HISTOCHEMICAL, IMMUNOHISTOLOGICAL AND ULTRA-STRUCTURAL STUDIES ON NEOPLASTIC ENDOCRINE CELLS IN CARCINOMAS OF THE GLANDULAR STOMACH AND SMALL INTESTINE OF RATS

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Neoplastic endocrine cells in the carcinomas of the stomach and small intestine of Wistar male rats, induced by MNNG or ENNG, were examined by histochemical, immunohistological and ultrastructural methods. Detection rates of argyrophil and argentaffin cells in the gastric cancers were 60% and 34.3%, respectively. MNNG induced small intestinal carcinomas contained argyrophil cells in 78.3% and argentaffin cells in 65.2%. Of 205 carcinomas of the small intestine induced by ENNG, 115 lesions (56.1%) contained argyrophil cells and 69 lesions (33.7%) contained argentaffin cells. In some of the cases, gastrin, glucagon and somatostatin were demonstrated in the tumor cells by the immunoperoxidase technique. Ultrastructurally, intracytoplasmic granules were observed, and a special form of endocrine cell which contained two different types of intracytoplasmic granules was also found (endocrine-exocrine cell). This latter finding indicates a multidifferentiating potentiality of tumor cell. Moreover, it would appear that these endocrine cells can arise from undifferentiated stem cells, and that the endocrine cells of the gastrointestinal tract are of the endodermal origin.

It is well known that argyrophil and argentaffin cells are scattered within the mucosal epithelium of the gastrointestinal tract. With respect to the origin of endocrine cells, an attractive hypothesis that they are derived from neural crest or neuroectodermal elements has recently been proposed by Pearse *et al.* They regarded the endocrine cells of the gut as cells of the APUD (amine and precursor uptake and decarboxylation) series (2, 18, 19). More recently Fujita *et al.* (5) have classified endocrine cells of the gastrointestinal tract and pancreas under one category of gastro-entero-pancreatic (GEP) endocrine system. The origin of the endocrine cells in the gastrointestinal tract is still a matter of debate. Some investigators observed these endocrine cells in carcinomas such as gastric (1, 6, 12, 14, 21, 31, 33), small intestinal (8), colonic (12, 25), pancreatic (34), pulmonary (16, 25) and breast (17) cancers etc.. There exists a considerable literature on endocrine cells concerning human materials (1, 8, 12, 14, 25, 31, 33), but little work has been done to study these cells in experimentally induced gastric (10, 11, 31) and colonic (30) carcinomas. A search of the literature fails to reveal a report of endocrine cells in experimentally induced carcinomas of the small intestine.

In this experiment the author studied the endocrine cells in adenocarcinomas

of the glandular stomach and small intestine of rats induced by chemicals histochemically, immunohistologically and ultrastructurally, and the origin of these cells was discussed.

MATERIALS AND METHODS

1. Carcinogens:

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), purchased from Aldrich Chemical Co. Inc., Milwaukee, U.S.A., were diluted in water at concentrations of 100 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$, respectively, with 0.4% Tween-60 (polyoxyethylene sorbitan monostearate).

2. Animals and experimental groups:

Six-week-old Wistar male rats were used in this investigation. The animals were divided into the following 4 groups.

Group I: Each of the animals received MNNG to drink freely for 35 weeks and then normal water for 5 to 15 weeks. Number of effective animals was 39.

Group II: Rats were given ENNG to drink for 17 weeks and killed 2 to 10 weeks later, and 43 rats were effective to study.

Group III: Fourteen rats received water containing 0.4% Tween-60 for 35 weeks and then normal water for 1 to 4 weeks.

Group IV: Ten rats were allowed to drink tap water only.

3. Histological and histochemical stainings:

For histological and histochemical examinations the specimens were fixed in 10% formalin solution, embedded in paraffin and cut serially for 4 μ . Each section was stained with hematoxylin-eosin, Grimelius' method for argyrophil reaction and Masson-Fontana's silver impregnation method for argentaffin reaction. PAS, mucicarmin and alcian-blue stains were performed in necessary cases.

