SHORT COMMUNICATION

Efficient induction of rat large intestinal tumors with a new spectrum of mutations by intermittent administration of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in combination with a high fat diet

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In the present study we have established novel intermittent protocols featuring a high fat (HF) diet for efficient induction of large intestinal tumors with a relatively small amount of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). In protocol 1, F344 male rats were first fed a diet containing 400 p.p.m. PhIP for 2 weeks, followed by continuous administration of a HF diet without PhIP for 108 weeks. In protocol 2, 2 week PhIP treatments were repeated three times with 4 week intervals on the HF diet alone, followed by continuous feeding of the HF diet for 42 weeks. At termination of the experiments, 16 (3 of 19) and 45% (9 of 20) of the rats had developed a total of three and 13 large intestinal tumors with protocols 1 and 2, respectively. The tumor incidence in protocol 2 was comparable with that observed with continuous feeding of 400 p.p.m. PhIP for 52 weeks, after exposure to only ~10% of the amount of carcinogen. Five of nine (55%) tumors harbored mutations in either the β-catenin or Apc gene, while all demonstrated accumulation of β-catenin protein in the cytoplasm and nucleus. This suggests that other unknown genetic alterations in the Wnt–Apc–β-catenin signaling pathway could have been involved in the development of tumors. By further modifying this intermittent protocol with HF diet, one could expect more efficient induction of lesions with much smaller amounts of PhIP in a shorter period. In addition, this model could provide a means to elucidate genetic alterations in large intestinal tumors induced by relatively low levels of carcinogenic insult, mimicking the cases of human colon carcinogenesis induced by exposure to environmental carcinogens.

The mode of carcinogenic action of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (1–3) and the mutation spectrum of large intestinal tumors induced by continuous feeding have been well characterized (4,5). Ito et al. have previously reported that when F344 rats were fed a basal powdered diet (Oriental MF; Oriental Yeast Co., Tokyo) containing 100 and 400 p.p.m. PhIP, 43 and 55% of male rats developed carcinomas in the large intestine within 104 and 52 weeks, respectively (2). No large intestinal tumors developed in either male or female rats with 25 p.p.m. PhIP within 2 years (6). PhIP is the most abundant heterocyclic amine in cooked meat and fish (7–9) and exposure doses for humans are mainly dependent upon individual lifestyle. Most colon carcinogenesis studies of PhIP have been carried out using continuous feeding protocols with relatively large amounts of the carcinogen and the effects of different administration protocols on the development of large intestinal tumors and also on mutation spectra have yet to be evaluated in detail. In addition, more efficient protocols need to be devised for the induction of tumors with much smaller quantities of PhIP, considering experimental costs. Based on our previous study that aberrant crypt foci (ACF), putative precancerous lesions of the colon, were induced within 4 weeks after 2 weeks administration of PhIP in AIN-93G diet and more efficiently with a high fat (HF) diet (10), we here employed a novel short-term and intermittent PhIP administration protocol in combination with a HF diet for induction of tumors.

Six-week-old F344 male rats were purchased from Clea Japan Inc. (Tokyo, Japan) and were allowed free access to water and diet. In protocol 1, which was designed as the follow-up of our previous study on the induction of ACF by PhIP (10), 26-week-old F344 male rats were administered 400 p.p.m. PhIP (Nard Institute, Osaka, Japan) in AIN-93G diet (Dyets Inc., Bethlehem, PA) for the first 2 weeks, followed by a HF diet without any carcinogen for 108 weeks. The HF diet was an AIN-93G-based diet supplemented with Primex (hydrogenated vegetable oil) so as to supply 59% fat-derived calories (Dyets Inc.), as described previously (10). In protocol 2, three cycles of 2 weeks PhIP with feeding of the HF diet during the 4 week intervals were conducted, with HF diet for 42 weeks thereafter (Figure 1). Control rats were fed the AIN-93G diet for 2 weeks followed by the HF diet for 108 weeks. The experiments were terminated at weeks 110 and 60 with protocols 1 and 2, respectively. Tumors developing in the large intestine were resected and immersed in neutralized 10% formalin overnight at 4°C. Paraffin sections were prepared by standard procedures and stained with hematoxylin and eosin for histopathological analysis. As summarized in Table I, the incidences of large intestinal tumors were 16% (3 of 19) with protocol 1 and 45% (9 of 20) with protocol 2, the first tumor in the latter case being detected at experimental week 35. Histological analysis revealed that 85% (11 of 13) of the tumors with protocol 2, including one in the cecum, were adenocarcinomas, while all three with protocol 1 were diagnosed as adenomas. The stained sections were examined for tumor histology according to the classification of Pozharisski (11), with minor modifications (Figure 2A and B). The tumor

Abbreviations: ACF, aberrant crypt foci; HF, high fat; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SSCP, single strand conformation polymorphism.
Fig. 1. Experimental protocol for PhIP administration. In protocol 1, 400 p.p.m. PhIP was administered in AIN-93G diet for the first 2 weeks (hatched box), followed by a HF diet for 108 weeks. In protocol 2, 2 weeks administration of 400 p.p.m. PhIP was repeated three times with 4 week intervals on the HF diet (open box), followed by the HF diet for 42 weeks. Control rats were fed the AIN-93G diet for 2 weeks followed by the HF diet for 108 weeks.

