The Effect of Exogenous Prostaglandin Administration on Tumor Size and Yield in Min/+ Mice

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ABSTRACT

This study set out to examine the effect of exogenous prostaglandin (PG) administration on tumor development in Min/+ mice. Mice were treated with the stable prostaglandin E2 analogue 16,16-dimethyl-PGE2 from 6–18 weeks of age. Mice were sacrificed, and tumor burden was assessed using morphometric techniques. Parameters measured were median tumor size, mean tumor size, the proportion of the area of the gastrointestinal mucosa covered with tumor, and the number of tumors per 1000 mm2 of gastrointestinal mucosa. In addition, proliferative and apoptotic indices were determined. These measurements were carried out for all regions of the small intestine (i.e., duodenum, jejunum, upper ileum, and lower ileum) and the large intestine (i.e., cecum and mid-colon/rectum). 16,16-Dimethyl-PGE2-treated animals showed a significant decrease in tumor burden (by approximately 50–70%), in comparison with those animals that were treated with vehicle alone (0.001% ethanol in 0.9% sterile saline), in all regions of the intestine (at P = 0.008 or better). This effect was contributed to by a reduction in the number of tumors (by approximately 20–50%) and a reduction in tumor size (by approximately 10–70%). An increase in tumor cell turnover was associated with this decrease in tumor burden, as determined by the changes in the levels of thymidine incorporation (significant at P = 0.003), apoptosis, and mitosis (nonsignificant).

INTRODUCTION

The Min/+ mouse is a well-characterized model of GI1 tumorigenesis (1–3) and has been used in many studies examining the efficacy of NSAIDs in inhibiting tumor cell growth (4–8). The mice are characterized by a mutation in the APC gene (the gene mutated in familial adenomatous polyposis) that results in the insertion of a premature stop codon (2). Loss of heterozygosity of the APC gene results in the development of small adenomas with a noninvasive phenotype, primarily within the intestinal mucosa. The precise number of tumors that an animal develops and their growth characteristics are determined by a number of genotypic modifiers (3, 9).

Over the last decade, much research has suggested that endogenous PGs can act as proliferative and survival factors for colorectal tumor cells as well as other tumor types. This is evidenced by a number of findings. NSAIDs, which inhibit COX (PG H2/G2 synthase), have been shown to reduce tumor cell proliferation both in vivo and in vitro and also to promote apoptosis of tumor cells (5–8, 10). COX has been proposed. One of the prime factors in promoting tumor cell growth and survival has been suggested to be PGE2, which can mimic the effects of COX-2 expression in vitro (31).

In this study, we have examined the effect of exogenous PGE2 on tumor development in Min/+ mice. We hypothesized that, given previous data, we should observe more rapid tumor development and perhaps tumors with an invasive phenotype. However, the results obtained suggest that the role of PGs as either paracrine or autocrine growth factors for tumor cells may be more complex than first proposed.

MATERIALS AND METHODS

Mice

Min/+ mice were bred in house, on a C57BL/6 background. PCR analysis was used to identify the genotype of the Min/+ mice used in this study (32). Both male and female Min/+ mice were used in these experiments. Previous studies in our laboratory have demonstrated no significant difference between the tumor burden of male and female animals (data not shown). Mice were kept on a 12-h light/12-h dark cycle with lights on at 7 a.m. and were allowed free access to food and water. Animals were monitored daily for physical well-being.

16,16dmPGE2

16,16dmPGE2 was obtained from Sigma-Aldrich (Poole, United Kingdom). 16,16dmPGE2 was obtained as a solution in methyl acetate. The methyl acetate was evaporated under a stream of nitrogen, and the 16,16dmPGE2 was immediately dissolved in ice-cold 100% ethanol (analytical grade) at a concentration of 1 mg/ml. The 16,16dmPGE2 was aliquoted into 30-μl volumes that were stored at −80°C until use.

Experimental Procedure

Experimental cohorts consisted of six mice [three males and three females (caged separately)]. Mice were injected with 16,16dmPGE2 from 6 until 18 weeks of age. The age of 18 weeks was chosen as an end point because our colony of Min/+ mice show well-developed adenomas at this age without...
significant morbidity. Injections were carried out using the following regimen: three i.p. injections of 16,16dmPGE₂ (0.25 ml; 40 ng/ml in physiological saline) were given weekly (Monday, Wednesday, and Friday at 2 p.m.). Parallel cohorts of mice were injected with 0.25 ml of ethanol (0.0004%)/physiological saline. Three separate experiments were carried out on three separate batches of animals. Experiments were carried out in a series with an interval of 2–4 weeks between each experiment.

