Malignant transformation of the esophageal mucosa is enhanced in p27 knockout mice

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Objective: In a previous study, we showed that experimentally induced gastroduodenal-esophageal reflux in mice treated with a carcinogen can result in Barrett esophagus and Barrett-associated adenocarcinoma. Since we have shown that most Barrett-associated adenocarcinomas in human beings have lost the tumor suppressor gene p27, we sought to determine whether cancer would be more likely to develop in p27 knockout mice than in p27 heterozygous or p27 wild type mice.

Methods: Three groups of mice were treated by esophagojejunostomy resulting in gastroduodenal-esophageal reflux and by a carcinogen (*N*-methyl-*N*-benzyl nitrosamine): group I (50 wild type), group II (45 p27 heterozygous), and group III (50 p27 knockout). The mice were killed 18 to 20 weeks after operation and studied macroscopically and histopathologically.

Results: Barrett esophagus developed in 7 (14%) mice in group I, 4 (8.9%) mice in group II, and 13 (26%) mice in group III. Cancers developed in 30 (60%) mice in group I, 31 (68%) mice in group II, and 43 (86%) mice in group III. Ten percent of the cancers in group I were adenocarcinomas, as were 16.1% in group II, and 23.3% in group III. The difference between rates of Barrett esophagus in groups I and II compared with group III was statistically significant ($P = .035$), as was true of the cancer rates ($P = .006$). The percentage of cancers that were adenocarcinomas was highest in group III, but not significantly different from groups I and II.

Conclusions: This experimental mouse model of Barrett esophagus and Barrett-associated adenocarcinoma is similar to what occurs in human beings and may be useful in developing methods to inhibit malignant transformation of Barrett esophagus.

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The rising prevalence of adenocarcinoma of the esophagus in the Western world is a widely recognized fact.¹⁻³ The development of metaplastic columnar epithelium of the esophagus, or Barrett esophagus (BE), is thought to be the result of reflux of acid and alkaline secretions into the esophagus. This metaplastic epithelium is susceptible to malignant transformation resulting in Barrett-associated adenocarcinoma (BAA). An additional source of this metaplastic epithelium may be from short tongues of intestinal-type epithelium in the region of the cardia, leading to the suggestion by Cameron and associates⁴ that 21% to 79% of cancers classified as cancers of the cardia may actually originate from short segments of Barrett epithelium.

The cyclin-dependent kinase inhibitor p27 acts as a negative regulator of the cell division cycle, regulating progression from the G1 to the S phase of the cell cycle. In a study of 58 resected specimens of BAA, using immunohistochemistry and in situ

hybridization, we found that loss of p27 expression occurred in most cases of BAA and was associated with a poor prognosis,⁵ as is the case in a variety of other tumors.⁶ Low p27 expression also correlated with metastases to the lymph nodes, depth of tumor invasion, and decreased survival.

Recently, BE and BAA have been produced experimentally in the rat by various surgical maneuvers designed to promote gastroduodenal-esophageal reflux.^{7,8} The availability of transgenic and knockout technology for manipulating gene function in the mouse model⁹ led us to attempt to duplicate in the mouse the results obtained in the rat model. We found that gastroduodenal-esophageal reflux produced by esophagojejunostomy plus administration of the carcinogen *N*-methyl-*N*-benzyl nitrosamine (MBN; Ash-Stevens, Inc, Detroit, Mich) resulted in BE, BAA, or both in 57.1% of the mice, a finding similar to that in the rat.¹⁰

The purpose of the present study was to determine whether a genetically altered mouse lacking the p27 gene is more susceptible to malignant transformation of the esophageal mucosal lining than is a mouse of the wild type with normal genetic structure.

Materials and Methods

Animals

Fifty Swiss-Webster mice with a normal genetic makeup (wild type) obtained from Taconic Farms, Inc (Germantown, NY), were used in this study. Two genetically altered male p27 knockout (KO) mice were obtained from The Fred Hutchinson Cancer Research Center, Seattle, Washington, and were used to establish a colony of p27 KO mice by mating them with p27 wild type female mice. From the offspring of these matings, a cohort of hybrids was selected: 45 p27 +/- heterozygous mice and 50 p27 -/- KO mice, the phenotypes of which were determined by polymerase chain reaction¹¹ performed on tail biopsy specimens.

