Suppression of Intestinal Polyposis in Apc^{Min/+} Mice by Inhibiting Nitric Oxide Production

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Abstract

Inducible nitric oxide synthase (iNOS) was shown to be expressed in normal mucosa and adenoma of small and large intestines of Apc^{Min/+} mice by reverse transcription-PCR and immunohistochemistry. Administration of the iNOS inhibitor aminoguanidine (1.5 g/liter) in drinking water or an l-arginine-deficient diet to Apc^{Min/+} mice resulted in a significant decrease in adenoma development in the small bowel but not the large intestine. Similarly, iNOS-gene knockout Apc^{Min/+} mice (Apc^{Min/+} iNOS^{-/-} or Apc^{Min/+} iNOS^{+/+}) developed significantly fewer adenomas in both small and large intestines than Apc^{Min/+} iNOS^{+/+} mice. These results suggest that iNOS-selective inhibitors could be used as a potential chemopreventive agent for colorectal cancers.

Introduction

NO2 functions as a mediator in inflammatory processes as well as being a physiologically important signaling molecule in virtually every tissue in the body. It causes DNA damage and cell death at high concentrations, whereas at lower concentrations, it exerts homeostatic effects in the cardiovascular and nervous systems (1). NO is generated by the conversion of l-arginine to l-citrulline in the presence of a constitutive eNOS or nNOS, acting in a Ca^{2+}-independent manner. Overproduction of NO has been associated with the pathogenesis of a variety of disorders including cancers. Numerous studies support the idea that iNOS is involved in carcinogenesis. Potential mechanisms for tumor-promoting roles of NO include DNA and tissue damage and gene mutations induced by reactive nitrogen species such as peroxynitrite and NOx, which are formed by the reaction of NO with superoxide anion (O2^{-}) or oxygen, respectively (1, 2). NO also stimulates tumor angiogenesis (3) and vascular permeability in solid tumors (4). Increased expression of iNOS has been associated with some pathological conditions such as ulcerative colitis (5), colon adenomas (6), and carcinomas (7) in human subjects as well as in large intestinal aberrant crypt foci (8) and tumors (9) induced by azoxymethane in rats. iNOS is also expressed constitutively in mouse ileum (10). Moreover, NO can activate the enzyme COX which plays pivotal roles in the progression of a variety of cancers via prostaglandin synthesis and angiogenesis (11, 12).

Min mice have a germ-line nonsense mutation at codon 850 of the Apc gene and spontaneously develop adenomatous polyposis coli (Apc) gene and spontaneously develop adenomas in the small and large intestines at the age of 10–12 weeks (13). Min mice are, therefore, considered to be a useful animal model for analysis and prevention of human familial adenomatous polyposis (FAP) and sporadic colorectal cancers.

In the present study, we investigated iNOS expression in normal and adenoma tissues obtained from Min mice by RT-PCR and immunohistochemistry. We have also studied the effects of iNOS inhibition on intestinal polyposis. Strategies to suppress iNOS included (a) pharmacological treatment with the iNOS-selective inhibitor aminoguanidine; (b) nutritional restriction of the iNOS substrate l-arginine; and (c) generation of iNOS knockout Min mice. Our results suggest that iNOS suppression could be an alternative chemopreventive strategy for colorectal cancers.

Materials and Methods

Animals. Male C57BL/6j-Apc^{Min/+} (13) and female C57BL/6j-Nos2^{+/-} (iNOS^{-/-}) mice (14) were obtained at 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME), and wild-type C57BL/6j mice were obtained from Iffa Credo (Lyon, France). All of the animals were raised in plastic cages under specific pathogen-free conditions. Animals were fed a standard diet for mice unless otherwise noted and had free access to water. All of the mice were killed at the age of 15 weeks with ether anesthesia. To increase the number of mice, male Apc^{Min/+} mice were mated with wild-type female mice. The genotype of the offspring for Apc^{Min/+} was analyzed by a PCR method described by Dietrich et al. (15).

Aminoguanidine Treatment. An experimental group of Apc^{Min/+} off-spring (4 males and 6 females) received drinking water containing aminoguanidine hemisulfate (Sigma Chemical Co.; A-7009) at 1500 ppm, from the age of 5 weeks. The control group (5 males and 7 females) received tap water. The water consumption of the animals was checked twice a week. On the basis of water consumption, it was estimated that the experimental group ingested a mean amount of 14.0 ± 1.7 mg (0.11 mmol) aminoguanidine/100 g body weight/day. This dose did not affect the water consumption compared with the control group.