4. Objects of examination:

Among the lesions developed in the glandular stomach and small intestine of rats, carcinomas, which had apparent structural and cellular atypisms and invaded to over the submucosal layer, were used in this investigation. Intramucosal carcinomas were excluded. Thirty-five gastric and 23 intestinal carcinomas induced by MNNG (group I), and 205 small intestinal carcinomas induced by ENNG (group II) were prepared. In control groups such as group III and IV, no tumors developed.

5. The criterion for evaluation of positive for silver reaction:

The criterion for evaluating carcinoma cell positive for silver reaction was applied to cancer tissue infiltrating from submucosa to the deep region. Argyrophil and argentaffin cells in areas of intramucosal carcinoma were excluded, since it could not completely be denied to include normal endocrine cells.

The cell was evaluated to neoplastic endocrine cell when it existed in neoplastic glandular epithelium or nests of cancer tissue and had atypism to define cancer cell cytologically with intracytoplasmic granules.

6. Immunohistological examination:

For immunohistological demonstrations of endocrine cells, the peroxidase-antiperoxidase (PAP) method (27, 28) was used. Some deparaffinized sections

were stained immunohistologically for gastrin, glucagon and somatostatin (DAKO PAP Kit, Kyowa medex).

7. Ultrastructural examination:

For an electron microscopic investigation of the endocrine granules, representative pieces were cut from the paraffin blocks and deparaffinized in xylene. Following a wash in phosphate buffer, the specimens were fixed in 2.7% glutaraldehyde, postfixed in 2.5% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. The ultrathin sections were examined in JEM-7A electron microscope.

RESULTS

Silver impregnation positive cells in normal intramucosal area were triangular-, columnar- or flattened-shaped, and existed commonly in the neck area and occasionally in the fundic area or the foveoral epithelium. Intracytoplasmic granules were recognized in infranuclear or perinuclear portion and often in the whole of the cytoplasm.

FIG. 1a. Neoplastic argyrophil cells in adenocarcinoma. Grimelius(G)'s method. $\times 400$

The incidence and distribution of neoplastic endocrine cells in carcinomas varied considerably and these cells were often found in several scattered cell colonies. Neoplastic endocrine cells were relatively similar to normal cells, but revealed more varied shapes such as triangular, flask-like, columnar, ovoid, round or irregular, etc.. Intracytoplasmic granules tended to be stained deeply in the whole cytoplasm, but few were dominant in infranuclear portion or rough and pale stained (Fig. 1).

1) Of the 35 adenocarcinomas of the glandular stomach induced by MNNG, Grimelius-positive, argyrophil cells were observed in 21 carcinomas (60%) and Masson-Fontana-positive, argentaffin cells were in 12 lesions (34.3%) (Table 1). No differences in structure were observed between the carcinomas with endocrine cells and without them. In the view of depth of invasion, neoplastic endocrine cells were observed to be more numerous in accordance with how deep they had invaded. The deeper tumors invaded, the more endocrine cells were tended to appear more in submucosal area than in most advanced areas. Both argyrophil and argentaffin cells were observed in metastasis into the lymph nodes (Fig. 2).

2) Of 23 carcinomas of the small intestine induced by MNNG, argyrophil cells were positive in 18 lesions (78.3%) and argentaffin cells were positive in 15

FIG. 1b. Neoplastic argentaffin cells in adenocarcinoma. Masson-Fontana(MF)'s method. $\times 400$

TABLE 1. Incidence of argyrophil and argentaffin cells in adenocarcinomas of the glandular stomach induced by MNNG

1) according to histologic type

Histologic type of adenocarcinoma	Number of carcinomas	Number of carcinomas with Argyrophil cells	Degree of occurrence of Argyrophil cells			Number of carcinomas with Argentaffin cells	Degree of occurrence of Argentaffin cells		
			+	++	##		+	++	##
Well differentiated	29	17 (58.6%)	12	5		11 (83.0%)	8		3
Moderately diff.	5	3 (60.0%)	2	1		1 (20.0%)	1		
Poorly differentiated	1	1 (100 %)		1		0			
Total	35	21 (60.0%)	14	7		12 (34.3%)	9		3

* (+ : few ++ : moderate ## : numerous)

