Changes in gastric endocrine cells in Balb/c mice bearing CT-26 carcinoma cells: an immunohistochemical study

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The distribution and density of gastric endocrine cells in Balb/c mice bearing CT-26 carcinoma cells were studied immunohistochemically employing specific antisera against serotonin, somatostatin, glucagon, gastrin, cholecystokinin (CCK)-8 and human pancreatic polypeptide (hPP). The animals were divided into two groups, a non-implanted sham group and a CT-26 carcinoma cell-implanted group. Samples were collected from two regions of the stomach (fundus and pylorus) at 28 days after implantation of the medium or the CT-26 cells (1×10^5 cells/mouse). Five of the 6 types of immunoreactive (IR) cells were identified, with only the hPP IR cells not being detected. The regional distribution of the gastric endocrine cells in the CT-26 implanted group was similar to that of the non-implanted sham group. However, the endocrine cells were significantly decreased in the CT-26-implanted group as compared to those of the non-implanted sham group. Serotonin- and somatostatin-IR cells in the fundus and pylorus , and gastrin- and CCK-8-IR cells in the pylorus of the CT-26 implanted groups were significantly decreased compared to those of the sham group. In addition, glucagon-IR cells were restricted only to the fundus of the sham animals. hPP-IR cells were not detected in either the T-26 implanted- or the non-implanted group. Since endocrine cells are the anatomical units responsible for the production of gut hormones, a change in their density may reflect a change in their capacity to produce such hormones. Implantation of the tumor cell mass induced severe quantitative changes in gastric endocrine cell density, an abnormality which may contribute to the development of gastrointestinal symptoms, such as anorexia and indigestion, frequently encountered in cancer patients.

Key words: Balb/c mouse; colon-26; implantation; gastric endocrine cells; immunohistochemistry.

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Gastrointestinal (GI) endocrine cells dispersed in the epithelia and gastric glands of the digestive tract synthesize various kinds of GI hormones and play an important role in the physiological functions of the alimentary tract. The investigation of GI endocrine cells is considered to be an important part of phylogenic studies (D'Este *et al.,* 1994) and the endocrine cells are regarded as the anatomical units responsible for the production of gut hormones; a change in their density would reflect a change in the capacity of producing such hormones (El-Salhy and Sitohy, 2001). Many studies have elucidated the regional distribution and relative density of different endocrine cells in the GI tract of various vertebrates. Extensive research on GI endocrine cells in mouse strains has been performed, including that on the normal Balb/c mouse (Ku *et al.,* 2004). The changes in GI endocrine cells in some diseases are also well demonstrated. An abnormal density of GI endocrine cells has been reported in patients with diabetes (El-Salhy and Sitohy, 2001), and proliferation of endocrine cells in the epithelium has been detected in pernicious anemia (Rode *et al.,* 1986). Some quantitative changes in endocrine cells have also been demonstrated in celiac sprue (Buchan *et al.,* 1984) and after pancreatectomy (Ravazzola *et al.,* 1977). In addition, changes of endocrine cells in colorectal carcinoma induced by chemicals has been demonstrated (Sitohy and El-Salhy, 2001).

Komurcu *et al.,* (2002) reported that dry mouth, weight loss, early satiety, taste changes, constipation, anorexia, bloating, nausea, abdominal pain and vomiting are the ten most common GI symptoms in patients with lung, breast and prostate cancer. In changes in the GI endocrine cells with tumor, most reports were related to the induction of GI endocrine tumor by chemicals (Solcia *et al.,* 1995; Wangberg *et al.,* 1995) or diseases (Sjoblom *et al.,* 1989). In addition, the composition, number and type of endocrine cells in the endocrine tumors

located in the GI tract are well recognized (Shimamoto *et al.,* 1983; Iwafuchi *et al.,* 1986; Yasui *et al.,* 1986).