Dietary l-Arginine Restriction. During the same period (5–15 weeks), Apc^{Min/+} mice for the experimental group (3 males and 6 females) and for the control group (3 males and 6 females) received a synthetic l-arginine-deficient diet (TD 91230) and complete amino acid diet (TD 86529), respectively, obtained from Harlan Teklad (Madison, WI). The arginine-free diet was formulated identically to the complete amino acid diet, except that it lacked arginine and was made isocaloric with the complete diet by the addition of more l-alanine.

Generation of Apc^{Min/+} iNOS^{-/-} and Apc^{Min/+} iNOS^{+/+} Mice. Apc^{Min/+} males were mated with iNOS^{-/-} females to generate Apc^{Min/+} iNOS^{-/-} mice. Such males were backcrossed with iNOS^{-/-} mice to generate Apc^{Min/+} iNOS^{-/-} mice. Genotyping of the iNOS knockout offspring was performed by PCR according to conditions kindly provided by Dr. V. Laubach, University of Virginia Health Sciences Center. In brief, mouse tail genomic DNA was extracted with the use of a QiAamp tissue kit (Qiagen Inc., Valencia, CA). Two different primer sets were used, which gave distinct products. The sequences of primer pairs for the wild-type allele (+) and targeted allele (−) were 5’-GAGGAGAGAGATCGATTTAGAGTCTTGG-3’ and 5’-TGAAAGC-CATGACCTTTCGATGATTAGCTGCCAGTCC-3’, respectively. A PCR product of ~1200 bp was generated in the presence of the targeted allele (−), and ~400 bp in the wild-type allele (+).

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2. The abbreviations used are: NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; COX, cyclooxygenase; Min, multiple intestinal neoplasia; RT-PCR, reverse transcription-PCR.

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The PCR was carried out as follows. Genomic DNA (~500 ng) was amplified in a 50-μl reaction mixture containing 1 μM primer pair, 0.4 mM dNTP, 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl and 1.25 units Taq DNA polymerase (PE Applied Biosystems, Norwalk, CT). Amplification conditions with the GeneAmp PCR System 2400 (PE Applied Biosystems) were 95°C for 3 min and 39 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 4 min. The PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide. In total, we used 9 (5 males and 4 females) Apc<sup>Min/+</sup> iNOS<sup>−/−</sup>, 28 (17 males and 11 females) Apc<sup>Min/+</sup> iNOS<sup>+/−</sup> and 7 (4 males and 3 females) Apc<sup>Min/+</sup> iNOS<sup>+/−</sup> mice.

**Multiplicities, incidences, and sizes of tumors were considered to be statistically significant at the level of** $P < 0.05$.

**Results and Discussion**

**Expression of iNOS mRNA and Protein**. As shown in Fig. 1, iNOS mRNA was expressed in most polyp tissues, but also sporadically in normal tissues. iNOS protein was constitutively expressed in ileal mucosal epithelium of wild-type mice, as reported by Hoffman et al. (10). In Min mice, iNOS protein was localized in epithelial cells comprising adenomas or normal mucosa in the small and large intestines (Fig. 2, A and B). Occasionally, iNOS expression varied even within individual adenomas. Specific positive superficial cells were clearly demarcated from negatively stained underlying tumor cells (Fig. 2C). Crypt epithelial cells and mucosal stromal cells did not express iNOS protein. Staining for iNOS protein was rarely seen in iNOS gene-knockout Apc<sup>Min/+</sup> mouse tissues (not shown). However, no difference in staining intensity between the tissues from control and from aminoguanidine-treated mice was detected.

**Tumor Multiplicity and Size**. We used three different approaches to suppress NO production in Min mice, including (a) pharmacological treatment with the iNOS-selective inhibitor aminoguanidine; (b) nutritional restriction of the iNOS substrate L-arginine; and (c) generation of iNOS knockout Min mice. Aminoguanidine is a relatively selective inhibitor for iNOS (IC₅₀ values for murine iNOS and rat nNOS are 5.4 and 160 μM, respectively; Ref. 17) and also inhibits the expression of iNOS protein induced by endotoxin in murine macrophages (18). Long-term administration of an L-arginine-free diet reduced NO production in rats as measured by nitrate excretion (19, 20).
In each experiment, there was no significant difference in body weight of mice between control and experimental groups. As shown in Fig. 3, iNOS inhibition by aminoguanidine, L-arginine-free diet, and genetic approaches reduced the small intestinal polyp numbers at significance levels of \( P = 0.09, P < 0.05, \) and \( P < 0.0005, \) respectively. The data on polyp sizes showed a decreasing trend with iNOS inhibition. The incidences and tumor multiplicities in large intestines were not different from those of controls after treatment with amino-guanidine or the arginine-free diet (Table 1). However, iNOS knock-out Min/+ mice (\( Apc^{Min/+} \) iNOS/–/– or \( Apc^{Min/+} \) iNOS/–/–) showed significantly lower tumor incidences (68 and 29%, respectively, compared with controls) and multiplicities (33 and 9%, respectively, compared with controls; \( P < 0.005, \) Table 1).