2) according to depth of invasion

Depth of invasion	Number of carcinomas	Number of carcinomas with Argyrophil cells	Deepest layer of presence of Argyrophil cells			Number of carcinomas with Argentaffin cells	Deepest layer of presence of Argentaffin cells		
			sm	pm	s		sm	pm	s
Submucosa	14	5 (35.7%)	5	*	*	2 (14.3%)	2	*	*
Muscularis propria	18	13 (72.2%)	12	1	*	10 (55.6%)	9	1	*
Serosa	3	3 (100 %)	1	1	1	0			
Total	35	21 (60.0%)	18	2	1	12 (34.3%)	11	1	

(sm: submucosa, pm: muscularis propria, s: serosa)

FIGS. 2a, b. Argentaffin cell in metastatic focus of lymph node (arrowed). a, H-E stain. $\times 100$
b, Masson-Fontana's method. $\times 400$

lesions (65.2%) with a high incidence (Table 2). Silver reaction positive cells were highly observed in the carcinomas invaded to serosa (subserosa), and they were presented more in the most advanced layers rather than in the submucosa, compared with the gastric carcinomas.

TABLE 2. Incidence of argyrophil and argentaffin cells in adenocarcinomas of the small intestine induced by MNNG

1) according to histologic type

Histologic type of adenocarcinoma	Number of carcinomas	Number of carcinomas with argyrophil cells	Degree of occurrence of Argyrophil cells			Number of carcinomas with Argentaffin cells	Degree of occurrence of Argentaffin cells		
			+	++	##		+	++	##
Well differentiated	22	17 (77.3%)	7	9	1	14 (63.6%)	8	6	
Moderately diff.	1	1 (100 %)	1			1 (100 %)	1		
Total	23	18 (78.3%)	8	9	1	15 (65.2%)	9	6	

* (+ : few ++ : moderate ## : numerous)

2) according to depth of invasion

Depth of invasion	Number of carcinomas	Number of carcinomas with argyrophil cells	Degree of occurrence of Argyrophil cells			Number of carcinomas with Argentaffin cells	Deepest layer of presence of Argentaffin cells		
			sm	pm	s		sm	pm	s
Muscularis propria	6	2 (33.3%)		2	*	2 (33.3%)		2	*
Serosa	17	16 (94.1%)		7	9	13 (76.5%)	1	6	6
Total	23	18 (78.3%)		9	9	15 (65.2%)	1	8	6

(sm : submucosa, pm : muscularis propria, s : serosa)

3) Of the 205 carcinomas of the small intestine induced by ENNG, 115 lesions (56.1%) contained argyrophil cells and 69 lesions (33.7%) contained argentaffin cells (Table 3). The greater parts of the carcinomas were the well-differentiated type, but in the areas of poorly differentiated adenocarcinoma, neoplastic endocrine cells were also recognized (Fig. 3). These endocrine cells were more highly observed as the tumors invaded deeper and deeper. They appeared more in the most invaded layer.

4) Representative sections were examined by PAP method for gastrin, glucagon and somatostatin. A relatively large number of gastrin-positive cells were recognized in the gastric carcinomas (Fig. 4), and a few of them were in the small intestinal carcinomas. A small number of somatostatin-positive cells were detected in both gastric and intestinal carcinomas (Fig. 5). Glucagon-positive cells were scarcely observed in carcinomas of the small intestine. These immunoreactive cells could be detected in the serial sections of the same materials.

5) Ultrastructurally intracytoplasmic secretory granules, 120 to 350 nm in

FIG. 3. Neoplastic endocrine cells in poorly differentiated adenocarcinoma. Upper left; H-E stain. $\times 100$ Upper right: Grimelius(G)' method. $\times 100$ Lower left: Masson-Fontana(MF)'s method. $\times 100$ Lower right: Masson-Fontana(MF)'s method. $\times 400$

Figs. 4a, b. Gastrin-immunoreactive cells in adenocarcinoma of the glandular stomach. PAP method. a $\times 100$, b $\times 400$

FIGS. 5a, b. Somatostatin-immunoreactive cells in adenocarcinoma of the glandular stomach (a) and small intestine (b). a, b $\times 400$

diameter, were observed. Various cell types were found electron-microscopically in adenocarcinomas of the stomach and small intestine. Electron microscopic characteristics of some cell types were the polymorphic, longish, dumbbells, which were similar to EC-cell (Fig. 6), and others were round of various sizes whose cell

FIG. 6. EC-cell with electron-dense, polymorphic secretory granules. $\times 3,700$

types could not be clearly detected (Fig. 7). A special form of the endocrine cell which contained two different types of intracytoplasmic granules was found. One were small, electron-dense endocrine secretory granules and the other were large, lucent granules similar to mucin droplets or zymogen granules (Fig. 8).