Although nearly one-half of the most frequently reported and most distressing symptoms in cancer patients are GI in nature (Komurcu *et al.,* 2002), studies on changes in GI endocrine cells have been restricted to regions of endocrine carcinoid tissues or non-neoplastic mucosa around the carcinoids. In addition, there has been no report dealing with changes of GI endocrine cell profiles after subcutaneous implantation of tumor cells except for 3LL cells (Ku *et al.,* 2005a,b), in which most of the GI endocrine cells, especially those related to GI motility, were markedly decreased after implantation of the 3LL cells. In addition, a marked decrease in chromogranin-IR cells, a common marker for endocrine cells (Cohn *et al.,* 1984), was detected after implantation of CT-26 cells (Ham *et al.,* 2005). However, there have been no reports revealing changes in individual GI endocrine cells in the stomach regions of Balb/c mice after subcutaneous implantation of CT-26 carcinoma cells of GI origin.

The object of this study was to clarify the changes of the individual endocrine cells in the stomach regions of Balb/c mice after subcutaneous implantation of CT-26 cells by specific immunohistochemistry employing antisera against serotonin, somatostatin, glucagon, gastrin, cholecystokinin (CCK)-8 and human pancreatic polypeptide (hPP).

Materials and Methods

Experimental animals

Twenty adult female Balb/c mice (6 weeks old, 21-26g b.w. upon receipt) were purchased from the Charles River Laboratories (Yokohama, Japan) and used in this study after acclimatization for one week. Animals were allocated 5 per autoclaved filter-top cage (Nalgene, Rochester, NY, USA) in a temperature- (20-25°C) and humidity- (50-55%) controlled room. Light:dark cycle was 12hr:12hr and sterilized feed (Samyang, Korea) and autoclaved water were supplied *ad libitum*. Animals were divided into two groups of 10 mice each: a CT-26 carcinoma cell implanted group and a non-implanted sham group. The experimental protocols were conducted in accordance with internationally-accepted principles for laboratory animal use and care as found in the Korea Food and Drug Administration guidelines.

Implantation of CT-26 cells

CT-26 cells were maintained as subcutaneous tumor mass. The subcutaneous tumor mass was excised under sterile conditions and single cell suspensions were prepared by collagenase type IV and Dnase I (Sigma, USA) in PBS (phosphate-buffered saline) followed by filtration of the resulting tumor cell suspension through a cell strainer (Costar, USA). After counting and adjusting the cell number (1×10^5 cells/mouse), the viable CT-26 cells were implanted under abdominal skin. In the non-implanted sham group, PBS only was injected s.c. at the same site.

Sampling

On the 27th day after implantation, the animals were fasted for around 24 hr. On the 28th day, the animals were anesthetized with diethyl ether and phlebotomized. Samples from two regions of the stomach, the fundus and pylorus, were removed and fixed in Bouin's solution.

Histology

After paraffin embedding, 3-4 μm serial sections were prepared. Representative sections of each tissue were stained with hematoxylin and eosin for light microscopic examination of the normal gastric architecture.

Immunohistochemistry

The representative sections were deparaffinized, rehydrated and immunostained with the peroxidase anti-peroxidase (PAP) method (Sternberger, 1979). The sections were incubated with normal goat serum for 1 hr at room temperature to block non-specific reactions.

After rinsing 3 times in 0.01M PBS, pH 7.4, the sections were incubated with the specific primary antisera listed in Table 1 for 18 hr at 4°C. After rinsing in PBS, the sections were incubated in secondary antiserum for 1 hr at room temperature. They were then washed in PBS buffer and incubated with the PAP complex for 1 hr at room temperature. The peroxidase reaction was carried out in a solution of 3,3'-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2 in 0.05M Tris-HCl buffer, pH 7.6. After immunostaining, the sections were lightly counterstained with Mayer's hematoxylin and the IR cells were observed under the light microscope.

Table 1. Antisera used in this study.

