Advances in Brief

Suppression of Intestinal Polyposis in \textit{Apc}^{\text{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 \textit{Apc}^{\text{Min/+}} mice by reverse transcription-PCR and immunohistochemistry. Administration of the iNOS inhibitor aminoguanidine (1.5 g/litter) in drinking water or a l-arginine-deficient diet to \textit{Apc}^{\text{Min/+}} mice resulted in a significant decrease in adenoma development in the small but not the large intestine. Similarly, iNOS-gene knockout \textit{Apc}^{\text{Min/+}} mice (\textit{Apc}^{\text{Min/+}} iNOS^{-/-} or \textit{Apc}^{\text{Min/+}} iNOS^{+/+}) developed significantly fewer adenomas in both small and large intestines than \textit{Apc}^{\text{Min/+}} \textit{iNOS}^{+/-} mice. These results suggest that iNOS-selective inhibitors could be used as a potential chemopreventive agent for colorectal cancers.

Introduction

NO 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 family of NOSs. iNOS can yield much larger amounts of NO than the 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 (O$_2^-$) or oxygen, respectively (1, 2). NO also stimulates tumor angiogenesis (3) and vascular permeability in solid tumors (4).

Materials and Methods

Animals. Male C57BL/6-j-Apc$^{\text{Min/+}}$ (13) and female C57BL/6-Nos2$^{\text{m1Lau}}$ (iNOS$^{-/-}$) mice (14) were obtained at 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME), and wild-type C57BL/6 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$^{\text{Min/+}}$ mice were mated with wild-type female mice. The genotype of the offspring for Apc$^{\text{Min/+}}$ was analyzed by a PCR method described by Dietrich et al. (15). Aminoguanidine Treatment. An experimental group of Apc$^{\text{Min/+}}$ offspring (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$^{\text{Min/+}}$ 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$^{\text{Min/+}}$ iNOS$^{-/-}$ and Apc$^{\text{Min/+}}$ iNOS$^{-/-}$ Mice. Apc$^{\text{Min/+}}$ mice were mated with iNOS$^{-/-}$ females to generate Apc$^{\text{Min/+}}$ iNOS$^{-/-}$ mice. Such males were backcrossed with iNOS$^{-/-}$ mice to generate Apc$^{\text{Min/+}}$ iNOS$^{-/-}$ animals. 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'-GAGGAGAGAGATCCGATTTAGAGTCTTGG-3', 5'-TGAAGCCATGAACCTTTCGCATTAGCATGG-3', and 5'-ACAGCCTCAGAGTTTCCCT-TCATGAAAGCAATGC-3', 5'-CAGAAAGACCTCGTCAAGAGGGCAT-AGAAAG-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.
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>/<sup>M</sup>, 28 (17 males and 11 females) Apc<sup>Min</sup>/<sup>M</sup> iNOS<sup>−/−</sup> mice, and 7 (4 males and 3 females) Apc<sup>Min</sup>/<sup>M</sup> iNOS<sup>−/−</sup> mice.

**Polyp Number Scoring and Histopathology.** Immediately after sacrifice, both ends of the small and large intestines were tied with thread, and the guts were inflated with 10% phosphate-buffered formalin. After 24 h, they were opened longitudinally to wash the formalin and digesta from the mucosal surface. The numbers and the major diameters of the polyps in small and large bowels were measured under the x10 power field of a stereoscopic microscope. For histological examination, formalin-fixed intestines were prepared by the Swiss roll method and embedded in paraffin, and 4-μm sections were prepared for H&E staining and immunohistochemistry.

**Immunohistochemistry of iNOS and RT-PCR of iNOS mRNA.** Briefly, after blocking endogenous peroxidase, sections were washed with PBS. They were incubated for 30 min in 1.5% goat normal serum. Sections were incubated for 2 h with anti-iNOS rabbit IgG (Santa Cruz Biotechnology; sc-651) diluted 1000-fold in PBS containing 0.1% BSA. Afterward, sections were rinsed twice in PBS. Incubations of slides in biotinylated secondary antibody and avidin-biotin-peroxidase complex in ABC Staining systems (Santa Cruz Biotechnology; sc-203) and visualization by diaminobenzidine were performed according to the manufacturer’s instructions.

Total RNA from polyps and normal intestinal tissues of Min/+ mice was isolated and analyzed for expression of iNOS mRNA by RT-PCR (16).

**Statistical Analysis.** Multiplicities, incidences, and sizes of tumors were compared between the control and experimental groups in respective experiments. The tumor incidences, numbers of large intestinal tumor-bearing animals per sample, were analyzed by the Mantel-Haenszel χ² test, whereas tumor multiplicity (the mean number of polyps per animal) and tumor size (the mean diameter of polyps) were analyzed by the Kruskal-Wallis test. Differences 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 aminoxyguanidine, 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 aminoxyguanidine or the arginine-free diet (Table 1). However, iNOS knock-}

\[ \text{out Min}^+/ \] 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 partially 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>Control amino acid diet</td>
<td>2/9 (22)</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>Min(^{+/}) iNOS(^{-/-})</td>
<td>9/9 (100)</td>
</tr>
<tr>
<td>Min(^{+/}) iNOS(^{-/-})</td>
<td>19/28 (68(a))</td>
<td>1.5 ± 0.3(a)</td>
</tr>
<tr>
<td>Min(^{+/}) iNOS(^{-/-})</td>
<td>2/7 (29(a))</td>
<td>0.6 ± 0.3(a)</td>
</tr>
</tbody>
</table>

\( a\) 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\(^{5}\)-1,4-phenylene-bis[1(2-ethanediyl)bis-isothiourea (K\(_1\)) values for human iNOS, nNOS, and eNOS: 7.4, 16, and 360 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-\(N_G\)-nitroarginine methyl ester (K\(_1\) values of L-\(N_G\)-nitroarginine: 4.4 \(\mu\)M, 15 nm, and 39 nm for iNOS (murine), nNOS (bovine), and eNOS (human), respectively). Therefore, more selective inhibitors of iNOS such as 1400W (K\(_1\) values 7 nm, 2 \(\mu\)M, and 50 \(\mu\)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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