DISCUSSION

Since the first report made by Hamperl (6) that silver-positive cells appeared in the carcinomas of the gastrointestinal tract, a considerable amount of research has been made on these endocrine cells (1, 12, 14, 21, 31, 33). But there has been only one report concerning the carcinoma of human small intestine (8). In the field of experimentally induced gastric carcinoma, some authors described endocrine cells in the tumor tissue (10, 11, 31, 32), but their reports were not detailed. No report was made on experimentally induced carcinoma of the small intestine. Spontaneous development of carcinomas in the glandular stomach of animals has rarely been observed except in Mastomys (22). Since Sugimura and Fujimura (29) established a method of inducing carcinomas of the glandular stomach of rats by MNNG, the induction of cancer in the stomach and small intestine of rats by

FIG. 7. Neoplastic endocrine cell with round, electron-dense secretory granules. $\times 5,000$

FIG. 8. Endocrine-exocrine cell with two different types of intracytoplasmic granules; small, electron-dense granules and large, mucin droplet-like granules of low electron density (arrowed). $\times 14,500$

FIG. 7.

FIG. 8.

oral administration of chemical carcinogens such as MNNG or ENNG has been widely observed (10, 11, 32). In the present paper, the author examined the endocrine cells which appeared in carcinomas of the glandular stomach and small intestine of rats induced by MNNG or ENNG. Sixty per cent of the gastric carcinomas induced by MNNG contained argyrophil cells, and 33.7% of them contained argentaffin cells. The incidence of argyrophil cells in the carcinomas of the small intestine induced by MNNG and ENNG was 78.3% and 56.1%, and that of argentaffin cells was 65.2% and 33.7%, respectively.

The difference of frequency between MNNG-induced small intestinal carcinomas and ENNG-induced ones is probably due to the difference of observation method rather than that of carcinogens. The former included several specimens which were cut from central and marginals of tumor, the latter included one specimen from the central cutting of cancer tissue. In view of the variability in distribution of these endocrine cells, it seems highly probable that the frequency of these cells would increase in number if the areas of examination were widened. Other authors have pointed out similar opinions (1, 8, 13, 33).

The silver-impregnated cells which were demonstrated in this study were considered neoplastic endocrine cells, i.e.:

- 1) Evaluation of the positivity for silver reaction was applied to the invasive portion of the tumor, and these cells in the area of intramucosal carcinoma and of carcinoma-in-situ were excluded, since these cells might include normal cells.

- 2) These cells had cellular atypism more or less.

- 3) Silver-impregnated cells were proven in metastatic foci of the lymph nodes.

- 4) Some gastrointestinal hormones were detected by immunohistochemical technique.

- 5) Secretory granules were observed by ultrastructural examinations.

Systematic studies of the gastric and intestinal carcinomas which contained endocrine cells have been reported by several investigators. Azzopardi and Pollock (1) reported that 13 per cent of gastric carcinomas (100 cases) contained argyrophil cells and 8% of them argentaffin cells. Kubo and Watanabe (12) observed argentaffin cells in 12 cases (3.1%) of 383 gastric cancers. Tahara *et al.* (31) described that argyrophil cells were found in 11 cases (6.9%) and argentaffin cells were noted in 4 cases (2.5%) of the 159 advanced human gastric carcinomas. Matsusaka (14) reported that the incidence of neoplastic argyrophil cells in gastric carcinomas was 62% in early carcinomas and 34% in advanced ones. More recently Proks and Feit (21) observed Grimelius-positive 8.1% (tubular 5.3%, diffuse 17.4%) and Fontana-positive 6.03% (tubular 2.65%, diffuse 17.4%) in 248 gastric cancers, respectively. The reported incidence of endocrine cells in gastric carcinomas has ranged from 0 (0/184 cases) (4) to as high as 62%.