Antisera raised	*Code*	*Source*	*Diluton*
Serotonin	B068082C	BioGenex Lab., San Ramon, CA, USA	1 : 20
Somatostatin	PU0421295	BioGenex Lab., San Ramon, CA, USA	1 : 20
Glucagon	927604	Dia Sorin, Stillwater, MN, USA	1 : 2,000
Gastrin	PU0190796	BioGenex Lab., San Ramon, CA, USA	1 : 20
CCK-8	750257	Dia Sorin, Stillwater, MN, USA	1 : 500
hPP	A610	DAKO Corp., Carpinteria, CA, USA	1 : 600

The antisera were raised in rabbits; CCK-8, cholecystokinin-8; hPP, human pancreatic polypeptide.

Table 2. Regional distribution and density of the endocrine cells in the stomach of Balb/c mice with and without implantation of CT-26.

Antiserum	*Non-implanted sham group*		*CT-26 implanted group*	
	Fundus	*Pylorus*	*Fundus*	*Pylorus*
Serotonin	10.10±1.85	23.00±5.93	3.40±1.07*	11.90 ± 4.25*
Somatostatin	3.80±1.75	20.60±6.20	1.40±0.70*	2.70 ± 2.54*
Gastrin	ND [1]	50.50±11.10	ND	9.90 ± 4.58*
CCK-8	ND	39.80±9.19	ND	8.50 ± 2.51*
Glucagon	1.10±0.32	ND	ND	ND
hPP	ND	ND	ND	ND

*Mean ± SD, Number of IR cells among 1000 epithelial and gastric acinar cells (1 field in each animal, total 10 fields); ND, not detected; hPP, human pancreatic polypeptide; CCK-8, cholecystokinin-8; *p<0.01 compared to the non-implanted sham group by the MW test.*

The specificity of each immunohistochemical reaction was determined as recommended by Sternberger (1979), including the replacement of specific antiserum by the same antiserum previously preincubated with its corresponding antigen.

Frequency

IR cells showing immunoreactivities against each antiserum were counted in the restricted view fields on a computer monitor using an automated image analysis process (Soft Image System, Germany) coupled to light microscopy; at least 1000 epithelial and gastric acinar cells per slide were considered. In each animal, the density of IR cells were calculated as mean ± standard deviation (S.D.) of total 10 fields per each gastric region.

Statistical analysis

The Mann-Whitney U-Wilcoxon Rank Sum W test (MW test) and SPSS for Windows program (Release 6.1.3, SPSS Inc., USA) were used for the determination of statistical significance of the data.

Results

No immunoreactivity was detected using antisera previously preincubated with their corresponding antigen. In this study, five types of IR endocrine cells were detected, i.e. against serotonin, somatostatin, glucagon, gastrin and CCK-8 in the stomach of Balb/c mice. hPP-IR cells were not detected in the stomach in this study.

Serotonin- and somatostatin-IR cells were identified in the fundus and pylorus of both the CT-26 implanted- and sham groups, while gastrin- and CCK-8-IR cells were found only in the pylorus of both the CT-26 implanted- and sham groups. On the other hand, glucagon-IR cells were restricted to the fundus of the non-implanted sham group. According to the location of the stomach, different regional distributions and densities of these IR cells were observed as shown in Table 2. Most of the IR cells in the epithelial regions were generally spherical or spindle- shaped, while occasionally rounded cells were found in the gastric gland regions.

Serotonin-IR cells

Serotonin-IR cells were observed in the fundus and pylorus of the sham and CT-26 implanted groups. Serotonin-IR cells were dispersed throughout the gastric mucosa of the fundus but were mainly located in the basal portion of the gastric mucosa of the pylorus, in both the sham and implanted groups. More numerous cells were detected in the pylorus compared to that of the fundus in both groups (Figure 1). The number of cells in the fundus of the CT-26 implanted group was decreased, by 66.3% ($p<0.01$), with respect to the non-implanted sham group. The density of serotonin-IR cells in the pylorus of the CT-26 implanted group was also decreased (by 46.3%, $p<0.01$), as compared to that of non-implanted sham group (Table 2).

