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Production of gastrin releasing peptide by medullary carcinoma of the thyroid

An immunohistochemical study*

Toru Kameya[1], Takashi Bessho[2], Masaru Tsumuraya[1], Ken Yamaguchi[3], Kaoru Abe[3], Yukio Shimosato[1], and Noboru Yanaihara[4]

[1] Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan
[2] Department of Surgery, Okura National Hospital, Tokyo, Japan
[3] Endocrinology Division, National Cancer Center Research Institute, Tokyo, Japan
[4] Laboratory of Bioorganic Chemistry, Shizuoka College of Pharmacy, Shizuoka, Japan

Summary. Forty medullary carcinomas of the thyroid (MCT) with documented calcitonin (CT) production were studied immunohistochemically for the production of gastrin releasing peptide (GRP), a mammalian counterpart of amphibian bombesin. GRP-positive cells, revealed by an unlabelled peroxidase-antiperoxidase immunoenzyme histochemistry were found in 81% (34/40) of the MCTs. Variable numbers of tumor cells in positive MCTs were immunostained for GRP. In 3 cases with Sipple's syndrome, cells in scattered microscopic MCT nodules and hyperplastic intrafollicular C cells of the thyroid were frequently positive for GRP as well as for CT. Non-neoplastic C cells (or CT-positive cells) of the human thyroids were also positive for GRP. In the neoplastic and non-neoplastic C cell system, some cells were confirmed to be immunoreactive with both anti-GRP and anti-CT. All these findings indicate that GRP and CT are closely associated peptide hormones produced by the C cell system.

Key words: Thyroid medullary carcinoma – C-cell – Gastrin releasing peptide – Calcitonin – Immunostaining

Medullary carcinoma of the Thyroid (MCT) is a clinically and pathologically distinctive neoplasm that is considered to arise from the neural crest-derived thyroid C cell system (Hazard et al. 1959; Williams 1966; Pearse

Offprint requests to: T. Kameya at the above address

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1974) and is known invariably to produce calcitonin (CT) (Tashjian et al. 1970; Sizemore and Go 1975) and to be occasionally associated with the production of adrenocorticotropin (ACTH) and its related peptides (Williams et al. 1968; Melvin et al. 1970; Bussolati et al. 1973; Birkenhäger et al. 1976; Kameya et al. 1977; Capella et al. 1978), somatostatin (Sundler et al. 1977; O'Briain et al. 1981; Sano et al. 1981), serotonin (Capella et al. 1978), and Substance P (SP) (Skrabanek et al. 1979). To the best of our knowledge, however, there is no previous report on the production of gastrin releasing peptide (GRP) by this tumor. GRP is a mammalian counterpart of bombesin, a peptide which possesses similar pharmacological actions and homologous amino acid sequence to GRP (Erspamer et al. 1975; Dockray et al. 1979; McDonald et al. 1979; Yanaihara et al. 1981). A recent report indicated that the bombesin-like immunoreactivity found in the porcine tissues including central nervous system was attributable to GRP or GRP-like materials (Yanaihara et al. 1981). Immunostaining with anti-bombesin or anti-GRP sera revealed that some nerve fibers (Polak et al. 1978; Yanaihara et al. 1981) and endocrine cells in the amphibian (Lechago et al. 1978), avian (Dockray et al. 1979; Timson et al. 1979) and mammalian (Polak et al. 1976; Tobe et al. 1982) as well as human fetal (Wharton et al. 1978) and adult bronchial epithelium (Cutz et al. 1981; Tsutsumi et al., personal communication) were positively stained. Recently, there have been a few reports suggesting the production of immunoreactive (IR) bombesin by lung cancers, especially by small cell carcinomas and carcinoids (Wood et al.; Moody et al. 1981; Gazdar et al. 1981; Erisman et al. 1982; Tamai et al. in press).

This is the first report demonstrating that most MCTs like non-neoplastic C cells are capable of production of IR-CT and IR-GRP.

Materials and methods

Tumor tissues of the primary focus from 40 cases of MCT were routinely fixed in formalin and/or Bouins's fluid and embedded in paraffin. The tumor cases included those which were the subjects of consultation by other hospitals for histological diagnosis and in which immunostaining was requested for hormones including CT. They were all confirmed to possess tumor cells that stained positively for CT. Metastatic foci in the liver and lymph nodes in 3 of the above 40 cases were also examined. Eight cases were described in a previous paper (Kameya et al. 1977). In 3 cases with Sipple's syndrome, horizontal sections at several levels of both lateral lobes of the thyroid were prepared, in order to examine microscopic foci of MCT and C cell hyperplasia. Human thyroid tissues were also used for the study of GRP production by non-neoplastic C cells or CT positive cells. Because of the paucity of C cells in the normal human thyroid, we used thyroid tissues of 2 cases of malignancy-associated hypercalcaemia, in which C cells were frequently encountered in the middle third of both lobes (Kameya et al. 1980).