According to only one report of human small intestinal carcinomas by Iwashita *et al.* (8), neoplastic argyrophil and argentaffin cells were found in 11 (68.8%) and 7 (43.8%) cases, respectively, of 16 tumors. The results of this study and some authors' (8, 14) indicate that these endocrine cells could be found with a high incidence by detailed observations.

With regard to the relationship between histologic type and frequency of endocrine cells, in gastric carcinomas, some authors described that these cells were more abundant in undifferentiated (poorly differentiated) carcinoma or diffuse carcinoma

than in differentiated adenocarcinoma (12, 31). On the contrary others reported endocrine cells to develop more in well-differentiated type (14, 33). In human small intestinal carcinomas, Iwashita *et al.* reported that these cells were recognized only in cases with well-differentiated adenocarcinoma (8). In the present study, there was no significant relation between the histologic type of carcinoma and incidence of neoplastic endocrine cells, similar to some reports (1, 14).

In the present study, endocrine cells in gastric carcinoma tended to appear more in submucosa than in the most advanced area of the cancer tissue. On the contrary, in small intestinal carcinomas, they were found more in the most invaded layer. The reason for this difference is uncertain. Some authors pointed out that endocrine cells tended to appear in carcinoma of intramucosal part in the stomach (1, 14, 33), and Watanabe (33) assumed the influence of the specific factor of mucosa *in situ*. In human small intestinal carcinoma, these cells were found almost equally within the tumor tissue by different layers of the intestinal wall (8). The high incidence of endocrine cells in serosa in the present study of small intestinal carcinomas might probably depend on the fact that the area of serosa (subserosa) in which cancer cells infiltrated was broad.

At present, more than 10 kinds of endocrine cell types of the gastrointestinal tract have been determined ultrastructurally or immunohistochemically (18, 23, 26). In this study the author examined a part of materials using the PAP method. Gastrin, glucagon, and somatostatin could be demonstrated in the induced cancer tissues. These cells seemed to appear with relative similarity in distribution and frequency to normal immunoreactive cells, although the author could not assert it because of a small number for examination. More cell types might be determined by further examinations.

With respect to the origin of endocrine cells, Pearse *et al.* proposed that the endocrine cells of the digestive tract originated from the neural crest (2, 18, 19). Contrary to this hypothesis, many authors have supported endodermal origin (1, 8, 12, 14, 25, 31, 33). Pierse *et al.* (20) made suspensions of adenocarcinoma cells and transplanted single cells into rats. The resulting cloned tumor contained vacuolated, mucinous, and argentaffin cells. Then they concluded that the undifferentiated colonic cell was a multipotential stem cell capable of differentiating into each of the three differentiated cells, and that the argentaffin cells were of endodermal origin. Similar results were obtained by others in transplantation of gastric cancer cells of rats induced by MNNG (11) or in stomach graft into subcutaneous tissue (15). Thus, undifferentiated stem cells might be assumed to have the multipotentiality of differentiating various cells. Autoradiographic examinations with ^3H -thymidine indicated that endocrine cells arose from immature precursor cells at the isthmus region, and that these cells might derive from common stem cells with mucosal cells (3, 4). In the present study, a special form of endocrine cell was found in ultrastructural observation, which contained two different types of intracytoplasmic granules, such as endocrine secretory granule and a large granule similar to a mucin droplet or a zymogen granule. This cell seems to coincide with the muco-argyrophilic tumor cell described by Schmid *et al.* in nasal carcinoma (24), or the endocrine-exocrine cell found by Hattori *et al.* in regenerated mucosa of rat stomach (7). This finding indicates a differentiating potential of the tumor cells, that is, a common precursor cell may give rise to both the endocrine cell and

the mucous cell. Endocrine cells and other tumor cells appear to derive from common stem cells. The author considers that neoplastic endocrine cells observed in this study may not necessarily derive from malignant transformation of normal endocrine cells. On the contrary, it would appear that these endocrine cells can arise from undifferentiated stem cells, and that the endocrine cells are of endodermal origin. In other words, adenocarcinomas with endocrine cells should not be assumed to consist of both proliferation of adenocarcinoma and neoplastic transformation of normal endocrine cells, but to be neoplastic cells which have differentiated to endocrine cells. In conclusion, these tumors which have silver-impregnated cells are not carcinoid but adenocarcinoma with endocrine differentiation.

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