Understanding the Interaction Between Prostaglandins and Toll-Like Receptors in Nicotine-Induced Rectal Tumor in Animals

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ABSTRACT

Background: *In the present study, we tried to understand the crosstalk between prostaglandins-COX-mediated rectal tumors and toll-like receptors in rats.*

Methods: *The tumor was induced using nicotine (100 µL/mL). Following the induction, the serum and rectal tissue were analyzed for Lipo-polysaccharides (LPS) and prostaglandin E2 in serum, and tissue expression of inflammatory mediators like TLR2,4, NFkB; cancer markers like Matrix metalloproteases 2 (MMP2), 9 and Cyclo-oxygenases 2 (COX-2) were estimated. The gut microflora analysis was carried out using the fresh fecal samples of both the study groups.*

Results: *In nicotine-induced group, there was a significant alteration in the gut microflora toward high Gram-negative strains and a decline in Gram-positive populations. All the inflammatory as well as cancer prognostic markers were significantly increased in the tumor-induced animals.*

Conclusion: *From the present study, it could be concluded that nicotine significantly induced rectal cancer in the mice model by modulating gut microflora and increasing COX-2 and prostaglandin E2 levels.*

Keywords: *COX-2, gut microflora, inflammation, nicotine, PGE3, rectal cancer*

INTRODUCTION

Rectal cancer is one of the leading causes of cancer with higher rates of death. Of the total number of new cases registered amounting to around 1.5 lakhs in the United States annually, the number of deaths has been reported to be around 51 000. It has been marked as the third leading cause of cancer and the second most common cause of cancer deaths.[1] Cigarette smoking has been indicated as one of the most prominent risk factors for the same. Smoking increases the incidences of rectal cancer more than in non-smokers.[2,3] The World Health Organization estimates, based on the trend of longevity, current smoking trends, and increasing adoption of unhealthy lifestyles, that the annual death toll will exceed 12 million and that there will be 15 million new cancer cases diagnosed annually by 2020.[4]

Tobacco and its products contain a wide range of chemicals, such as nicotine and other carcinogens. Nicotine in combination with these carcinogens is responsible for devastation and for all the deaths worldwide. Nicotine is the principal component of all types of tobacco products and smoke. Nicotine is one of the component of tobacco which is non-carcinogenic but at the same time addictive[5]; Carcinogenic components of tobacco include nicotine-derived nitrosamines, which has been widely reported to induce cancer in both animals and humans.[6,7] Nitrosamines, like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN), are human carcinogens.[8]

The rectal tumor has been well characterized. Despite our current understanding of the molecular mechanisms for inducing angiogenesis, multiple regulatory factors and receptors which are extensively involved have not been fully and clearly understood. Eicosanoids, especially arachidonic acid, are released from tumor cells. The cyclooxygenases, that is, COX-1 and COX-2, are responsible for generating prostaglandins and thromboxanes.[9,10] COX-2 is not expressed under normal conditions but is upregulated by inflammatory signals, such as TLR4 signaling and cancer[11] and elevated levels of reactive oxygen species

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(ROS).[12] Gut microbiota has been reported in the progression and development of rectal tumors. The microbiome is involved in induction through multiple pathways like inducing chronic inflammation, altered metabolism in the host, and bioactivation of metabolites as a carcinogenic compound.[13,14,15] TLR4 plays a key role in the intestinal innate immune system as the first line for the recognition of intestinal tract bacteria. TLR4 has a cell surface pathogen associated molecular patterns (PAMPs) recognition which induces inflammation generating innate immune responses to pathogens by inducing signaling cascades of kinase and transcription factor activation.[16] The inflammation through the gut microbiome is regulated by the Gram-positive and Gram-negative diversity wherein the Gram-negative are controlled by higher Gram-positive strains. More specifically, *Bifidobacterium* to *Escherichia* (B/E) ratio is a crucial indicator of an inflammatory state. During rectal tumor, *Bifidobacterium* count has been reported to be decreased significantly with an increase in *E. coli*. In the present investigation, attempts have been made to understand the role of COX-2, prostaglandins, and TLRs in the nicotine-induced rectal tumor.