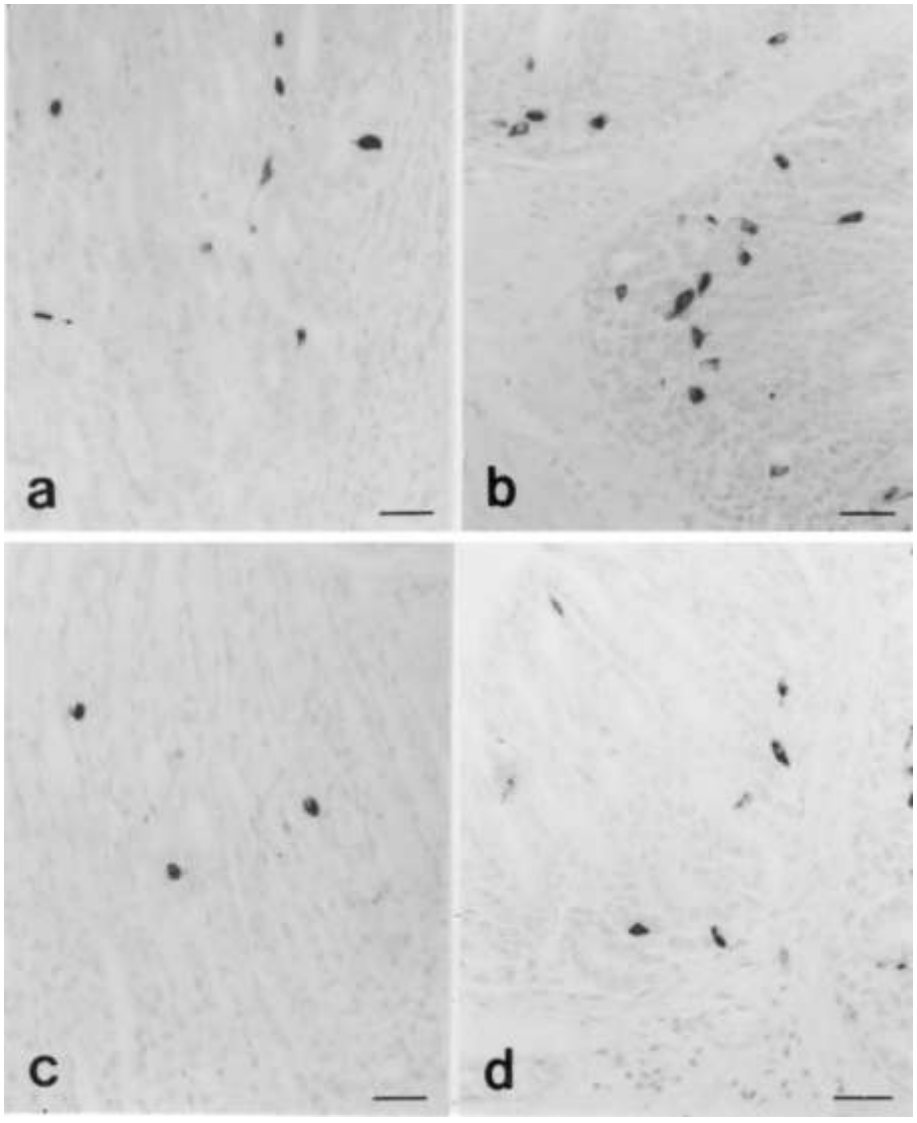

Figure 1. Serotonin-IR cells in the fundus a, c) and pylorus b, d) of the non-implanted sham group a, b) and the CT-26 implanted group c, d). In the CT-26 implanted group, serotonin-IR cells were strongly decreased in the fundus and pylorus. Scale bars = 33 μm; PAP methods.