The p27 KO mice were confirmed by polymerase chain reaction amplification (94°C for 30 seconds, 56°C for 45 seconds, 72°C for 2 minutes) of the unique mutant transgene with primers: K3: 5'-TGG AAC CCT GTG CCA TCT CTA T-3' and N1: 5'-CCT TCT ATG GCC TTC TTG ACG-3', obtaining a 0.5-kilobase (kb) band. The wild type phenotype was identified by polymerase chain reaction amplification of a 1.0-kb band with primers K3 (see above) and K5: 5'-GAG CAG ACG CCC AAG AAG C-3'. The heterozygous phenotype carried both the 1.0-kb and the 0.5-kb bands when run on 1% agarose gel.

The animals were housed, one animal per cage, in an environment with a 12-hour light/dark cycle, at a temperature of 70°F with a humidity of 41% ± 4%. The mice were fed commercial mouse chow with water provided ad libitum. Solid food was withdrawn 24 hours before surgery, which was performed approximately 8 weeks after birth. Esophagojejunostomy was performed under general anesthesia with intraperitoneally administered sodium pentobarbital at a dose of 18.0 mg/kg. This study protocol was approved by the Standard Committee on Animals of Harvard Medical School, and animals were cared for according to their recommendations, which are in compliance with the guidelines of the Institute of Laboratory Animal Resources, National Research Council.

Surgical Procedure

The experimental procedure used was performed when the mice were 8 weeks old and was designed to result in gastroduodenal-esophageal reflux of gastric, bile, and pancreatic juices.¹² This intervention was accomplished through a midline abdominal incision with division of the esophagus immediately proximal to the esophagogastric junction, which was closed with a through-and-through suture of 6-0 polypropylene. An ellipse of anterior wall of jejunum was excised a few millimeters distal to the ligament of Treitz. The distal esophagus was then anastomosed to the jejunum at the enterostomy site with 4 to 6 interrupted through-and-through 7-0 polypropylene sutures. The animals were given water 2 hours after the operation and mouse chow the next day.

Carcinogen

MBN, a carcinogen with a predilection for inducing esophageal cancer, was the agent used in the animals scheduled to receive carcinogen.¹³ Treatment began 1 week after the operation. The MBN was diluted in a saline solution and given by intraperitoneal injection at a dose of 2.5 mg/kg. Injections were continued once a week for the duration of the experiment.

Experimental Groups

The animals were divided into 3 separate groups. Group I consisted of 50 +/- wild type mice that underwent esophagojejunostomy plus administration of the carcinogen. Group II consisted of 45 p27 +/- heterozygous mice that also underwent esophagojejunostomy and received the carcinogen, as did 50 p27 -/- KO mice in group III.

Tissue Preparation and Histopathologic Analysis

Eighteen to 20 weeks after the operation, the mice were killed with an overdose of pentobarbital. The entire esophagus of each mouse was removed, including the esophagojejunostomy site. The jejunum was opened longitudinally on its mesenteric border, and the patency of the anastomosis was determined. The esophagus was then opened longitudinally and examined macroscopically to determine the presence or absence of esophagitis, tumor, or both. The specimen was then photographed and prepared for histopathologic study.

Tissues were fixed in 10% formalin solution for 8 hours and embedded longitudinally in paraffin. Next, 5-μm sections were cut along a longitudinal axis and stained with hematoxylin and eosin. Slides were reviewed without knowledge of treatment group assignment. Three sections of each esophagojejunostomy specimen were examined. When the anastomosis was not seen, deeper sections were obtained until the region of the anastomosis was identified. Barrett-type mucosa was defined as intestinal-type mucosa with goblet cell metaplasia present proximal to the anastomosis and bound on both the proximal and distal ends by squamous mucosa, stricter criteria than are used in human specimens. Barrett epithelium was thus unequivocally distinguishable from the mucosa of the small bowel (with prominent villi) to which the esophagus was anastomosed. Presence or absence of Barrett-type epithelium was recorded. No examples of high-grade dysplasia of Barrett epithelium were seen. High-grade dysplasia/carcinoma in situ of the squamous mucosa was defined by full-thickness, high-grade squamous cell dysplasia without surface maturation, in the absence of submucosal invasion. Invasive carcinomas were classified as squamous cell carcinomas, adenocarcinomas, or mixed

Figure 1. Postmortem specimen from a p27 KO mouse after esophagojejunostomy and administration of a carcinogen. Note dilated esophagus proximal to esophagojejunostomy where a tumor (arrow) is clearly visible.

adenosquamous carcinomas. Both high-grade dysplasia/carcinoma in situ and invasive carcinomas were included in calculating the cancer rate of this experimental model.