Fig. 2. β-catenin accumulation in rat large intestinal tumors. Tumor cells showed the typical features of well-differentiated adenocarcinoma. They are glandiform structures with tubular cavities of varying, irregular shape, lined with a high cylindrical epithelium arranged in one or several rows in hematoxylin and eosin staining (A and B). Normal tissues are observed in the upper left parts in the same sections. Accumulation of β-catenin protein was observed in both nucleus and cytoplasm (C and D). Levels of accumulation were assessed as weak in (C) and dense in (D). (A) and (C), tumor 1; (B) and (D), tumor 4a.

Table I. Incidences and numbers of large intestinal tumors induced by PhIP in F344 male rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Experimental period (wk)</th>
<th>Tumor incidence (%)</th>
<th>No. of tumors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adenoma</td>
</tr>
<tr>
<td>Control</td>
<td>110</td>
<td>0/20 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Protocol 1</td>
<td>110</td>
<td>3/19 (16)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>60</td>
<td>9/20 (45)</td>
<td>2 (15)</td>
</tr>
</tbody>
</table>

*One rat developed tumors in both colon and cecum. Among 11 adenocarcinomas, one was in the cecum.
and was located at the splicing acceptor site (Table II). This mutation was detected at the splicing acceptor site of intron 10. Three of nine (33%) tumors were found to harbor mutations in both codon 32 and codon 34 (Table II). Two mutations in both codon 32 and codon 34 (Table II).

Apc gene was located at the splicing acceptor site (Table II). This mutation may affect normal splicing of exon 11, resulting in a decrease in wild-type Apc transcript. No mutation was found for APC or β-catenin in the non-tumorous counterpart tissues. Immuno-histochemical analysis was also carried out to assess accumulation of β-catenin protein in tumor cells. A mouse monoclonal antibody for mouse β-catenin (Transduction Laboratories, Lexington, KY) was used as the primary antibody and biotinylated goat anti-mouse IgG (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) as the secondary antibody. All of the tumors demonstrated accumulation of β-catenin protein compared with the surrounding normal-looking tissue (Figure 2C and D), as well as colon tissues from age-adjusted control animals without PhIP treatment (data not shown), although only half harbored mutations. This result suggests that other molecules involved in the Wnt–Apc–β-catenin signaling pathway (17), such as Axin1 (18–20) and Axin2 (21,22), could be mutated or down-regulated in these tumors.

In the present study we were able to establish a novel protocol of intermittent PhIP administration in combination with a HF diet for efficient induction of large intestinal tumors with relatively small amounts of PhIP. Repeating the intermittent protocol, or setting more frequent intervals with much shorter periods of PhIP administration without increasing the total carcinogen exposure, might allow large intestinal tumors to be induced more efficiently. Tsukamoto et al. recently reported that administration of PhIP intragastrically three times a week for 7 weeks at a dose of 100 mg/kg body wt could also efficiently induce large intestinal tumors within 50 weeks, six tumors being found in 10 F344 rats, although the daily exposure to PhIP was much higher than in our current study (23). Intermittent intragastric administration of PhIP with a HF diet also induced mammary cancers in female Sprague–Dawley rats as effectively as a continuous administration of PhIP.
protocol (24). One possible explanation for the enhanced induction of tumors with a small amount of PhIP in combination with a HF diet could be that PhIP was able to induce single or multiple mutations in cells, largely depending upon the total amount of exposure, and that these mutations might stimulate cells to proliferate selectively. However, when cells are repeatedly exposed to large amounts of genotoxic agents, as with continuous PhIP administration, the chance of generating multiple, disadvantageous mutations might also be high. Some of the mutations might interfere with growth of focal populations, perhaps resulting in cell death. Under these circumstances, only a subset of transformed cells might survive and proliferate. Introduction of intervals between PhIP exposure periods could result in less chance of lethal mutations occurring. Furthermore, the HF diet could directly promote cell growth (10,25,26). All of the colon tumors developing with long-term continuous feeding of PhIP harbored mutations in either exon 14 or 15 of the Apc gene (4) or in exon 3 of the β-catenin gene (5). All the mutations found in Apc were a one G deletion in a GGGA stretch, which is therefore considered a signatures mutation for PhIP (13). In contrast to these previous studies, fewer mutations but a new spectrum of mutations were observed in the Apc and β-catenin genes in tumors induced with the current intermittent protocol. Further mutational analyses should be conducted to clarify these points. In conclusion, the present protocols could provide us with a useful approach more closely mimicking the actual mode of human exposure to PhIP. By extension of this idea, one might expect even more efficient protocols for the induction of neoplasms. Our results also suggest that researchers should be cautious and take experimental protocols seriously into consideration when assessing human hazard risks with various environmental carcinogens.

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References


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