MATERIALS AND METHODS

Test Animals and Sample Collection

All the procedures conducted in the present study were approved by the Institutional Animal Care and Use Committee, Nirma University, Ahmedabad under the CPCSEA guidelines of the Ministry of Environment and Forest, New Delhi (Protocol No. IS/BT/FAC-19-2516). Male Wistar rats of 8-10 weeks were obtained from Zydus Research Centre (Ahmedabad, India). The animals were housed in standard cages, and three rats in each cage. Diets and water were provided ad libitum.

Diets and Experimental Design

The animals were randomized into 2 groups: the control and nicotine-induced rectal tumor administered group with 5 animals each. Both the groups were fed with contained normal rodent diets (Amrut agro foods, Mumbai). The rectal tumor was induced using nicotine (100 µL/mL) mixed in drinking water for 25 days.

Weekly body weight and food consumption were recorded. Freshly excreted fecal samples were collected 24 hours prior to the autopsy from both groups. Fecal samples collected were stored at –80°C before DNA extraction. Blood and colonic tissue were collected from the experimental animals at the time of autopsy.

Blood and Tissue Sample Collection

About 1 mL of blood was collected from the retro-orbital plexus under mild anesthesia into the microfuge tubes for collection of serum on day 0 (for baseline values) and the last day of the experiment. Plasma LPS was analyzed using Pierce LAL chromogenic endotoxin quantitation kits as per the protocol mentioned by the manufacturer.

Fecal Collection and DNA Extraction

DNA extraction was carried out from fecal samples using the QIAamp DNA stool mini kit according to instructions given by the manufacturer (Qiagen, Hilden, Germany). The DNA quantity was determined by taking the absorbance at 260 nm (A260), while quality was estimated by determining the A260/A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Microbial Quantification by qPCR

The specific 16s rDNA primers targeting different bacterial phyla and genera were used to quantify the fecal microbiota by qPCR. Standard strains of *Lactobacillus casei* (MTCC 1423), *E. coli* (MTCC 443), and *Clostridium perfringens* (MTCC 450) were obtained from the microbial type culture collection (MTCC, Chandigarh, India), whereas *Bifidobacterium bifium* (NCDC229) was obtained from the national collection of dairy culture (NDRI, Karnal, India). The strain of *Bacteroides vulgatus* (ATCC 25285) was obtained from the American Type Culture Collection (ATCC, USA). The standard strains were used for the construction of standard curves for qPCR. The data presented are the mean values of duplicate qPCR analysis.

Tissue Gene Expression

RNA was isolated from fresh rectal tissue and collected at the time of autopsy using TRI reagent (Sigma-Aldrich), according to the manufacturer's protocols. RNA was quantified by OD_{260}/OD_{280}. The expressions of TLR2, TLR4, NF-kB, MMP-2, MMP-9, and COX-2 were carried out by RT-PCR. β-actin was used as a normalization control.

Prostaglandin E2 Quantification

Tissues were homogenized in PBS with 10% 2,6-di-*tert*-butyl-*p*-cresol. Prostaglandin E2 (PGE2) levels were measured using Agilent 6460 tandem mass spectrometry with Agilent 1200 liquid chromatography system (LC-MS-MS, Agilent Technologies), normalized with protein concentration.

Statistical Analysis

All the values are expressed as mean ± standard deviation. Statistical analysis was carried out using Graphpad Prism software version 6. One-way ANOVA followed by Tukey's multiple comparison test was used to determine the statistical significance between various groups. Differences were considered to be statistically significant when $P < .05$. The significant value in comparison with the control group was indicated with an asterisk (*).

RESULTS

In the present study, there was a significant decrease in diet intake and body weight was observed in the nicotine-induced rectal tumor as compared to the control group.

The role of nicotine in modulating the gut microflora modulation was studied on 3 major gut dominant phyla, that is, Firmicutes, Bacteroidetes, and Proteobacteria. Nicotine significantly modulated the microbial phyla. A major effect was observed on the firmicutes and bacteroidetes. Firmicutes showed a significant decrease while bacteroidetes significantly increased. Proteobacteria showed an increase but it was nonsignificant (Figure 1A).

