Understanding the Role of Microbes in Intestinal Tumor Pathogenesis

Biomedical and Health Sciences

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Rationale

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, with 1 million diagnoses yearly. The chronic inflammation of the GI tract is associated with changes in the gut microbiome, which can promote physiological functions associated with cancer, including cell proliferation (Sears & Garrett, 2014). Various inflammatory cells produced during chronic inflammation lead to the formation of a microenvironment that provides nutrients that support tumorigenesis (Kaur et al., 2018). In addition, the gut microbiome is a regulator of CRC pathogenesis through pro-inflammatory responses (Rooks & Garrett, 2017). Celecoxib is a selective NSAID used to directly target COX-2. Elevated COX-2 expression was found in approximately 85% of adenocarcinomas (Ogino et al., 2008). Treatment with celecoxib has been found to significantly reduce the number of polyps in people with FAP (Marnett & DuBois, 2002). Also, celecoxib can reduce proliferation in the base of normal-appearing ileal and colonic crypts of $Apc^{Min/+}$ mice (Montrose et al., 2016). Mice treated with broad-spectrum antibiotics before and during AOM injection and DSS treatment had significantly fewer, smaller tumors than untreated mice, hinting that tumor incidence and penetrance were dependent on the gut microbiome (Zackular et al., 2013).

Inflammation-linked carcinogenesis is a concept often observed within the gastrointestinal tract, but the underlying mechanisms driving microbially mediated tumorigenesis remain to be elucidated. The goals of this study were to examine the mechanisms by which the administration of antibiotics reduces tumor burden, whether the attenuation of the proliferation-suppressing effects of celecoxib under antibiotics is mediated via the stem cells, and whether the bacterial waste and the gut microbiome could directly manipulate inflammation

within the gut. Comparing celecoxib in the presence and absence of antibiotics could provide information as to whether celecoxib within the gut microbiome more as an anti-inflammatory, an antibacterial, or both.

Research Questions/Hypotheses/Expected Outcome

Inflammation-linked carcinogenesis is a concept often observed within the gastrointestinal tract, but the underlying mechanisms driving microbially mediated tumorigenesis remain to be elucidated. Despite recent research, there are significant gaps in the understanding of how antibiotics increase the risk of colorectal cancer. Therefore, the main goals of this study are to examine the mechanisms by which the administration of antibiotics reduces tumor burden, whether the attenuation of the proliferation-suppressing effects of celecoxib under antibiotics is mediated via the stem cells, and whether the bacterial waste and the gut microbiome could directly manipulate inflammation within the gut. Comparing celecoxib in the presence and absence of antibiotics could provide information as to whether celecoxib acts within the gut microbiome more as an anti-inflammatory, an antibacterial, or both.

Procedures

<u>Mouse Treatments</u>

All of the mouse treatment and tissue isolation will be done by another member of the lab. Mice were given a purified chow diet with 1,000 ppm celecoxib or a control chow diet with 0 ppm celecoxib. At 6 weeks of age, a broad-spectrum antibiotic cocktail of metronidazole, vancomycin, and streptomycin will be administered to Groups 3 and 4 through their drinking water. Mice will be sacrificed at 16 weeks of age. Their ileal content will be collected and fixed on slides for quantification of polyp burden by another member of the lab.

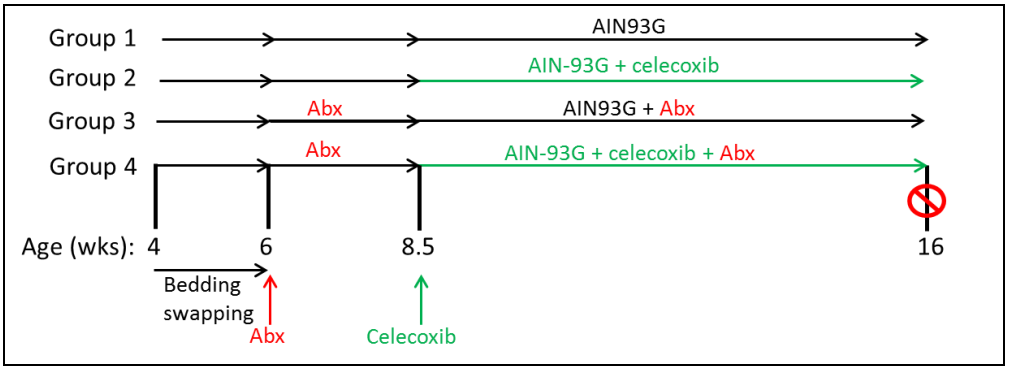

Fig 2. Schematic representation of the experimental design.

Immunohistochemistry for Ki-67

1. Required Reagents

    a. 1X PBS

    b. 1% Hydrogen Peroxide: add 8 mL of 30% H_2O_2 to 232 mL of 1X PBS using the electric pipette or P1000. If doing 3 slides only: 2 mL H_2O_2 to 58 mL PBS.

    c. 10 mM Citrate solution: dissolve 735 mg (.735g) of Na-Citrate salt into 250 mL of MilliQ water (in 500 mL beaker), stir, and adjust pH to 6.0 by adding 4 drops of 37% HCl with a transfer pipette.

    d. Blocking Buffer: add 10% serum of the host of the secondary antibody in 1X PBS

    e. ABC reagent: To 250 µl of PBS, add 4.4 µl of Reagent A and then 4.4 µl of Reagent B (in order) and vortex immediately. Let sit at room temperature for at least 30 mins before use.

    f. DAB solution: To 250 µl of MilliQ water, add 4.4 µl of buffer; vortex immediately. Then add 8.8 µl of DAB, vortex, and finally add 4.4 µl of H_2O_2 and vortex again. Make fresh before use and store solution in dark at room temperature until use.