In the present study, we have demonstrated that iNOS is expressed especially in epithelial cells of normal and adenoma tissues throughout the intestines, predominantly in distal parts. However, no crypt cells and not all adenoma cells showed expression. Although the precise role of iNOS in these cells is not known, it is possible that iNOS may play an important role in tumorigenesis from the early stage via paracrine or autocrine signaling by normal or tumor cells. Moreover, the fact that strong positive staining of superficial epithelial cells was clearly demarcated from underlying unstained tumor cells in some adenomas means that NO is partly participating in the cellular differentiation pathway. Interestingly, our immunohistochemical results indicate that Min mice possessing a heterozygous iNOS gene (\( Apc^{Min/+} \) iNOS/+) rarely expressed iNOS protein. This fact can explain the low tumor multiplicities in \( Apc^{Min/+} \) iNOS/–/– mice as well as in \( Apc^{Min/+} \) iNOS/–/– mice (see above).

Table 1: Effects of inhibition of NO production on adenoma formation in the large intestine of Min mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incidence (%)</th>
<th>No. of polyps/mouse (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4/12 (33)</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Aminoguanidine-treated</td>
<td>5/10 (50)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control amino acid diet</td>
<td>2/9 (22)</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Arginine-free diet</td>
<td>3/9 (33)</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min/+ iNOS/–/–</td>
<td>9/9 (100)</td>
<td>4.6 ± 1.0</td>
</tr>
<tr>
<td>Min/+ iNOS/–/–</td>
<td>19/28 (68)</td>
<td>1.5 ± 0.3*</td>
</tr>
<tr>
<td>Min/+ iNOS/–/–</td>
<td>2/7 (29)</td>
<td>0.6 ± 0.3*</td>
</tr>
</tbody>
</table>

* Significantly different (\( P < 0.05 \)) from the control group.

Various roles of NO in carcinogenesis have been reported (1). Excess NO causes DNA damage and inhibits DNA repair, thus increasing gene mutation. NO can also have diverse effects on tumor biology, including angiogenesis, invasion, immunosuppression, and so forth, all of which facilitate tumor growth (1). It also activates the enzyme COX-2, which has a pivotal role in the progression of colorectal cancer by enhancing angiogenesis or other unknown mechanisms (11, 12). Elevated levels of COX-2 have been shown in Min mouse adenomas (21, 22). The tumor-promoting role of COX-2 has been indirectly demonstrated by suppressing it with selective inhibitors or by gene knockout in Min or \( Apc \) gene-deleted mice (23). Thus, the suppression of NO could reduce NO-mediated-DNA or -tissue damage, angiogenesis, and/or COX-2 activation in adenoma tissues, thus giving a beneficial effect.

In conclusion, our results suggest that NO produced by iNOS plays an important role as an endogenous factor in the development of intestinal polyposis in Min mice and that an iNOS-selective inhibitor can be used as a chemopreventive agent for colorectal cancers. In support of these results, the iNOS-selective inhibitor \( S,S^*\)-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea (\( K \)) values for human iNOS, nNOS, and eNOS: 7.4, 16, and 360 \( \text{nm} \), respectively; Ref. 8) suppressed development of azoxymethane-induced aberrant crypt foci in rats. However, both inhibitory (24) and enhancing (25) effects on development of azoxymethane-induced aberrant crypt foci have been reported in rats treated with the nonselective iNOS inhibitor L-\( N^2 \)-nitroarginine methyl ester \( [K \] values of L-\( N^2 \)-nitroarginine: 4.4 \( \mu \text{m} \), 15 \( \mu \text{m} \), and 39 \( \mu \text{m} \) for iNOS (murine), nNOS (bovine), and eNOS (human), respectively). Therefore, more selective inhibitors of iNOS such as 1400W (\( K \) values 7 \( \mu \text{m} \), 2 \( \mu \text{m} \), and 50 \( \mu \text{m} \) for human iNOS, nNOS, and eNOS, respectively; Ref. 26) should be further tested for chemopreventive effects on colon carcinogenesis.

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References


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