Dietary Microbes Modulate Transgenerational Cancer Risk

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Abstract

Environmental factors are suspected in the increase of obesity and cancer in industrialized countries but are poorly understood. Here, we used animal models to test how future generations may be affected by Westernized diets. We discover long-term consequences of grandmothers’ in utero dietary exposures, leading to high rates of obesity and frequent cancers of lung and liver in two subsequent generations of mice. Transgenerational effects were transplantable using diet-associated bacteria communities alone. Consequently, feeding of beneficial microbes was sufficient to lower transgenerational risk for cancer and obesity regardless of diet history. Targeting microbes may be a highly effective population-based approach to lower risk for cancer. Cancer Res; 75(7); 1197–204. © 2015 AACR.

Introduction

Inflammation-associated, metabolic, and neoplastic diseases have increased in frequency in the industrialized world due to numerous factors, including reduced activity levels, increased total energy intake, environmental carcinogens, and immune status (1, 2). Compelling hypotheses aim to explain this phenomenon and reshape preventive medicine. According to one microbe immune-centric hypothesis, inhabitants of developed countries have immune systems of reduced lifelong regulatory capacity due to societal practices in the form of refined diets, antibiotics, and Caesarian births with insufficient beneficial microbe exposure during perinatal life (1–3). Impaired regulatory capacity leads to uncontrolled immune inflammatory responses, obesity, and cancer later in life (2–3). Our recent studies in mice have shown that Westernized diet (NWD)-associated obesity and cancer coincide with changes in gastrointestinal tract microbial communities and immunoregulatory capacity preventable by dietary enrichment with beneficial bacteria (4–7). We test here whether these postulated effects of gut microbes transcend generations. We discover that mother mice consuming NWD during pregnancy convey detrimental long-term consequences to subsequent generations, in particular high rates of lung and liver cancer with obesity and premature senescence. We show this transgenerational predisposition is mediated by intestinal bacteria, highlighting a key role for microbial flora in reducing the risk of cancer.

Materials and Methods

Animals

Outbred conventional or germ-free CD-1 Swiss stock mice (Charles River) were housed and handled in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities with diets, experimental methods, and housing as approved by the Institutional Animal Care and Use Committee. A genetically outbred stock with robust breeding capacity and absent transgenic predilections to cancer was selected for these transgenerational studies. To test the transgenerational impact of dietary microbes on progeny, the experimental design was to expose F0 (grandmother) mice to diets starting at the age of 8 weeks. Special dietary treatment continued until the birth of their pups. Progeny were later examined to determine health risks to subsequent generations. Euthanasia was performed using carbon dioxide overdose at 1 year of age, unless otherwise specified. Because of early-life morbidity, F2 progeny (grandchildren) were euthanized at 6 months of age or younger according to institutional humane criteria and clinical disease. Tissues were collected upon necropsy and then examined histologically. Each experiment included 5 to 10 animals per sex per treatment group, performed in duplicate, as described in detail below.

Special diets for animals

Outbred Swiss CD-1 mice were placed on experimental diets starting at 8 weeks of age: control AIN-76A (Harlan-Teklad) and a NWD with high fat and low fiber with substandard levels of vitamins B and D (TD.96096; Harlan-Teklad; Supplementary Table S1) as previously described (7). Subsets of CD-1 mice who had been treated with NWD in utero subsequently received in their drinking water an anti-inflammatory strain of Lactobacillus reuteri ATCC-PTA-6475 cultivated as described elsewhere (6, 9), with live organisms supplied at a starting dosage of 3.5 × 109 organisms/
mouse/day in drinking water (4). Live bacterial counts in water bottles were calculated to be $1.4 \times 10^6$ colony-forming units (CFU) per mouse on day 1, $4.1 \times 10^5$ CFU on day 2, and $1.1 \times 10^5$ CFU on day 3; and L. reuteri was detectable by PCR in feces and bowel of mice undergoing the dosing regimen, as described in detail in Lakritz and colleagues (4). Control mice received regular drinking water. Fresh drinking water for both groups of animals was replaced twice weekly throughout the experiments.