The above-mentioned regimen was chosen on the basis of results from a pilot experiment that assessed three different dosing schedules (data not shown).

Assessment of Tumor Burden

**Tissue Processing.** When the mice were 18 weeks old, they were sacrificed by cervical dislocation. The intestines were excised and flushed with ice-cold PBS (pH 7.4). The intestines of two male and two female mice from each cohort were taken for morphometric analysis of tumor size. The small intestine was cut into four regions (duodenum, jejunum, and upper and lower ileum), and the large intestine was cut into two regions (cecum and mid-colon/rectum). Each length of intestine was opened out by longitudinal incision and pinned flat, using entomology pins, on to a wax base in a large plastic Petri dish. Next, the intestinal segments were fixed in 4% formaldehyde in PBS overnight at 4°C and transferred to 70% ethanol.

**Methylene Blue Staining.** After initial processing, as outlined above, the tissue was rinsed twice in 70% ethanol and left for 1 h. The 70% ethanol was then replaced by 40% ethanol (in PBS), and the dishes were placed on an orbital shaking table for 10 min. The tissue was then washed in PBS for an additional 10 min before staining the tissue with 0.1% methylene blue (in PBS) for 10 min. After staining with methylene blue, the tissue was washed three times in ice-cold PBS (10 min/wash). Each region of tissue was cut into three approximately equal pieces that were then mounted individually in glycerol on glass microscope slides.

**Morphometry.** A Zeiss Axiohome microscope was used in conjunction with a personal computer and Zeiss software to make morphometric measurements. Specifically, we measured the area of the whole mount and the area of all individual tumors. Whole mounts were viewed using a ×4 magnification objective and ×10 magnification eye pieces, with bright-field illumination.

When making the incision along the gut during the initial preparation of the whole mounts, occasional tumors were cut into two pieces. In such a case, the two pieces of the same tumor appear on opposite sides of the whole mount and can be easily identified and matched up. The areas of the two pieces of tumor are determined separately and then summed to give the true area for the whole tumor.

The following figures were recorded and calculated: (a) the area of each intestinal region (area); (b) the area of each tumor within each region; (c) the sum of the area of all tumors within each region (areaₜ); (d) the proportion of intestinal surface area covered by tumor (areaₜ/area) for each region (pₜ); (e) the total number of tumors within each region (T); and (f) the number of tumors per 1000 mm² area for each region (TP₁K).

Assessment of DNA Synthesis by Autoradiography

The incorporation of tritiated thymidine was used as a measure of DNA synthesis to indicate the proliferative state of the tissue. Briefly, at the end of each experiment, two mice from each group (one male mouse and one female mouse) were selected and injected i.p. with 25 μCi of tritiated thymidine (in 0.1 ml of physiological saline). Mice were sacrificed 40 min later, and the intestines were excised, flushed with ice-cold PBS, and fixed in 4% formaldehyde in PBS overnight at 4°C. The intestine was cut into six regions, as previously described. Tissues were processed for wax embedding, and 3-μm-thick sections were cut. Tissue sections on microscope slides were coated with nuclear fasttrack emulsion (Ilford, Mobberley, United Kingdom) and exposed for 3 days before developing, as described previously (33). After development, cells with three or more silver grains over the nucleus were classified as being positive for thymidine incorporation, and a labeling index was determined.

