Effect of the Bowman-Birk Protease Inhibitor on the Expression of Oncogenes in the Irradiated Rat Colon

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ABSTRACT

In this study, we tested the influence of i.p. Bowman-Birk protease inhibitor (BBI) administration on oncogene expression in unirradiated and irradiated rat colon mucosa. Total cellular RNA was collected from the colonic mucosa, and the levels of c-myc, c-fos, c-Ha-ras, c-EGFR, and c-actin mRNA were examined by standard dot and Northern blot analyses. The data demonstrate that BBI is capable of preventing radiation-induced overexpression of c-myc and c-fos without interfering with the constitutive expression of these 2 genes. It was also determined that BBI did not interfere with either radiation-induced overexpression of c-Ha-ras and c-EGFR or the constitutive expression of c-Ha-ras, c-EGFR, or c-actin. The data demonstrate that the anticarcinogenic BBI selectively inhibits the overexpression of c-myc and c-fos while not affecting crypt cell proliferation. These results suggest that a protease is involved in the pathway for enhanced c-myc and c-fos expression and that protease inhibitors such as BBI can interrupt this pathway.

INTRODUCTION

Studies have indicated that both naturally occurring and synthetic protease inhibitors have the capacity to inhibit carcinogenesis in vivo and in vitro (reviewed in Refs. 1 and 2). For example, the soybean-derived BBI, an inhibitor of both trypsin and chymotrypsin (3), has been shown to suppress experimentally induced cancers in animals. BBI in the diets of animals has been shown to suppress dimethylhydrazine-induced colon and liver carcinogenesis (4, 5), dimethylbenz[a]anthracene-induced cheek pouch carcinogenesis in hamsters when applied topically (6), and 3-methylcholanthrene-induced lung tumors when administered by gavage or i.p. injection (7). In vitro studies have demonstrated that protease inhibitors including BBI are also capable of preventing transformation of cultured cells induced by either physical or chemical carcinogens (8–13).

These same anticarcinogenic protease inhibitors also suppress a number of other phenomena that have been associated with animal carcinogenesis and malignant transformation of cultured cells, such as: c-myc expression (14–16), ras-induced cellular transformation (17), chromosomal aberrations occurring in cells from Bloom’s syndrome patients (18), and H2O2 formation in phorbol ester activated neutrophils (19). Previous studies have shown that the level of c-myc mRNA is reduced in proliferating C3H/10T½ cells that were grown in a medium containing anticarcinogenic protease inhibitors, such as antipain, BBI, and leupeptin (14, 15, 20). Protease inhibitors that do not have the ability to reduce radiation-induced transformation, such as soybean trypsin inhibitor, elastatinal, and α1-antitrypsin, had little or no effect on the expression of c-myc.

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The abbreviation used is: BBI, Bowman-Birk protease inhibitor.

The colonic epithelium of rodents has been utilized as a model for the investigation of cellular proliferation, migration, differentiation, and carcinogenesis (reviewed in Refs. 21 and 22). It is well recognized that ionizing radiation disrupts the normal homeostasis of the gut mucosa. Radiation-induced epithelial cell killing is followed by a compensatory increase in crypt cell proliferation (21–24). Radiation has also been used to induce colonic carcinomas in rodents (25, 26). We have previously shown that abdominal irradiation led to a compensatory hyperplasia of the colonic mucosa of mice and an overexpression of c-myc during the time of increased crypt cell proliferation (16). BBI administered by gavage in conjunction with abdominal irradiation inhibited the overexpression of c-myc RNA but had no effect on radiation-induced crypt cell proliferation (16). Although both in vivo and in vitro studies demonstrate that chemopreventive protease inhibitors prevent c-myc expression, little is known about the effect of protease inhibitors on the expression of other oncogenes that are thought to be relevant for cellular proliferation and carcinogenesis.