Response to the microfloral alteration was also studied on 4 gut dominant genera, that is, *Lactobacilli*, *Bifidobacteria*, *Escherichia*, and *Clostridia* (Figure 1B). Nicotine significantly altered gut dominant microbiota. Significant reduction (*$P < .05$) was observed on both the beneficial strains, that is, *Lactobacilli* and *Bifidobacteria*. *Clostridia*, however, did not show any significant response. A most significant increase was observed in *Escherichia* (***$P < .001$). Nicotine-induced rectal tumor animals showed elevated *E. coli* (Figure 1B). Lipopolysaccharides present on the cell wall of Gram-positive strains are potent endotoxins. As observed in Figure 1B, there was a significant increase in the *E. coli*, and LPS was determined in both the study groups. In the nicotine-induced rectal tumor group, there was a significant increase in the serum LPS levels indicating high-grade inflammation (Figure 1C).

The degree of cancer induction and inflammation was determined by quantifying the expression of inflammatory mediators such as TLRs, NF-kB, MMP2, 9, and rectal cancer markers like COX-2 and prostaglandin E2. TLR-2 expression showed a non-significant decline. This is in concurrence with the drop in *Lactobacilli* and *Bifidobacteria* count in the rectal tumor group (Figure 2a).

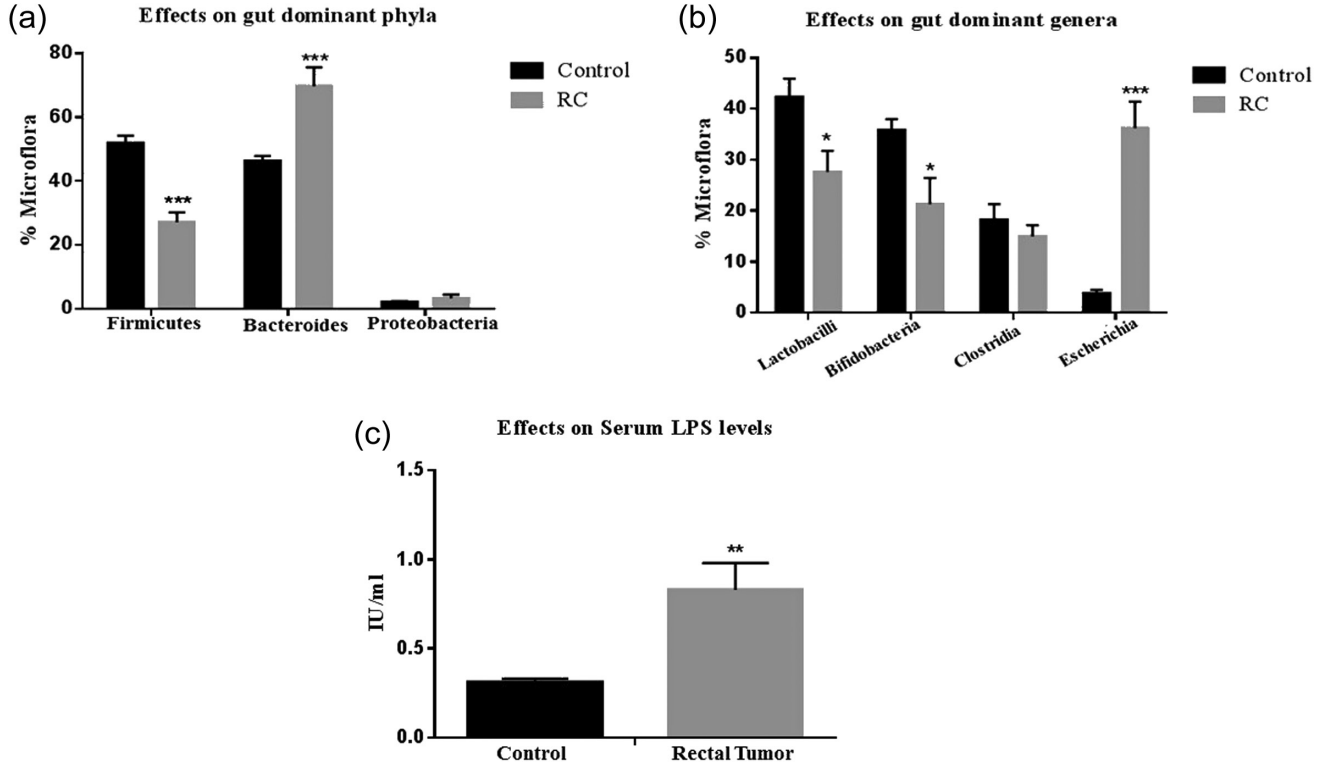

Figure 1. Fecal microbial and serum LPS analysis in control and nicotine-induced rectal cancer-induced group.