Results

All 145 mice survived the treatment schedule and were available for study 18 to 20 weeks after the operation. Dilatation of the distal esophagus of varying degree just proximal to the anastomotic site was present in all specimens, presumably related to moderate obstruction at the anastomotic site (Figure 1). Macroscopic evidence suggesting esophagitis consisted of redness of the mucosa, irregularity of surface epithelium, or thickness of the esophageal wall with or without ulceration (Figure 2). Some or all of these changes were noted in 28 (56%) of the 50 mice in group I, 35 of which have been reported on previously,¹⁰ as well as in 13 (28.9%) mice in group II and 18 (36%) mice in group III. Macroscopic evidence suggestive of neoplasm was noted in 25 (50%) mice in group I, 32 (71.1%) mice in group II, and 42 (84%) mice in group III. The tumors, for the most part, were located at or near the site of esophago-

Figure 2. Postmortem specimen from a p27 KO mouse showing transition (arrow) from pale squamous epithelium to pink epithelium of Barrett esophagus. While not visible in the gross specimen, islands of squamous mucosa were present at the anastomotic site.

jejunostomy. Four mice in group III had macroscopic evidence of 2 tumors. In each case, a more proximally located lesion was found in addition to that in the vicinity of the anastomotic site (Figure 3). No tumors were identified elsewhere in the body of any mouse.

The histopathologic findings are summarized in Table 1. Because of the similarities of results in mice of groups I and II, these results have been combined for purposes of statistical analysis. The rates of BE and of cancer developing in this model, not the rate of mice in which these changes developed, was determined. BE (Figure 4, A) was identified in 7 (14%) mice in group I (associated with cancer in 5 mice), 4 (8.9%) mice in group II (associated with cancer in 1 mouse), and 13 (26%) mice in group III (associated with cancer in 8 mice). None of the BE observed was associated with suture material. The rate of BE in groups I and II as compared with that of group III was significantly different ($P = .035$; Fisher exact test). Cancers were identified in 30 (60.0%) mice in group I, 31 (68.9%) mice in group II, and 43 (86%) mice in group III.

Figure 3. Postmortem specimen of a p27 KO mouse after esophagojejunostomy and administration of a carcinogen. Two tumors are visible (arrows). The distal tumor was an adenocarcinoma, and the more proximal tumor was a squamous cell cancer.

The difference in the cancer rates in groups I and II as compared with that in group III was highly significant ($P = 0.006$; Fisher exact test).

The number of cancers that occurred among the 3 groups of experiments (109 cancers) exceeds that of the mice in which they developed (104 mice) because 2 cancers developed in 5 mice, all in group III (Figure 3). Four of these mice had an adenocarcinoma as well as a squamous cell cancer, and another had an adenosquamous cancer as well as a separate squamous cell cancer. Only 4 of these multiple cancers were identifiable macroscopically, however.

Most cancers were of the squamous cell type (66 cancers) and predominated in the mice of group III (31 cancers) (Figure 4, B), but 18 cancers were adenocarcinomas (Figure 4, C) and 25 cancers were adenosquamous cancers (Figure 4, D). Adenocarcinomas accounted for 10% of the cancers in group I, 16.1% in group II, and 23.3% in group III. Although the difference in the rates between groups I and II compared with group III (13.1% compared with 23.3%) was not significant, the trend is highly suggestive.

All cancers arose in the mucosa and were often ulcerated. Of the invasive tumors, 20% were well differentiated, 63% were moderately differentiated, and 5% were poorly differentiated. Twelve percent were in situ squamous cell carcinomas (invasive only into the lamina propria) and were not graded for differentiation.

When depth of invasion was assessed, 55% were invasive into the submucosa, 19% into the muscularis propria, and 14% invaded through the serosa into adjacent structures, usually the liver. Remarkably, all but one of the cancers that invaded through the serosa were either adenocarcinomas or adenosquamous carcinomas. This is particularly important in this model of BAA. Once again, 12% were only invasive into the lamina propria.

Sixty-two percent of cancers were located at or near the anastomosis (within one intermediate power field by light microscopy—20 $\times$) and 43% were proximal, most of which were squamous cancers. The total exceeds 100% because 2 cancers developed in 5 mice.

Discussion

Decreased expression of the tumor suppressor gene p27 has been identified in a number of human cancers, including cancers of the breast,¹⁴ colorectum,¹⁵ lung,¹⁶ and stomach,¹⁷ and has been associated with an aggressive behavior and a poor prognosis. Our group was the first to identify a similar phenomenon in resected BAA.⁵ Subsequently, others have reported similar findings in cases of BAA¹⁸ and squamous cell cancers of the esophagus.¹⁹ We have also shown that in the colon,¹⁵ lung,¹⁶ and prostate (unpublished data, M.L.), the reduced expression of the tumor suppressor gene p27 is the result of increased proteasome-mediated degradation.