The present study was designed to further evaluate the effect of i.p. BBI administration on oncogene expression in the colonic mucosa of rats following abdominal irradiation. Results of this study demonstrate that BBI administration did not affect compensatory crypt cell proliferation but prevented the overexpression of c-myc and c-fos in the regenerating colonic mucosa following abdominal irradiation. The expression of c-Ha-ras and c-EGFR was unaffected by the protease inhibitor treatment.

MATERIALS AND METHODS

General Procedures. Five- to 6-week-old Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN) were used in this investigation. Upon arrival at the laboratory, the rats were housed in an environment-controlled room and had free access to food and water. Following a 1-week acclimation period, 5 rats were randomly assigned to each of 4 treatment groups. Rats assigned to groups A and B received i.p. injections of sterile saline every other day for 20 days or until they were killed. Rats assigned to groups C and D received i.p. injections of a soybean extract containing BBI (hereafter referred to as BBI, 25 mg/kg, filter sterilized; Central Soya Company, Fort Wayne, IN) every other day for 20 days or until the rats were killed. One day after the first saline or BBI injection, all rats were anesthetized with an i.p. injection of sodium pentobarbital (40 mg/kg), and the rats of groups A and C were sham irradiated. The rats of groups B and D were whole-abdomen irradiated with 11 Gy in a specially constructed jig that shielded the remainder of the body. The radiation was delivered by a modified Mark IV Cs-137 γ-iradiator (J. L. Shepard and Associates, San Fernando, CA) at a dose rate of 9.63 Gy/min.

dpm/Crypt. One h prior to being killed, the rats received 0.5 μCi/g body weight of tritiated thymidine (200 μCi/ml solution; 60 Ci/mmol) via i.v. injection. After the labeling period, the rats were killed by an overdose of pentobarbital and the entire colon removed and rinsed with chilled saline. Approximately 0.5 cm of transverse colon was removed, fixed in Clarke’s solution, and at a subsequent time hydrated, hydrolyzed in 1 N HCl, then stained with Schiff’s reagent. Fifty colon crypts were isolated in triplicate from each segment by a microdissection.
technique originally described by Hagemann et al. (27) and modified by Hanson et al. (28). The isolated crypts were placed into miniscintillation vials containing 150 µl 0.5 N NaOH. After the crypts were solubilized, 3 ml scintillation cocktail (Ecolume; ICN Biomedical, Inc., Irvine, CA) were added, each vial counted, and the results expressed as dpm/crypt.

RNA Isolation. The remainder of the colons were split open longitudinally and the colonic mucosa removed from the underlying submucosa and muscularis with a microscope slide. The colonic mucosa from the animals of each treatment group were pooled and total cellular RNA was collected by the method of Chirgwin et al. (29) and then stored at -80°C until used.

Northern and Dot Blot Hybridization. Fifty µg of total cellular RNA were blotted onto a nitrocellulose filter using a dot blot manifold (Schleicher and Schuell, Keene, NH). Filters were baked at 80°C in a vacuum oven for 2 h, then hybridized with 32P-labeled cDNA using standard methods (30) and subsequently exposed to X-ray film. Dot blots were used to select time points to be compared by Northern blot analysis. The probes used in this investigation c-myc, v-fos, v-Ha-ras, and v-erbB (EGFR), and actin were obtained from Oncor, Inc. (Gaithersburg, MD).

For Northern analysis, 3–5 µg poly (A+) RNA were isolated from total RNA using an oligo dT column and size fractionated on a 1% formaldehyde agarose gel (31) and transferred to a nitrocellulose filter. The filters were hybridized to a 32P-labeled c-myc, or v-fos probe as described above.

RESULTS

General Observations. Fig. 1 shows the effect of the various treatment protocols on rat body weight. Rats receiving i.p. administration of saline or BBI gained weight steadily throughout the investigation. Rats receiving 11 Gy of abdominal irradiation in addition to i.p. administration of saline or BBI exhibited a decline in body weight that reached its minimum value 6–10 days after irradiation. Body weights of the irradiated rats then gradually increased toward that of the unirradiated rats.