Somatostatin-IR cells. Somatostatin-IR cells were observed in the fundic and pyloric regions of both non-implanted sham and CT-26 implanted groups. The cells were dispersed in the gastric mucosa, and were more numerous in the pylorus regardless of implantation (Figure 2). The number of cells in the fundus of the CT-26 implanted group was significantly ($p<0.01$) lower than that in the sham group by 63.16%. The density of somatostatin-IR cells in the pylorus of the CT-26 implanted group was also lower than that of the sham group by 86.89% ($p<0.01$) (Table 2).

Glucagon-IR cells

A few glucagon-IR cells were found in the fundus of the sham group (Figure 3), but were not observed in the CT-26 implanted group (Table 2).

HPP-IR cells

No hPP-IR cells were observed in the gastric regions of either the CT-26 implanted or non-implanted groups (Table 2).

Gastrin-IR cells

Gastrin-IR cells were restricted to the pyloric regions in both groups. Most of these IR cells were dispersed in the basal portion of the gastric mucosa of the pylorus, regardless of implantation of CT-26 cells or not (Figure 4). The density of gastrin-IR cells in the CT-26 implanted group was lower than that of non-implanted group by 80.55% ($p<0.01$) (Table 2).

CCK-8-IR cells

CCK-8-IR cells were restricted to the pylorus of

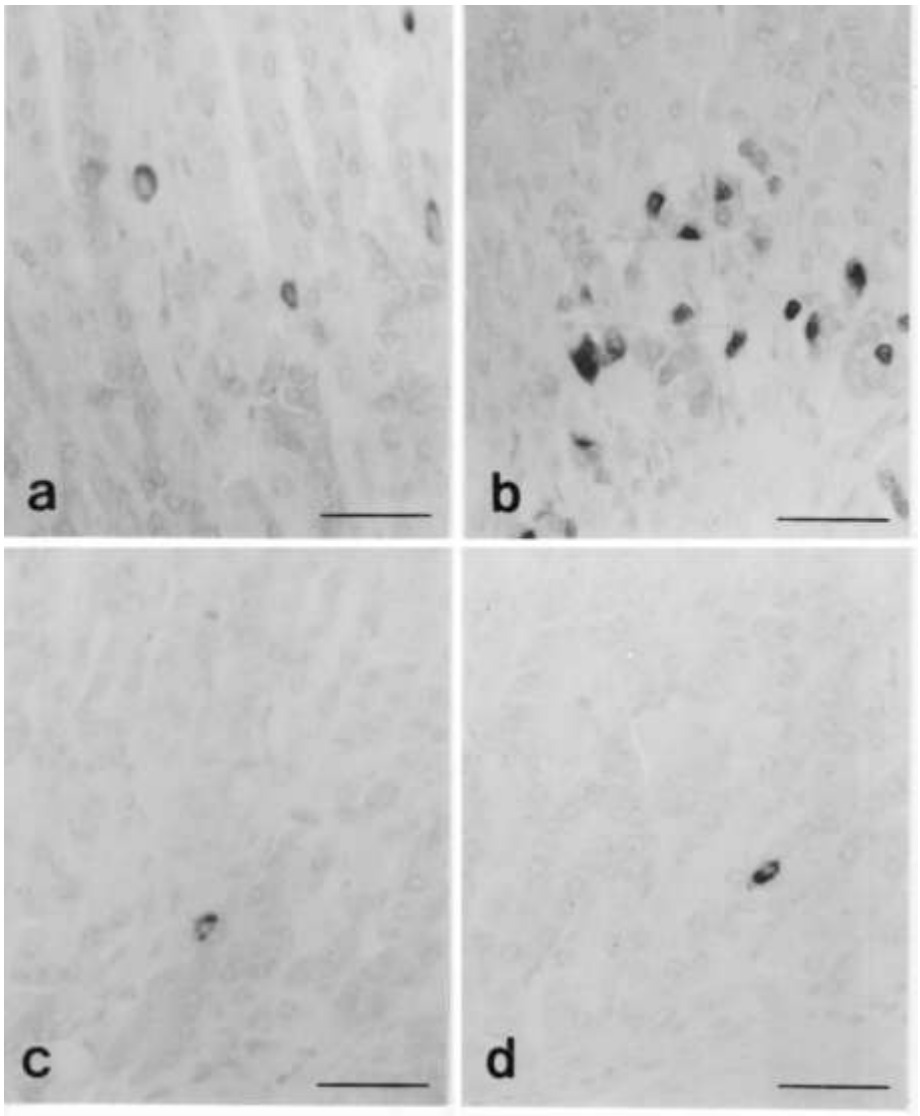

Figure 2. Somatostatin-IR cells in the fundus a, c) and pylorus b, d) of the non-implanted sham group a, b) and the CT-26 implanted group c, d). In the CT-26 implanted group, somatostatin-IR cells were strongly decreased in the fundus and pylorus. Scale bars = 33 μm; PAP methods.

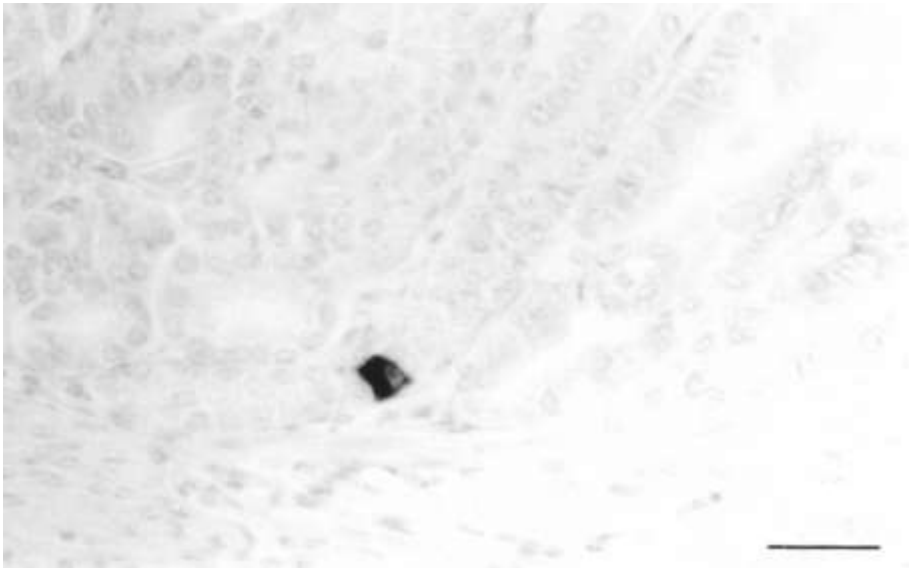

Figure 3. Glucagon-IR cells in the fundus of the non-implanted sham group. These cells were not detected in the CT-26 implanted group. Scale bars = 33 μm; PAP methods.

