Ph.D. thesis

DROSOPHILA TYPE IV COLLAGEN MUTATION ASSOCIATES WITH IMMUNE SYSTEM ACTIVATION AND INTESTINAL DYSFUNCTION

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2016

Introduction

The structure and function of the mucosal epithelia is determined and supported by the underlying basement membrane (BM). The BM functions in separation, epithelial cell polarization, absorption, sensation and secretion in various tissues and body compartments. The BM is a specialized form of the extracellular matrix, composed of numerous components with a predominance of type IV collagens COL4A1 and COL4A2. Mammals, including humans, harbor three pairs of type IV collagen genes (*COL4A1-A6*). The inherited disorder of renal, ocular and cochlear basement membranes associates with mutations of the X-linked *COL4A5* gene in the majority of patients with Alport syndrome, whereas lesions in the autosomal *COL4A3* and *COL4A4* genes are responsible for the symptoms of Alport syndrome in about 20% of patients. The majority of the mammalian BM is composed of building units of type IV collagen trimers with $(COL4A1)_2COL4A2$ composition. Clinical manifestations associated with *COL4A1* mutations include perinatal cerebral

hemorrhage and porencephaly, hereditary angiopathy, nephropathy, aneurysms, and muscle cramps (HANAC), ocular dysgenesis, myopathy and Walker-Warburg syndrome. The latest reports demonstrate systemic tissue degeneration and pleiotropy associated with *COL4A1* mutations and confirm the experimental observations that the phenotypes of *COL4A2* mutations are phenocopies of *COL4A1* gene mutations.

The mucosal epithelia of the gastrointestinal tract are constantly challenged by the microbiome resulting in various types of interactions including commensalism, symbiosis and pathogenicity. Host-microbe interactions in the gut are studied only in a few animal models including *Drosophila melanogaster*. Antimicrobial defense in *Drosophila* is facilitated by phagocytosis of pathogenic microorganisms, by the synthesis of antimicrobial peptides (AMPs) and by the generation of reactive oxygen species. Gut-associated bacterial community in *Drosophila* is scarce, harboring 1-20 bacterial phylotypes. Recent studies identified five dominating commensal species in the gut of wild-type

flies: *Lactobacillus plantarum, Lactobacillus brevis, Acetobacter pomorum, Gluconobacter morbifer* and *Commensalibacter intestini*. Overexpression of AMPs, demonstrated in *caudal* hypomorphic RNAi mutants, resulted in restructuring of the commensal bacterial population with the dominance of the pathogenic *Gluconobacter morbifer* accompanied by degradation of gut epithelial cells and high mortality of the host. These observations suggested a potential role for high levels of AMPs in epithelial cell degeneration, gut pathogenesis, and increased mortality. In aging flies, dysfunction of the intestinal barrier that normally permits the absorption of nutrients and solutes and hampers host contact with harmful entities including microorganisms has been, accordingly, tightly linked to overexpression of AMPs. Compromised intestinal barrier function was also noted in *big bang* (*bbg*) null *Drosophila* mutants. The BBG protein is localized in the gut epithelial septate junctions that, in the absence of BBG, are compromised and result in reduced lifespan and chronic inflammation of the anterior midgut epithelium in mutant animals.

We have recently reported an allelic series of conditional, dominant, temperature-sensitive (DTS) mutations in the type IV collagen gene *col4a1* in *Drosophila.* The *col4a1*mutant heterozygotes are viable and fertile at permissive temperature (20^{o}C), but perish at restrictive temperature (29^{o}C). The phenotype associated with mutations of *col4a1* include severe myopathy resulting from massive degradation of striated muscle fibers and degeneration of both the circular smooth muscle cells and epithelial cells of the gut that occurs following detachment from the BM. While there are signs of some repair, the capacity of the scavenger system, and/or the kinetics of cell renewal and regeneration fail to keep up with the ongoing cell degeneration in these mutants. Based on these observations, we hypothesized that *col4a1* mutants may suffer from extensive cell damage-induced chronic inflammation and demonstrate a robust immune response. In *Drosophila*, during inflammation the immune response involves the immediate synthesis of AMPs. Therefore, we have carried out a series of tests in order to determine whether AMP induction is associated

with mutation-induced aberrant *col4a1* functions in *DTS-L3* mutants selected for analysis based on the presence of confirmed cell degeneration and its lowest survival rate among the *col4a1* mutant lines at 29^{o}C.

Methods

TUNEL assay was performed according to the manufacturer's instructions to detect certain signs of apoptosis.

We used the Primerfox algorithm to design primers for the *col4a1* gene and capillary sequencing to determine the mutation sites. BLAST was used to align the acquired nucleotide sequences with NCBI database entries and ClustalW2 and Clustal Omega for protein sequences.

Trizol reagent was applied on 3 day old flies to extract total RNA which was then purified by adding Ambion RNA-free DNase and went through Qiagen RNeasy column cleansing. The raw processed signals from the microarray were subject to a series of strict filtering steps then statistically evaluated by two-sided t-test ($p<0,01$). Gene ontological analysis was based on

Panther database associations; post-hoc tests were applied, where it was possible. In the QRT-PCR measurements the very same RNA samples were probed as in the case of the microarray experiment. For all genes tested the OreR 18°C samples were used as the reference level of expression.

The levels of reactive oxygen species were normalized to protein content and measured spectrophotometrically.

After isolating individual bacterial strains from the guts, we performed the widely used 16S RNA sequencing technique and analyzed the results with the aforementioned BLAST algorithm and the NCBI nucleotide database.

For the examination of the intestinal barrier function we mixed Patent Blue V dye to the medium, and fed the flies with the mixture of ampicillin and tetracycline or the pure, liquid culture grown cells of the isolated bacteria.

Results

After identifying the mutation sites in the *col4a1* gene in each of our alleles we focused on the *DTS-L3* mutant. We have noticed a TUNEL positive reaction in the intestinal tract of these animals at restrictive temperatures.

Our results demonstrated overexpression of the AMP genes *Metchnikowin, Diptericin, Diptericin B, Attacin A, Attacin C* and *edin,* compared to their down-regulation in control animals. These experiments were fortified by the *in vivo* expression of GFP-tagged ATT proteins in the intestines in accordance with the expression pattern demonstrated at the mRNA level. Phylogenetic analysis of the gut bacteria identified in both mutant and control animals, *Lactobacillus*

plantarum and *Acetobacter cerevisiae* as cultivable members of the intestinal microbial community with decreased CFU values under restrictive conditions. Antibiotic treatment resulted in lengthened maximal lifespans, but similar half-lives.

High dose diet of either *Lactobacillus plantarum* or *Acetobacter cerevisiae* was detrimental at high temperatures, but can be tolerated at 20°C. The same bacilli were less harmful when applied at lower CFUs.

Data from this study support the conclusion that in *col4a1* mutant *Drosophila* compromised BM function, gut epithelial cell detachment cause enlarged mid-gut with multiple diverticula, extensive cell degeneration, overexpression of AMP genes, collectively leading to intestinal dysfunction and shortened lifespan.

References

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Author's impact factor: 8.845