**Fig. 1.** Graphical representation of changes in tumor burden in animals receiving either vehicle control (□; n = 10) or 16,16dmPGE₂ (●; n = 12) at 18 weeks of age. Changes in the proportion of epithelial surface area covered with tumors (pₜ; A), the number of tumors per 1000 mm² of intestinal surface area (TP₁K; B), average tumor area (C), and median tumor area (D) are shown. Data were analyzed by two-way ANOVA. Pₜ for statistical significance are indicated.
Immunohistochemistry

Immunohistochemical analysis of p21^{WAF-1/CIP1} expression was carried out as described previously (33), in parallel to assessment of thymidine incorporation. Developed slide autoradiographs were rinsed overnight in deionized water before incubation with rabbit polyclonal antibody to p21WAF-1/CIP1 at 0.5 μg/ml (PC55; Calbiochem, Nottingham, United Kingdom). Sections were subsequently incubated with biotinylated goat antirabbit IgG (1:200 dilution; Vector Laboratories, Peterborough, United Kingdom) and avidin/biotin-horseradish peroxidase complex reagent (ABC Elite; Vector Laboratories). Immunodetection was carried out by incubation with 3,3'-diaminobenzidine (Sigma). Slides were counterstained with thionin blue before dehydration and mounting.

Assessment of Mitosis and Apoptosis

Mitotic and apoptotic events within tumor epithelium and normal, uninvolved epithelium were assessed and recorded using well-established and previously reported morphological scoring techniques (33). For scoring of tumor cross-sections, the Zeiss Axiohome system allows a grid to be superimposed on the image and allows the user to place specific symbols (corresponding to specific cellular features, i.e., apoptosis, mitosis, label, and so forth) over each cell scored, which are then autotallyed by the computer.

Statistical Analysis

Statistical analysis was carried out using two-way ANOVA with respect to morphometric measurements. No significant interaction between variables was observed. For the analysis of cell kinetic parameters, three- or four-way ANOVA was used. Analysis was carried out using SPSS for Windows.

RESULTS

Morphometry

In total, 6967 tumors were measured [2922 from 16,16dmPGE2-treated animals (n = 12) and 4045 from control-treated animals (n = 10)]. In all three experiments, it was observed that, contrary to our expectations, mice treated with 16,16dmPGE2 had less tumor burden at 18 weeks of age, as judged by the proportion of the epithelial area that was covered in tumors (pT; Fig. 1A). The effect of 16,16dmPGE2 on tumor growth was observed in all regions of the small intestine and the mid-colon/rectum. The reduction in tumor burden was found to be due both to a decrease in the number of tumors observed (TP1K; Fig. 1B) and a reduction in tumor size (Fig. 1, C and D). A plot of tumor frequency, with tumors assigned to size bins with a 0.25-mm² increment, demonstrated tumor size to have a...
Poisson-like distribution, as demonstrated in Fig. 2. The proximal small intestine (duodenum) had the tumors with the largest area (largest tumor measured = 23.5 mm²), whereas the distal region (upper and lower ileum) had the highest frequency of tumors (approximately 35% of all tumors occurred in the upper ileum). There were very few small tumors in the mid-colon/rectum, hence the high average tumor size in this region (Fig. 1C). Some colorectal tumors had a different morphological appearance in comparison to the flat, mushroom-shaped adenomas of the small intestine and were spheroidal. Two mice in control groups from separate experiments were culled before they reached 18 weeks of age because they became moribund. They were found to have large, spheroidal tumors that appeared to have occluded the colon. None of the other mice displayed any outward signs of distress or discomfort during the course of the experiments. No tumors were recorded in the cecum.

**Cell Kinetic Studies**

Measurements of parameters such as thymidine labeling and apoptosis were performed to assess how 16,16dmPGE₂ might have been exerting its effect. From all of the mice used for the cell kinetics studies in all of the experiments [total, 12 (6 treated and 6 untreated mice)], a total of 177 individual tumors were analyzed (85 tumors from untreated animals and 92 tumors from treated animals). For this part of the study, we chose to examine the duodenum because it had the tumors with the largest areas and the upper ileum because it had the greatest numbers of tumors per unit area. The mid-colon rectum was not included in this analysis due to the low frequency of tumors within intestinal cross-sections (<3% of tumors occurred within this region). For this part of the analysis, tumors were assigned to three categories: (a) cystic tumors, small, ring-shaped tumors in the cross-section; (b) small tumors, tumors that are more “glandular” in appearance than cystic tumors but are compact, with a maximum cutoff size of 0.4 mm² in cross-section (as measured using the Zeiss Axiohome); and (c) large tumors, large tumors (>0.4 mm² cross-section) with “tubulovillous” and “glandular” morphology. Representative tumor sections are illustrated in Fig. 3, showing typical patterns of thymidine labeling and p21WAF-1/CIP1 immunoreactivity and high magnification views of apoptotic and mitotic cells.