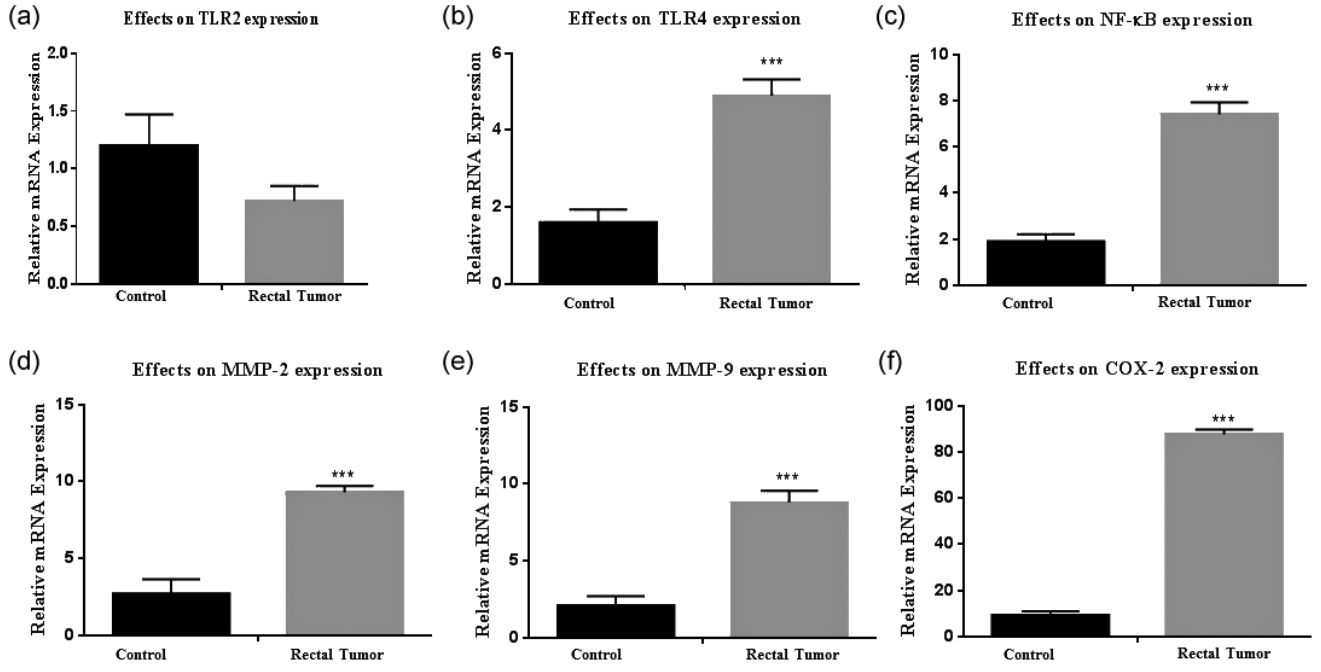

Figure 2. Expression of Inflammatory mediators and tumor markers in colonic tissue of control and nicotine-induced rectal cancer-induced group.

TLR-4 was significantly increased in the tumor group; LPS are TLR-4 ligands. As found in the elevation of LPS hence corresponding increase in TLR-4 confirms the results (Figure 2B). TLR-4 results in inflammation by upregulating NF-kB expression. In the present study, NF-kB was also significantly increased in the tumor group (Figure 2C). MMP2 and MMP9 are important markers for tumor induction. Both MMP-2 and MMP-9 were significantly increased in the tumor group (Figure 2E and F). COX-2 and prostaglandin E2 have been reported to be involved in the progression of the rectal tumor. In the present study, COX-2 has been significantly increased in the tumor group (Figure 2G). Prostaglandins and COX-2 are interdependent in their upregulation. Prostaglandins showed an interdependent increase in the expression in tumor tissues (Figure 3).