**Experimental design**

**Experiment 1.** Six eight-week-old CD-1 female mice were fed ad libitum a NWD diet TD.96096 mimicking fast-food starting at 8 weeks of age (Fig. 1A). Females who ultimately served as mothers and grandmothers were immediately arranged into breeding pairs to induce pregnancy. Special diets were replaced with control AIN-76A chow upon birth of F1 progeny. To serve as controls, 6 separate 8-week-old CD-1 female mice were fed ad libitum a control chow AIN-76A and similarly arranged into breeding pairs to produce experimental control progeny animals. All animals had ad libitum access to diets with unrestricted exercise. Their progeny were also examined for obesity and cancer when they reached 1 year of age, including body weights along with tissue collections upon necropsy for histologic examination.

**Experiment 2.** To test whether gut microbes were sufficient for transgenerational effects, 5 germ-free female Swiss CD-1 mice were fed by gastric gavage the feces from NWD-fed mother mice in Experiment 1 (Fig. 1B). Five germ-free mice received comparable stool collected from control diet-fed moms in Experiment 1 to serve as controls. To achieve this, the germ-free mice were dosed 3 times every other day by gastric gavage with 0.05 grams of fresh fecal slurry per mouse per dose. Subsequently, both groups of fecal transplant-recipient moms were co-housed with a male mouse in autoclaved caging and fed only control chow AIN-76A diet to produce progeny for future examination. Progeny were examined for cancer at 1 year of age when tissues were collected upon necropsy.

**Experiment 3.** Six F1 female progeny with in utero exposure to NWD from Experiment 1 (above) were randomly arranged into breeding pairs at 8 weeks of age to produce offspring (grandchildren) for further observation (Fig. 1C). Six F1 progeny from control diet-fed moms in Experiment 1 were similarly arranged to F1 progeny from NWD-fed moms in Experiment 1 to serve as controls. To achieve this, the germ-free mice were dosed 3 times every other day by gastric gavage with 0.05 grams of fresh fecal slurry per mouse per dose. Subsequently, both groups of fecal transplant-recipient moms were co-housed with a male mouse in autoclaved caging and fed only control chow AIN-76A diet to produce progeny for future examination. Progeny were examined for cancer at 1 year of age when tissues were collected upon necropsy.
Experiment 4. To further test the microbe-centric hypothesis, 3- (10 males and 10 females per treatment) and received in their histologically to con
nasia. Tissues were collected upon necropsy and then examined parent's cohort) due to unexpected morbidity requiring eutha-
examined for cancer starting at 6 months of age (earlier than their
Experiment 1 that received NWD while
Illumina sequencing were constructed using a 2-step PCR ampli-
upon necropsy at 1 year of age.

Scurfy-like thymic atrophy and failure-to-thrive in 9 of 20
control AIN-76A diet throughout Experiment 3. Unanticipated
were arranged in matings (Fig. 1B). Control
mice consistent with our previous
findings and also those of other groups who have studied obesity and metabolic syn-
Fecal transplant from NWD-fed donors is suf-
Firmicutes and lower abundance of
Bacteroidetes in NWD-treated mice consistent with our previous findings and also those of other groups who have studied obesity and metabolic syn-
Statistical analyses
The Mann–Whitney U test was used for whole body and thymic weight analyses. Tumor incidence in experimental groups was compared with the Fisher exact test. All analyses were performed with the GraphPad Prism version 5.0 for windows, GraphPad software. \( P < 0.05 \) was statistically significant.

Results
Offspring of mice consuming Western diet have high risk for cancer
Lifestyle changes, including Westernized diet, appear to underlie many of the chronic inflammatory diseases, including obesity, diabetes, heart disease, autoimmune diseases, and cancer (11). To test whether NWD diet-induced obesity (7) and neoplasia (4) may be passed to subsequent generations, we fed NWD to mother mice (F0) during pregnancy and then examined health outcomes in their children reared eating a regular control chow diet (Fig. 1a). For these studies, we used outbred white Swiss CD-1 stock without additional exposure to carcinogens. Mother mice of generation F0 consuming NWD for several weeks during pregnancy were not themselves affected by cancer and obesity in this study. However, we found that F1 progeny of moms consuming NWD during pregnancy had more frequent cancers when compared with offspring of mother mice eating control diet during pregnancy (Table 1).