Because protease inhibitors may lead to pancreatic hypertrophy, the pancreas from each animal was weighed at the time of autopsy. Table 1 lists the mean pancreas weights from rats in each treatment group. Rats treated with 11 Gy of abdominal irradiation exhibited a reduction in pancreas weight that reached a nadir at 7 days after irradiation. The irradiated pancreases slowly gained weight throughout the remainder of the investigation. BBI administration had no effect on the pancreas weight of irradiated or unirradiated rats.

dpm/Crypt. To examine the influence of the various treatments on crypt cell proliferation, the uptake of [3H]thymidine by the colonic crypts was monitored at the time of autopsy. Abdominal irradiation led to a time-dependent change in the proliferative activity of the colonic epithelium, as indicated by the uptake of [3H]thymidine in the colonic crypts (Table 2). Rats subjected to 11 Gy of abdominal irradiation exhibited a marked but transient increase in crypt cell proliferation (dpm/crypt), which peaked at 7 days postirradiation. The compensatory increase in crypt cell proliferation then rapidly returned to control levels. Administration of BBI i.p. had no influence on either the control level of crypt cell proliferation or the radiation-induced compensatory increase in crypt cell proliferation as measured by the uptake of tritium (Table 2).

RNA Expression. Hybridization analysis of dot and Northern blots revealed that 11 Gy of abdominal irradiation led to an increase in the expression of c-myc, c-fos, c-Ha-ras, and c-EGFR mRNA. Dot blots of total cellular RNA were used to determine when oncogene expression was most enhanced and whether BBI would affect the expression of the oncogenes. Expression of c-myc was greatest 7 days postirradiation. Northern analysis confirmed that c-myc mRNA was elevated 7 days after irradiation; a representative blot is shown in Fig. 2. Densitometry indicated that c-myc expression in the colonic mucosa exhibited a 2.2-fold increase at 7 days after irradiation when compared to untreated rats (Table 3). Expression of c-myc from the colonic mucosa of rats treated with BBI alone or radiation plus BBI resulted in no change relative to the untreated animals (Table 3). Thus, following abdominal irradiation, the peak in c-myc mRNA expression occurred simultaneously with increased crypt cell proliferation. Northern blots of mRNA collected 3 days after irradiation revealed that radiation-induced overexpression of c-myc was preceded by a substantial increase in c-fos expression (Fig. 2). Densitometry of Northern blots from 3 days after treatment revealed that 11 Gy led to a 6.3-fold increase in c-fos expression, whereas BBI alone and 11 Gy plus BBI produced no change in c-fos expression relative to the
A considerable body of evidence indicates that certain protease inhibitors are able to suppress carcinogenesis and cellular transformation (4–13). However, little is currently known regarding the mechanism by which protease inhibitors function to suppress carcinogenesis. Because protease inhibitors suppress both physically and chemically induced carcinogenesis in a variety of experimental systems, it suggests that protease inhibitors may have the ability to suppress oncogene expression.

Table 3  
<table>
<thead>
<tr>
<th>Treatment</th>
<th>c-myc*</th>
<th>c-fos*</th>
<th>c-Ha-ras*</th>
<th>c-EGFR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>BBI</td>
<td>1.13 ± 0.09*</td>
<td>0.91 ± 0.11*</td>
<td>0.99 ± 0.03*</td>
<td>1.30 ± 0.24*</td>
</tr>
<tr>
<td>11 Gy</td>
<td>2.18 ± 0.09</td>
<td>6.34 ± 0.16</td>
<td>1.83 ± 0.16</td>
<td>2.96 ± 0.42</td>
</tr>
<tr>
<td>11 Gy + BBI</td>
<td>1.01 ± 0.05*</td>
<td>0.99 ± 0.22*</td>
<td>1.82 ± 0.08</td>
<td>3.35 ± 0.53</td>
</tr>
</tbody>
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* Message RNA levels of c-myc as evaluated at 7 days irradiation.
* Message RNA levels of c-fos, c-Ha-ras, and c-EGFR as evaluated at 3 days after irradiation.

untreated group (Table 3). BBI administration appeared to have little effect on the constitutive level of c-myc or c-fos mRNA (Figs. 2 and 3). However, i.p. administration of BBI completely prevented the radiation-induced overexpression of both c-myc and c-fos (Figs. 2 and 3) without interfering with the compensatory increase of crypt cell proliferation (Table 2).

