV-R	0.021±0.004		0.035±0.001		0.055±0.005		0.350±0.049		0.589±0.110	
GF	0.005±0.002	0.01, GF vs CONV-R	0.024±0.002	0.001, GF vs CONV-R	0.029±0.003	0.01, GF vs CONV-R	0.165±0.049	0.04, GF vs CONV-R	0.219±0.074	0.04, GF vs CONV-R
GF-FT	0.014±0.003	0.23, GF-FT vs CONV-R	0.036±0.006	0.65, GF-FT vs CONV-R	0.050±0.008	0.53, GF-FT vs CONV-R	0.273±0.036	0.23, GF-FT vs CONV-R	0.385±0.066	0.23, GF-FT vs CONV-R
		0.04, GF-FT vs GF		0.02, GF-FT vs GF		0.02, GF-FT vs GF		0.17, GF-FT vs GF		0.17, GF-FT vs GF

Values shown represent mean±SEM. CONV-R indicates conventionally raised; GF, germ-free; GF-FT, GF after fecal transplantation.

Table 2. Media Area in Uninjured Right Carotid Arteries in CONV-R, GF, and GF-FT Cohorts

	Media Area (mm^2)	*P* Value
CONV-R	0.019±0.001	
GF	0.018±0.002	0.71, GF vs CONV-R
GF-FT	0.020±0.004	0.50, GF-FT vs CONV-R
		0.40, GF-FT vs GF

Values shown represent mean±SEM. CONV-R indicates conventionally raised; GF, germ-free; GF-FT, GF after fecal transplantation.

transplantation. As shown in Table 2, the media areas of the uninjured right carotid areas were similar between the 3 groups, suggesting that the relative smaller post-injury media area in the GF mice compared with CONV-R and GF-FT mice represents a difference in remodeling response rather than an intrinsic difference in baseline vessel morphology. While the larger study includes mice from both sexes, females will be analyzed and reported separately as there is known sexual dimorphism in both neointimal hyperplasia susceptibility[7] and the microbiome.[8] Microbial load at the time of carotid ligation was similar between CONV-R and GT-FT mice (16S rDNA gene copies$\times 10^5$: 2.3±0.6 [CONV-R] versus 4.2±1.2 [GF-FT]; *P*=0.3). Notably, however, there was a significant difference in beta diversity, indicating the differential presence or absence of some sequence variants, between the CONV-R and GF-FT fecal samples (Figure 1C), suggesting that there was incomplete transfer of microbiota between the 2 cohorts. This incomplete transfer was likely caused by differences in colonization versus natural acquisition of microbiota and by immune differences in GF animals, which can reduce the fidelity of transplants into GF animals, though this fidelity is higher than transplants into conventional or antibiotic-treated animals.[9] ANCOM analysis to search for specific microbes which differed between samples was inconclusive, indicating the difference in beta diversity was not caused by drastic differences in the abundance of a few organisms but rather small changes in many. Nonetheless, finding a core set of microbes which can restore the hyperplasia phenotype is of high priority and may be possible with future GF models.

Discussion and Conclusions

This study provides the first direct demonstration of the impact of gut microbiota on the remodeling response of peripheral arteries after injury, with reversal of attenuated neointimal hyperplasia in GF mice after fecal transplantation compared with CONV-R mice. Possible mechanisms include direct or indirect modulation of the local arterial inflammatory response by microbiota-derived components, ie,

lipopolysaccharide and/or microbe-generated metabolites. Whereas we previously identified significant differences between CONV-R and GF mice in systemic concentrations of inflammatory cytokines and chemokines and in arterial infiltration of inflammatory cells,[2] preliminary investigation comparing acute inflammation in CONV-R, GF, and GF-FT cohorts using multiplex immunoassays of inflammatory cytokines revealed non-linear relationships between individual cytokines, microbial colonization, and neointimal hyperplasia (data not shown) that will require further investigation to fully understand the inflammatory cell dynamics in post-injury arteries driven by microbial colonization. Further studies are also required to unravel and refine the mechanistic link between peripheral arterial remodeling and gut microbiota and to elucidate whether modulation of the gut microbiome represents a novel therapeutic target for prevention and treatment of arterial restenosis.

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Disclosures

None.

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