Specifically, the most profoundly increased cancer types arising in mice with in utero exposure to NWD included pul-
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F2 progeny display increased cancer and accelerated aging

Recognizing extensive pathology existing in F1 mice, we next tested whether subsequent generations are also at increased risk for cancer arising from ancestral in utero dietary indiscretions. Thus, we performed mating experiments among the F1 children of mother mice who had consumed NWD or control chow during pregnancy (Fig. 1C). Initially, infertile mating of Swiss F1 progeny of pregnant (50%) vs. 0 of 6; not significant) made it difficult to produce the necessary F2 grandchildren from NWD-fed grandmothers. Upon further examination of this generation, F1 female nulliparous littermate progeny exhibited not only obesity and insulin resistance in daughters of NWD-fed mothers, hepatocellular carcinoma, and females manifested as failure-to-thrive (9 of 20 vs. 0 of 20; P < 0.05) in 8-week-old F2 progeny (Fig. 3C), affecting both male [50% (5 of 10)] and female [40% (4 of 10)] animals. The syndrome included thymic involution and inflammatory infiltrates throughout vital organs resembling "scurfy" mice with immunoregulatory defects (16).

Recognizing that pathogenic gut bacteria trigger accumulations of neutrophils and host regulatory cells in mice with gut dysbiosis and found increased numbers of intravascular and extravasated neutrophils, an observation supported by myeloperoxidase (MPO)-specific IHC in liver, adipose tissue, and mesenteric arteries (Fig. 3C). In contrast, the presence of neutrophils in control diet- and LR-treated mouse tissues was minimal. It was previously shown that recipient offspring exposed to NWD "stress" is linked with underlying epigenetic mechanisms. Ancestral exposures to NWD were also manifested as failure-to-thrive (9 of 20 vs. 0 of 20; P < 0.05) in 8-week-old F2 progeny (Fig. 3C), affecting both male [50% (5 of 10)] and female [40% (4 of 10)] animals. The syndrome included thymic involution and inflammatory infiltrates throughout vital organs resembling "scurfy" mice with immunoregulatory defects (16).

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Grandchildren (F2 progeny) emerging from fertile F1 mating ultimately exhibited lung (P < 0.0001) and liver (P < 0.0001) cancers with neoplasms arising at younger ages than those seen in their parents (Table 1). Similar early-onset transgenerational phenomena have been described in F2 progeny of rats undergoing experimental stressors while in utero (15), raising the possibility that in utero microbial "stress" is linked with underlying epigenetic mechanisms. Ancestral exposures to NWD were also manifested as failure-to-thrive (9 of 20 vs. 0 of 20; P < 0.05) in 8-week-old F2 progeny (Fig. 3C), affecting both male [50% (5 of 10)] and female [40% (4 of 10)] animals. The syndrome included thymic involution and inflammatory infiltrates throughout vital organs resembling "scurfy" mice with immunoregulatory deficits (16).

Recognizing that pathogenic gut bacteria trigger accumulations of neutrophils (17), we examined non-neoplastic tissues from descendents with gut dysbiosis and found increased numbers of intravascular and extravasated neutrophils, an observation supported by myeloperoxidase (MPO)-specific IHC in liver, adipose tissue, and mesenteric arteries (Fig. 3C). In contrast, the presence of neutrophils in control diet- and LR-treated mouse tissues was minimal. It was previously shown that reciprocal relationships exist between neutrophils and host regulatory...
capacity (10, 18). Additional studies are needed to ascertain whether perinatal changes in microbe communities serve to modulate immunoregulatory capacity later in life. Taken together, these findings led us to conclude that microbial dysbiosis leads to transgenerational effects, including cancer, obesity, and accelerated aging. Knowing NWD-associated obesity and cancer are preventable by dietary enrichment with beneficial bacteria (4, 7), we hypothesized that oral microbe therapy may similarly reduce transgenerational cancer burdens.