**Thymidine Labeling.** It was observed consistently that tumors from treated mice contained a significantly higher percentage of cells demonstrating tritiated thymidine incorporation, i.e., in S phase. This was true for all tumor types in both the duodenum and upper ileum. Overall, the percentage of thymidine-incorporating cells increased from a mean of 9.6 ± 0.2% (mean ± SE) to 18.5 ± 0.4% in the duodenum and from 9.4 ± 3.4% to 13.5 ± 2.4% in the upper ileum. Data were analyzed using a three-way ANOVA, which showed that the effect of the treatment was significant at P = 0.003, with neither the region (P = 0.31) nor the tumor type (P = 0.69) having any significant effect on thymidine incorporation.

**Apoptosis.** The AI = (total number of apoptotic bodies)/(total number of nonapoptotic cells + total number of apoptotic bodies) × 100.

When tumors were assessed on a regional basis, AI was significantly increased by 16,16dmPGE₂ treatment in all tumor types of upper ileal origin (P = 0.041), as assessed by unpaired t-test assuming unequal variance; however, this was not observed in duodenal tumors, with only small tumors showing an increase in AI, and cystic and large tumors showing a decrease in AI (Fig. 5). Overall, there was no significant effect of treatment on the levels of apoptosis observed in duodenal tumors, as assessed in a three-way ANOVA (P = 0.59); however, there was a significant relationship between tumor type and
apoptosis ($P = 0.009$), with large tumors demonstrating a smaller percentage of apoptotic cells than either small or cystic tumors.

**Mitosis.** The MI = (total number of mitotic cells)/(total number of nonmitotic cells + total number of mitotic cells) × 100, where the total number of nonmitotic cells includes all apoptotic bodies.

As would be expected from the thymidine incorporation data, a moderate increase in mitosis was also observed in tumors of treated animals ([mean ± SE, 0.8 ± 0.1% (treated animals) versus 0.6 ± 0.1% (controls)]; however, the effect of 16,16dmPGE$_2$ on the levels of mitosis was nonsignificant, as assessed in a three-way ANOVA ($P = 0.34$). The fact that the increases observed are nonsignificant may be explained by the fact that there are few mitotic cells ($\leq$1% of total cells) and that there was great variability in the number of mitotic cells between the fields that were scored. Although the effect of the treatment was not significant, the analysis did show that levels of mitosis were significantly higher in upper ileal tumors (1 ± 0.1%) than in duodenal tumors (0.7 ± 0.1%; $P = 0.030$, Mann-Whitney U test).

**AI:MI.** The ratio of apoptosis to mitosis was calculated because it can give a better indication of any shift in the dynamic status of the tumors. The results obtained were essentially similar to those obtained for apoptosis alone. A consistent increase in the AI:MI ratio was observed in all upper ileal tumors (Fig. 6); no increase was observed in any duodenal tumor groups (data not shown).

**p21$^{\text{WAF-1/CIP1}}$ Labeling.** An increase in p21$^{\text{WAF-1/CIP1}}$-positive cells in small and large tumors was observed in treated animals (Fig. 7). In contrast, the number of p21$^{\text{WAF-1/CIP1}}$-positive cells in cystic tumors decreased in animals treated with 16,16dmPGE$_2$. ANOVA showed this relationship between type and p21$^{\text{WAF-1/CIP1}}$ labeling and treatment to be significant ($P = 0.042$).

**Effects on Normal Epithelium.** In parallel to its effect on tumor cells, 16,16dmPGE$_2$ also caused a modest increase in the number of crypt epithelial cells demonstrating thymidine incorporation (S-phase cells) in areas of normal epithelium showing no tumor involvement. Fig. 8 shows the frequency distribution of thymidine incorporation on a cell positional basis, with cell position 1 being the base of the crypt (see Ref. 33). Overall, the percentage incorporation increased from 16% to 21% in the duodenum and from 16.1% to 19.4% in the upper ileum in treated animals. We were unable to detect any significant changes in apoptosis or mitosis.