2. Procedure
    a. Turn on and set the incubator to 60º C (will take ~45 minutes). This translates to 55º C inside the incubator.
    b. Bake the slides for 20 minutes.
    c. Deparaffinization and Rehydration
        i. Xylene (i) 5 mins (During the 5 mins make the 1% hydrogen peroxide in PBS and keep it covered)
        ii. Xylene (ii) 5 mins
        iii. 100% Et-OH (i) 3 mins
        iv. 100% Et-OH (ii) 3 mins
        v. 95% Et-OH 3 mins
        vi. 70% Et-OH 3 mins
        vii. Wash slides in running water for 2 mins.
        viii. Incubate the slides in 1% Hydrogen peroxidase for 20 mins and keep covered.
        ix. Wash slides in running water for 2 mins.
    d. Antigen Retrieval
        i. Put the slides in the container filled with the citrate buffer pH 6.0. Place this in the decloaking chamber for 10 mins at 115°C.
        ii. Seal the chamber properly and press start.

iii. Once done, wait until the pressure reaches 0, let the pressure release, turn off chamber, and then remove the lid and leave slides inside for 20-30mins (goal is to cool the solution gradually).

iv. Remove the container holding the slides from the decloaking chamber and leave on the benchtop and cool for another 20-30 mins.

v. Wash slides in running water for 2 mins.

vi. Dab off the excess water on a paper towel and circle the tissue with PAP pen (a pen that creates a hydrophobic barrier around the tissue).

e. Blocking and Primary Antibody Incubation

i. Incubate in blocking buffer for 30 minutes at room temperature in a humidified chamber (created by placing one paper towel sprayed with MilliQ water on each side of the enclosed chamber).

ii. Dab tissue slides on a paper towel, and add primary antibody (mouse) diluted in blocking buffer.

iii. Place the slides in a humidified chamber and incubate overnight at 4°C.

f. Secondary Antibody Incubation

i. Tap buffer off of slides and wash 3X in 1X PBS for 5 mins each (place on standard analog rocker).

ii. Incubate in blocking buffer (serum of the host of the secondary antibody, 100 microliters) for 30 mins at room temperature in humidified chamber.

iii. Dab tissue slides on a paper towel, and add biotinylated secondary antibody diluted 1:200 in blocking buffer. Incubate in a humidified chamber for 30 minutes at room temperature.

iv. While incubating in 2° Ab prepare the Vectastain Avidin-Biotin Complex reagent and incubate at room temperature for 30 mins prior to use.

v. Wash slides 3X in 1X PBS for 5 mins each.

vi. Add 100-150 µl of ABC reagent (depending on size of section) until the tissue is covered. Incubate in a humidified chamber for 30 mins at room temperature.

vii. Wash slides 3X in 1X PBS for 5 mins each.

viii. At the third wash step, prepare the DAB solution (3,3′-Diaminobenzidine, routinely used to stain nucleic acids and proteins) and store in the dark at room temperature.

ix. Add DAB solution and incubate until light brown color appears (time of incubation will vary for each antibody but typically between 30 sec and 2 mins).

x. Immediately stop reaction by immersing the slides in MilliQ water. Staining should immediately be visible as dark brown patches.

g. Hematoxylin Staining

i. Wash murine tissue section slides in running water for 2 mins.

ii. Incubate with hematoxylin for 5 mins by adding solution onto slide using transfer pipet.

iii. Wash slides in running water until clear.

iv. Immerse slides in Bluing Reagent in slide container for 3 mins (pour back solution into stock bottle after use).

v. Wash slides in running water for 2 mins.

h. Dehydration and Slide Preparation

i. 70% Et-OH 3 mins

ii. 95% Et-OH 3 mins

iii. 100% Et-OH (ii) 3 mins

iv. 100% Et-OH (i) 3 mins

v. Xylene (ii) 5 mins

vi. Xylene (i) 5 mins

i. **Do not let the xylene dry out.** After the last xylene incubation, take out the slide and immediately mount with coverslips using Permount.

j. Let the slides dry overnight.

Additional experiments will be run on HCT116 cells with qRT PCR. The tissue culture cells were obtained from ATCC and are BSL-2. After harvesting cells as a cell pellet, RNA will be purified using the RNeasy Plus Mini Kit (Qiagen). The concentration of total RNA for each sample will be measured using a Nanodrop spectrophotometer (Thermo Fisher) and used to calculate the amount needed to be put into each PCR plate well. B-actin will be used as an internal control. CT values, the PCR cycle numbers where signals become discernible above background noise, will be used to calculate the change in the CT value of COX-2 relative to the

change in the CT value of β-actin, the calibrator. The relative expression of the COX-2 gene will be calculated from CT values using the formula below.

$$\text{Relative Expression} = 2^{-\Delta\Delta CT}$$

Risk and Safety

This project will be completed in a BSL-2 lab with a BSL-2 hood. Materials will be disposed of in a biohazard bin when appropriate. Proper personal protection equipment will be worn, such as lab coats and gloves. Tissue culture waste will be properly inactivated and disposed of.

Data Analysis

A negative binomial regression and general linear hypothesis test will be used to analyze the data for tumor number and size.

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NO ADDENDUMS EXIST