DISCUSSION

In the present study, following induction with nicotine, there was a decrease in the body weights of the animals which were resultant due to reduced intake of food. Bodyweight has been widely reported to be declining following the induction of cancer.[17,18]

Nicotine and its derivatives, that is, nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) found extensively in cigarette smoke have been widely and extensively reported to cause lung cancer,[19,20,21] and liver cancer in animals.[8,22] It has been widely documented that the majority of tumors can be induced independent of the route of administration and some completely unrelated organs.[23] In the present study, oral administration of nicotine-induced rectal tumor. Statistically, rectal cancer has also been reported in individuals with oral intake of nicotine.[24]

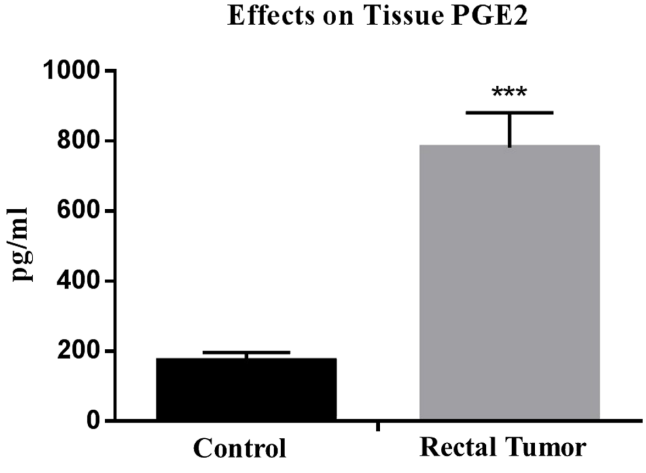

Figure 3. Tissue PGE2 in control and nicotine-induced rectal cancer-induced group.

Gut microbiota has been widely reported to be playing a crucial role in tumor pathogenesis.[25] Gut microbial dysbiosis is one of the major precursors for rectal tumors.[26,27] In the present investigation, there has been a significant increase in the Bacteroides phyla and *E. coli* species and a decrease in firmicutes and beneficial strains like *Lactobacilli* and *Bifidobacteria*.

Gram-negative strains which are predominantly in the Bacteroides phyla induce the inflammation via their membrane-bound LPS which are ligands for TLR-4. Louis et al.[28] reported that chronic inflammation is aggravated by pathogenic bacteria predominantly gram-negative strains, and activation of pro-inflammatory cytokines. In the present study, TLR-4 and NF-kB expressions were increased in tumor tissues with decreased TLR-2 expression.

MMPs have been widely reported in aggravating angiogenesis by degrading basement membrane and other extracellular matrices. MMP-2 initiates integrin signaling and enhancing endothelial cell survival and proliferation resulting in tumor progression.[29] Both MMP-2 and MMP-9 have been extensively reported in various cancer models.[30] In the present study, both MMP2 and 9 have been increased significantly.

Overexpression of COX-2 has been directly implicated in a variety of tumors, such as colorectal tumors, pancreatic, lung, and breast cancers.[31] Prostaglandin E2 is a major pro-inflammatory mediator, which has been reported in different types of human malignancies including colorectal, lung, breast, head and neck cancer, and is often associated with a poor prognosis.[32] COX-2 and PGE2 are interdependent in their expression. Increased production of PGE2 results in induction and even overexpression of COX-2. This interaction resulted in modulation of cell proliferation, decreased cell death, and tumor invasion. In the present study, COX-2 has been significantly increased and PGE2 is also found to be significantly elevated compared to the control group.

In conclusion, nicotine-induced animals showed significant alteration in the gut microflora and inflammatory mediators, and tumor markers. The results thus obtained would open newer avenues to target PGE2 and gut microflora as a prospective treatment target for the treatment of rectal tumors. Identification of crosstalk between microbial modulation and inflammation would be key in the identification of newer therapeutic targets.

Ethics Committee Approval: Ethical committee approval was received from the Institutional Animal Care and Use Committee, Nirma University, Ahmedabad under the CPCSEA guidelines of the Ministry of Environment and Forest, New Delhi (Protocol No. IS/BT/FAC-19-2516).

Informed Consent: N/A.

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Declaration of Interests: The authors declare that they have no competing interest.

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