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**Fig. 4.** Tritiated thymidine incorporation in duodenal tumors (A) and upper ileal tumors (B) from animals receiving either vehicle control (○) or 16,16dmPGE$_2$ (■). Data were pooled from three separate experiments and analyzed by three-way ANOVA. Overall, 16,16dmPGE$_2$ treatment resulted in a significant increase in labeling ($P = 0.003$). N values on the X axis under the respective bars represent the number of tumors scored.

**Fig. 5.** AI (percentage of apoptosis) in duodenal tumors (A) and upper ileal tumors (B) from animals receiving either vehicle control (○) or 16,16dmPGE$_2$ (■). Data were pooled from three separate experiments and analyzed by three-way ANOVA. Overall, there was no significant effect of treatment on AI, although upper ileal tumors as a whole showed a significantly greater AI in treated animals ($P = 0.041$, Mann-Whitney U test). N values on the X axis under the respective bars represent the number of tumors scored.

**Fig. 6.** AI:MI ratio for upper ileal tumors from animals receiving either vehicle control (○) or 16,16dmPGE$_2$ (■). Data were pooled from three separate experiments and analyzed by three-way ANOVA. There was no significant effect of treatment on the AI:MI ratio. N values on the X axis under the respective bars represent the number of tumors scored.
The data obtained from this study demonstrate that exogenous PG administration can significantly inhibit GI tumor development using the Min/+ tumor model. This finding was unexpected and perhaps counterintuitive because previously published data suggested that PGE2, specifically PGE2, can act directly as growth and survival factors for normal, intestinal crypt epithelial cells (34) and intestinal tumor cells (30, 31). There has also been a large amount of work that has indirectly supported the role of PGE2, a product of COX-mediated arachidonic acid metabolism, in GI tumorigenesis. Elevated levels of mucosal PGE2 are associated with colon tumors in man (27, 28), and elevated levels of COX-2 are observed in primary human tumors, i.e., of COX-2 function through either pharmacological means (21) results in reduced tumorigenesis. In contrast, ablation (11–17). Diet-induced tumor promotion in murine models of intestinal disease is also associated with elevated levels of PGE2, a product of COX-mediated arachidonic acid metabolism, in GI tumorigenesis. Elevated levels of mucosal PGE2 are associated with colon tumors in man (27, 28), and elevated levels of COX-2 are observed in primary human tumors, colorectal tumor cell lines, and murine models of tumorigenesis (11–17). Diet-induced tumor promotion in murine models of intestinal disease is also associated with elevated levels of PGE2 (35). Ablation of COX-2 function through either pharmacological means [i.e., NSAIDs and selective COX-2 inhibitors (5, 6, 10, 24, 25)] or gene deletion of COX-2 (21) results in reduced tumorigenesis. In contrast, enforced expression of COX-2 in colorectal tumor cells results in enhanced metastatic potential associated with PG synthesis (20).

However, there is now a growing body of evidence that suggests that the role of COX-2 and PGE2 in malignancy may not be so simple. There are numerous reports that the antitumor effect of NSAIDs (including selective COX-2 inhibitors) is independent of their ability to inhibit PGE2 synthesis (8, 36–39). Also, COX-2 expression does not correlate with proliferative status or tumor stage in primary colorectal cancers (40), and the selective COX-2 inhibitor NS-398 has been shown to induce apoptosis in colorectal cell lines independently of their COX-2 expression status (41). COX-2 has even been hypothesized to be a tumor suppressor gene (42). In addition, nontransformed, immortalized cells derived from mice null for either COX-1 or COX-2 show up-regulation of the remaining gene, enhanced expression of cPLA2, and increased PGE2 synthesis (43). PGE2 has been reported to inhibit the proliferation of certain colorectal cancer cell lines (42). All of these data suggest that the effects resulting from the manipulation of COX function and PG biosynthesis depend on the experimental context.

The data presented here support a role for PGE2 in promoting cell proliferation, with increased thymidine incorporation observed in normal and tumor epithelium from all mice treated with 16,16dPGE2. Because 16,16dPGE2 also reduced the tumor burden, this increased proliferation must be viewed as reflecting an increase in overall cell turnover in the GI epithelium. For tumor burden to decrease in parallel to increased cell proliferation, cell loss through apoptosis or extrusion into the gut lumen must also increase. We were able to observe an increase in apoptosis in upper ileal tumors from treated animals; however, we were not able to demonstrate a significant increase in apoptosis between treated and untreated animals when pooled experimental data (for all regions) were analyzed as a whole. The failure to do so may be related to the intrinsic experimental difficulties in trying to quantify apoptotic events in the tumor cross-sections. The absolute numbers of apoptotic cells are small, and the number of apoptotic events that can be observed in tumor cross-sections is highly variable.