Figure 2.
Neoplastic phenotypes encountered in progeny of Swiss CD-1 mice eating NWD chow during pregnancy. A, pulmonary adenocarcinoma. A large raised mass occupies the major part of the left lobe. The expansile, unencapsulated mass shows a mixed acinar and papillary histologic growth pattern with closely packed lobules and cords supported by sparse stromal elements. Alveolar septa-like structures are lined by atypical cuboidal cells with eosinophilic cytoplasm and large, round euchromatic nuclei. B, hepatocellular carcinoma. A large irregular mass with superficial focal necroses obliterates normal liver shape and occupies approximately two thirds of the organ. The large well-circumscribed liver tumor compresses the normal liver parenchyma on the right and displays a solid growth pattern. The diagnosis is moderately well-differentiated hepatocellular carcinoma with large neoplastic hepatocytes and a high degree of nuclear pleomorphism. C, lymphoma. White lobulated mesenteric mass composed of solid sheets of neoplastic cells that infiltrate the mesenteric fat. The uniform, medium-sized lymphocytes have a scant cytoplasm and round nuclei with fine chromatin. Their histomorphology is consistent with that of lymphoblastic lymphoma. D, large, irregular, lobulated mammary tumor. Solid prominent cords and large nests of neoplastic cells are separated by moderate amounts of fibrovascular stroma. Occasional nests show central area necrosis (comedo-like pattern). Neoplastic mammary epithelial cells show marked atypia and pleomorphism and abundant mitotic figures, including abnormal ones. Hematoxylin and eosin. Scale bars, 500 μm (middle column); 25 μm (right column).
microbe communities, we applied a dietary enrichment strategy using beneficial bacteria originally isolated from human breast milk. Experiment 4 used F1 progeny of NWD-fed mothers (Fig. 1D), such that upon weaning at 3 weeks of age, experimental mice consumed a model microbial organism *L. reuteri* continuously in their drinking water. Control mice consumed regular water throughout the study. Upon later examination at 1 year of age, F1 children consuming *L. reuteri* had significantly lower risk for obesity (Fig. 3A) and cancer than did their untreated control counterparts (Table 1; Supplementary Fig. S3). It is promising but unknown to what extent other microbe cocktails or life stage interventions may be effective, although oral administration of purified *L. rhamnosus* was previously shown to suppress obesity in human adults (19). Mouse models and diets do not precisely mimic human conditions; however, these findings further supported a gut microbe immune-centric hypothesis and the possibility that diet-associated bacteria may be engineered to mitigate emerging inflammatory disease epidemic in Westernized societies.

**Discussion**

In summary, we show that in utero effects of gut microbes may transcend generations. We discover that mother mice consuming a Westernized diet during pregnancy convey to their children and grandchildren detrimental long-term consequences, including high risk of obesity and cancer. In humans, there is precedent for maternal dietary folate (vitamin B9) deficiency leading to leukemia in offspring (20); although, the cancer outcome in the present study was reproducible using...
Dietary habits during pregnancy shape disease susceptibility. The transgenerational hypothesis suggests that Westernized individuals raised in a highly controlled environment exhibit sex hormones together with immune dysregulation in transgenerational effects; however, this remains to be explicitly tested. Importantly, fecal microbiome transplant was sufficient for the transgenerational cancer effect. Grandchildren were most severely affected, exhibiting a premature aging scurfy-like syndrome and high rates of cancer at a young age, when their grandmother had consumed Western diet during her pregnancy. The scurfy-like syndrome suggests immune T regulatory (Treg) cell insufficiency (16) previously implicated in obesity and cancer (4–7). Taken together, our data suggest that dietary stress leads to gut microbe dysbiosis that elevates the risk of heritable cancers. Intestinal bacteria were sufficient to remedy the transgenerational effect, pointing to novel microbe-based personal or public health preventative medicine strategies. Intestinal bacteria were sufficient to remedy the transgenerational effect observed in mice, and further studies of novel microbe-based mechanisms in the role of human cancer and obesity prevention are warranted.

In conclusion, it is now clear that profound changes in our environment have contributed to a societal health crisis. Our previous data have built upon the perinatal microbiome-driven concept to include neoplastic diseases (3, 5). Our current data expand this microbe-centric view further to include mammalian progeny and suggest that the microbial flora of a mother animal affects the predisposition of her progeny to cancer. We have shown here that a key mediator of diet-induced transgenerational effect observed in mice, and further studies of novel microbe-based mechanisms in the role of human cancer and obesity prevention are warranted.

References

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Development of methodology: B.J. Varian, S. Mirabal, S.E. Erdman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Poutahidis, T. Levkovich, S. Mirabal, Y.M. Ibrahim, S.E. Erdman
Writing, review, and/or revision of the manuscript: T. Poutahidis, B.J. Varian, S.M. Kearney, S.E. Erdman
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Study supervision: S.E. Erdman
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