Recently, the availability of p27 KO mice has permitted animal research regarding the role of p27 in the development of malignant diseases. Nakayama and associates²⁰ showed that p27 KO mice exhibited only hyperplasia resulting in increased body size, multiple organ dysplasia, and pituitary tumors, which was similar to the findings of Fero and associates.¹¹ However, when Fero and associates²¹ challenged both p27 KO and p27 heterozygous mice with irradiation and a chemical carcinogen, tumors developed in multiple tissues, although the mortality rate was higher in the KO mice than in heterozygous mice.

The results we are reporting are similar in some respects to those of Fero and associates.²¹ The importance of a carcinogen is clear from our studies, as evidenced by the high cancer rate in group III mice. In another group of p27 KO mice (not reported on here) that had esophagojejunostomy without MBN, the cancer rate was only 29.2%, and the cancers were not adenocarcinomas. In a previous study of ours,¹⁰ the importance of gastroduodenal reflux in addition to the use of a carcinogen in the development of a BE and BAA was clearly evident. BE rarely developed in wild type

Figure 4. Hematoxylin and eosin stains of representative pathologic entities in p27 knockout mice. **A,** Metaplastic Barrett epithelium present within esophageal squamous mucosa. **B,** Invasive squamous cell carcinoma. **C,** Invasive adenocarcinoma. **D,** Invasive adenosquamous carcinoma. (Original magnification $\times 400$.)

TABLE 1. Histopathologic findings

Finding	Group I (n = 50)	Group II (n = 45)	Group III (n = 50)	P value, groups I and II vs group III
Barrett esophagus	7 (14%)	4 (8.8%)	13 (26%)	.035
Cancer	30 (60.0%)	31 (68.9%)	43 (86%)	.006
Squamous cell	15	20	31*	
Adenosquamous	12	6	7*	
Adenocarcinoma	3 (10%)†	5 (16.1%)†	10* (23.3%)†	NS

Group I, Esophagojejunostomy + carcinogen, p27 wild type; group II, esophagojejunostomy + carcinogen, p27 heterozygous; group III, esophagojejunostomy + carcinogen, p27 KO; KO, knockout; n, number of mice; NS, not significant.

*The sum of these numbers is greater than 43 because 5 mice had 2 cancers (see text).

†Percent of total cancers.

mice given only a carcinogen, and adenocarcinoma developed in none of them.

In contrast to the results reported by Fero and associates,²¹ no deaths occurred among our mice, but they were killed only 4 to 5 months after treatment, whereas those of Fero and associates²¹ were followed up much longer. In addition, no tumors developed elsewhere in the body in our mice, proba-

bly as a result of the fact that the carcinogen (MBN) that we used has a predilection for inducing esophageal cancer. In contrast to their study, we did not find a significantly higher cancer rate in the heterozygous mice (group II), although this may be due to the limited sample size.

The high cancer rate in p27 KO mice that underwent esophagojejunostomy and administration of MBN (group III)

was highly significant ($P = .006$) when compared with the cancer rate in mice in groups I and II. The percentage of cancers that were adenocarcinomas was higher in this group (23.3%) and, although not significantly so, certainly exhibited a trend in that direction. Because it is generally accepted that adenocarcinomas of the esophagus in human beings are BE related, the rate of adenocarcinomas in group III mice might have been even higher had these mice been killed after a longer postoperative interval, particularly in view of the higher rate of BE in mice of this group. Why the rate of BE was highest among mice in group III is not clear, but it is possible that p27 may be involved in some fashion with epithelial protection.²² The strict criteria we used in the definition of BE, namely metaplastic epithelium bounded proximally and distally by squamous epithelium, might have underestimated somewhat its prevalence in the 3 arms of this study.

In conclusion, the experimental production of gastroduodenal-esophageal reflux in p27 KO mice treated with MBN results in a high rate of BE and of cancer. Interestingly, the rate of adenocarcinomas in this group was higher than that of adenocarcinomas in p27 wild type and p27 heterozygous mice, although the difference was not quite statistically significant, probably because of the limited sample size. All the same, some of the mice in group III had pathologic changes similar in some respects to the disease that occurs in some human beings. This model, therefore, may be useful in developing methods to inhibit malignant transformation of BE to BAA. The high rate of squamous cell cancers suggests that this model could also be valuable in studying this form of esophageal cancer. However, the addition of experimental gastroduodenal-esophageal reflux to administration of carcinogen would be unnecessary since in an earlier study of ours,¹⁰ 11 of 15 (73%) cancers developing in wild type mice treated only with a carcinogen were squamous cell cancers of the esophagus, and none was adenocarcinoma.

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