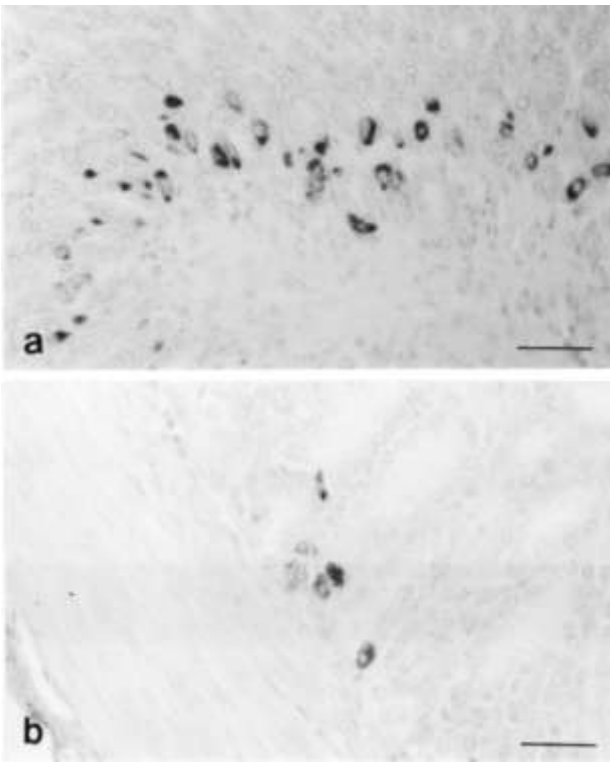

Figure 4. Gastrin-IR cells in the pylorus of the non-implanted sham group a) and the CT-26 implanted group b). In the CT-26 implanted group, gastrin-IR cells were strongly decreased. Scale bars = 33 μm; PAP methods.

both groups. Most of the IR cells were dispersed in the basal portion of the gastric mucosa of the pylorus, regardless of implantation or not of CT-26 (Figure 5). The number of CCK-8-IR cells in the CT-26 implanted group was lower than that of the sham group by 78.64% ($p<0.01$) (Table 2).

Discussion

CT-26 cells are rectal carcinoma cells and are established by administrating NMO to Balb/c mice. It is transplantable to Balb/c mice and is one of the most widely used types of tumor cells for the study of antitumor agents (DeWys, 1972; Moon *et al.,* 1999).

In the present study, the changes of the endocrine cells in the stomach regions of Balb/c mice bearing CT-26 murine carcinoma cells were followed by specific immunohistochemistry. The distribution and frequency of the endocrine cells in the stomach regions of non-implantation group showed patterns similar to those already reported for normal Balb/c mice (Ku *et al.,* 2004). As a result of CT-26 implantation, most of the IR cells were significantly decreased in both the fundus and pylorus. In the pylorus, more marked changes were demonstrated, with the greatest changes being detected for the somatostatin-IR cells. These changes might be responsible for inducing some GI disorders observed in cancer patients (Komurcu *et al.,* 2002).

Serotonin is a monoamine widely distributed in the nervous system and the gastro-entero-pancreatic endocrine cells; in the GI tract, its main functions are inhibition of gastric acid secretion and contraction of smooth muscle (Guyton, 1988). Serotonin-IR cells are markedly decreased in the duodenum of diabetic mice (Spangeus *et al.,* 2001) and the number and area they occupy are significantly decreased in ethanol-induced damage of gastroduodenal regions of mice (Penissi *et al.,* 2000). In the present study, serotonin-IR cells were significantly lowered in the stomach of the mice bearing the CT-26 cells. These changes might be responsible for GI problems, especially some clinical signs related to gastric motility and gastric acid secretion. Such changes were previously detected after implantation of 3LL cells (Ku *et al.,* 2005a).

Somatostatin, a 14 amino acid peptide consisting of a straight chain and a cyclic chain which was first isolated from the hypothalamus of sheep (Brazeau *et al.,* 1973). This peptide inhibits the secretion of other neuroendocrine hormones (Kitamura *et al.,* 1984). It is well known that somatostatin-IR cells are the most widely distributed endocrine cells in the GI tract, except for the large intestine, of all vertebrate species, including the primitive agnathans (Ku *et al.,* 2003). Somatostatin-IR cells are significantly increased in cancer adjacent mucosa as compared to that of cancer distant mucosa of colorectal endocrine cancer patients (Zhao *et al.,* 1997); and a decrease of these IR cells was demonstrated in duodenal ulcer patients with Helicobacter pylori; levels which returned to normal after eradication of the Helicobacter pylori (Queiroz *et al.,* 1993). In the present study, somatostatin-IR cells were markedly decreased in the stomach of the CT-26 implanted group compared to that of non-implanted sham group; this was quite similar to the results found after implantation of 3LL cells (Ku *et al.,* 2005a). The decrease in the somatostatin-IR cells may be considered to induce somewhat serious problems in GI physiology as regards digestive functions.