Abdominal irradiation also led to an increased expression of c-Ha-ras and c-EGFR in the colonic mucosa. A representative dot blot shows that the expression of c-Ha-ras peaked at 3 days after irradiation (Fig. 4); similar results were found for c-EGFR (data not shown). Densitometry of c-Ha-ras dot blots at 3 days posttreatment demonstrated a 1.8-, 1.0-, and 1.8-fold change in c-Ha-ras expression in the groups treated with 11 Gy alone, BBI alone, and 11 Gy plus BBI, respectively, when compared to the untreated group (Table 3). Analysis of RNA dot blots from 3 days after treatment revealed a 3.0-, 1.3-, and 3.4-fold change in c-EGFR expression relative to the untreated group, in the groups treated with 11 Gy alone, BBI alone, and 11 Gy plus BBI, respectively (Table 3). BBI administration had no effect on the constitutive or radiation-induced expression of c-Ha-ras or c-EGFR (Fig. 4). Expression of β-actin, a normal structural gene, was found not to be affected by radiation and/or BBI administration and demonstrated approximately equal loading of RNA (Fig. 5).
The results of this study indicate that BBI prevented the overexpression of c-myc and c-fos in the colonic mucosa by a mechanism that did not influence crypt cell proliferation.

An interesting feature of the investigation reported here is the spectrum of gene expression affected by BBI administration. BBI prevented radiation-induced overexpression of c-myc and c-fos without interfering with the constitutive expression of these 2 genes. This indicates that enhanced expression of c-myc and c-fos is dependent upon a protease that is inhibited by BBI, whereas the constitutive expression of these 2 genes is independent of this proteolytic regulation. The mechanism by which BBI prevents the overexpression of c-myc and c-fos has not been defined. However, there are a number of points in the regulatory cascade of c-myc and c-fos that potentially may be affected by protease inhibitors. Signal transduction from the cytoplasm to the nucleus primarily involves a translocation of proteins between the 2 compartments (reviewed in Ref. 37). For example, steroid hormones are shuttled from a receptor at the membrane through the cytoplasm into the nucleus. Peptide growth factors, such as PDGF, bind to a cellular receptor and initiate the transduction of a signal that ultimately results in a change in RNA transcription (37). Growth factors have been shown to rapidly increase the transcription of c-myc and c-fos through a signal transduction pathway (38–40). Thus, BBI may act on a proteolytic dependent step along the signal transduction pathway to prevent the overexpression of c-myc and c-fos. In addition, negative transcription factors have been postulated to exist for both c-myc and c-fos (reviewed in Refs. 41 and 42). Perhaps BBI suppresses the overexpression of these 2 oncogenes by preventing the proteolytic degradation of repressor proteins. Thus, there are a number of candidate sites for BBI to act in preventing the overexpression of c-myc and c-fos.

In contrast, both the constitutive expression and the overexpression of the 2 membrane genes evaluated in this study, c-Ha-ras and c-EGFR, were unaffected by BBI administration. The results of this study are consistent with the possibility that BBI administration may suppress the overexpression of one “type” of oncogene, thereby blocking the pathway of carcinogenesis. Elucidation of the biochemical mechanism by which protease inhibitors interact with oncogenes should provide valuable information regarding the nature of the carcinogenesis process and methods for its arrest.

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REFERENCES


Fig. 5. Northern analysis of c-actin expression in the colonic mucosa at day 7 from (A) untreated rats, (B) rats treated with 11 Gy abdominal irradiation, (C) rats treated with BBI, or (D) rats treated with BBI and 11 Gy abdominal irradiation.


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