We have previously addressed the many difficulties related to the interpretation of AlIs (44). Some of the variability associated with the identification of the denominator cell population can be eliminated by calculating the ratio of apoptotic to mitotic cells (Al/Mi). An increase in Al/Mi was found consistently in all upper ileal tumors from treated animals, although the magnitude of the increase did not reach significance. In contrast, a decrease in Al/Mi was observed in cystic (in particular) and large duodenal tumors.

Although changes in the Al/Mi ratio may be small, they can have a profound influence. For example, in the small intestinal crypts, there are approximately 250 cells/crypt and 3500 cells/villus derived from just four to six individual stem cells (45). Therefore, each stem cell makes a substantial contribution, and the loss of just one (in the absence of homeostatic compensatory mechanisms) would have a significant effect on crypt and villus size. The same may be true for tumor stem cells and tumor size.

Another complication associated with assessing apoptosis is that the relationship between the absolute number of apoptotic events and the number of apoptotic bodies observed is unclear. We have demonstrated previously that different apoptotic stimuli can result in different degrees of apoptotic cell fragmentation in normal small intestinal crypt epithelial cells (Ref. 44 and the references therein). When scoring apoptosis in the Min/+ tumor cross-sections, one has to count the total number of apoptotic bodies because it cannot be said with certainty how many individual apoptotic events are represented by several adjacent apoptotic bodies. It is also possible that drug treatment could reduce the half-life of apoptotic cells (44); as we can only observe apoptosis in a single time frame, a false appreciation of the true frequency of apoptotic events would be gained. Only further experimentation will be able to address all of these questions.
cell proliferation in response to 16,16dmPGE$_2$. A parallel increase in the frequency of p21$^WAF-1$/CIP1 expression was also observed in treated animals. There are two plausible explanations for this result. Firstly, that 16,16dmPGE$_2$ is driving tumor cell proliferation and, subsequently, differentiation, given that p21$^WAF-1$/CIP1 expression in epithelial cell types is associated with the early stage of commitment to cell differentiation (49, 50). However, p21WAF-1/CIP1 expression was not observed within the differentiated villus epithelial cell population in “normal” epithelium of Min$^/+$ mice. Alternatively, 16,16dmPGE$_2$-driven proliferation may ultimately result in cells becoming senescent; p21$^WAF-1$/CIP1 expression is also associated with cellular senescence (51, 52).

It is reasonable to assume, given the previously published results from in vitro studies (30, 31, 34), that 16,16dmPGE$_2$-induced cell proliferation is mediated directly through the E prostaglandin receptor subtypes localized to the intestinal epithelial cells, i.e., EP2 and EP4 receptors (53, 54). However, we cannot rule out the fact that metabolites of 16,16dmPGE$_2$ may also contribute to the observed effects. 16,16dmPGE$_2$ undergoes extensive metabolism before elimination. The parent compound shows biphasic clearance from the plasma with an initial half-life of 4.5 min and a subsequent half-life of 20–30 min, although it takes 24 h to eliminate approximately 50% of the radioactivity of a single labeled dose from the plasma (55, 56). Metabolism occurs via $\alpha$-oxidation and $\beta$-oxidation, giving rise to a variety of compounds, of which the dinor and tetranor metabolites are the most abundant (55, 56). We have found no literature concerning the action of these compounds in any epithelial cell systems.

It is possible that the antitumor effect of 16,16dmPGE$_2$ is mediated through a mechanism other than binding to the EP receptors on the epithelial cells. Certain prostanoids are known to be ligands for the PPAR family (57–59). Within the intestinal epithelium, two PPAR subtypes are expressed, PPAR-$\gamma$ (colon > small intestine) and PPAR-$\alpha$ (small intestine > colon; Ref. 60). Recently, PPAR-$\gamma$ agonists were demonstrated to promote tumor formation in Min$^/+ $ mice.
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