Glucagon is synthesized in the A cells of the pancreas and regulates serum glucose levels. Glucagon-IR cells have been demonstrated in various mammals, but there are no reports dealing with changes

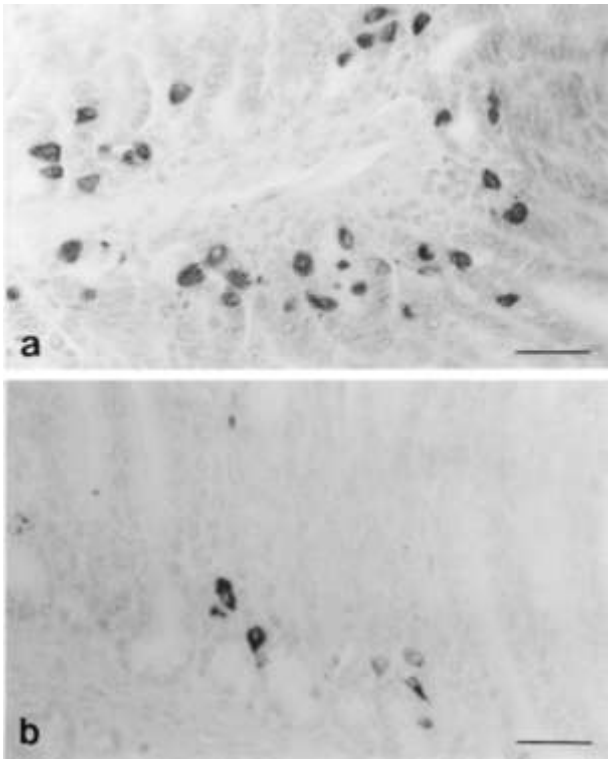

Figure 5. CCK-8-IR cells in the pylorus of the non-implanted sham group a) and the CT-26 implanted group b). In the CT-26 implanted group, CCK-8-IR cells were strongly decreased. Scale bars = 33 μm; PAP methods.

in glucagon-IR cells in cases of disease or in abnormalities induced by chemical or other manipulation.

In the present study, glucagon-IR cells were restricted to the fundus of the non-implantated sham groups.

It is generally accepted that gastrin and CCK-8 originate from same precursor. In the human duodenum, a large fraction of these cells, besides reacting with non-C terminal CCK antibodies and C-terminal gastrin/CCK antibodies, also show immunoreactivity with C-terminal gastrin-34 antibodies co-localised with CCK in a variable fraction of secretory granules (Solcia *et al.,* 1989). Gastrin, secreted by intestinal G cells, promotes gastric acid secretion, and CCK secreted by intestinal I cells stimulates pancreatic enzyme secretion. Gastrin-IR cells were increased by long-term omeprazole treatment in children (Pashankar *et al.,* 2001) but were decreased in unilateral cervical vagotomized mice (Qian *et al.,* 1999) and in patients with peptic gastric ulcer (Stave *et al.,* 1978). In addition, enteric CCK-8-IR cells were significantly increased in celiac sprue (Buchan *et al.,* 1984). In the present study, gastrin- and CCK-8-IR cells were markedly decreased after implantation of CT-26 cells compared to non-implanted sham.

PP was isolated from insulin extracts of pancreas at 1961. The regional distribution of PP-IR cells in mouse strains has become relatively well known since then and strain-dependant differences have been shown to exist among mice (Ku *et al.,* 2003). Although the exact function of PP is unknown, it is generally accepted that it is related to functions of the exocrine pancreas. In Balb/c mice, hPP-IR cells were not detected in the stomach regions (Ku *et al.,* 2004). In agreement, in the present study, no hPP-IR cells were detected in either the CT-26 implanted or non-implanted groups.

In conclusion, endocrine cells are the anatomical units responsible for the production of GI hormones, and the change in their density reflects a change in the capacity of producing these hormones. Implantation of tumor cell mass (CT-26) induced severe quantitative changes of gastric endocrine cell density; the abnormal density may contribute to the development of gastrointestinal symptoms such as anorexia and indigestion, frequently encountered in patients with cancer.

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