Four μ serial sections were prepared for haematoxylin-eosin, and immunohistochemical examination for CT and GRP. In immunohistochemistry, the unlabeled peroxidase-antiperoxidase (PAP) method (Sternberger et al. 1970) was performed. The antisera against human CT (antiserum 172-2) (Abe et al. 1977), and porcine synthetic GRP (antiserum R-6902) (Yanaihara et al. 1981) were raised in rabbits and characterized in our institutes. Anti-rabbit immunoglobulin (the second layer of the reaction) and PAP complex (the third layer of the reaction) were purchased from Dakopatts (Copenhagen, Denmark). The latter two were used in dilutions

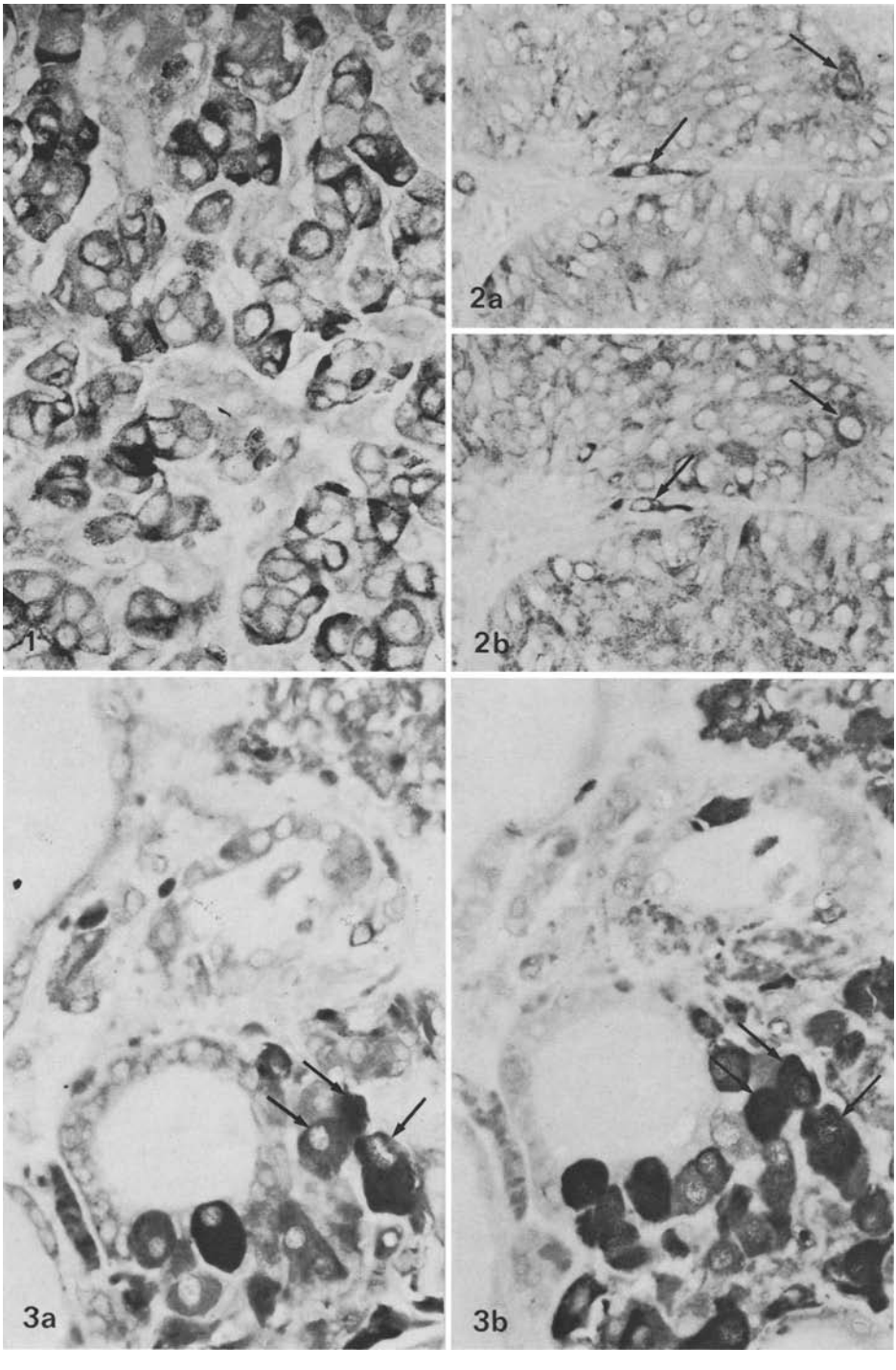
of 1:50 and 1:100, respectively. Peroxidase activity was demonstrated with 3, 3′-diaminobenzidine according to the method of Graham and Karnowsky (1966). Reactivity of the primary antisera used was confirmed by known specific distribution of positively stained cells in normal rat tissues including thyroid for CT, and human fetal lungs for GRP. The specificity of each antiserum was also confirmed immunohistochemically by an absorption test, which consisted of the treatment of tissue sections by working diluted antiserum (1:800 for anti-CT, and 1:400 for anti-GRP) absorbed by an excess of the corresponding antigen (50–100 μg/ml of working solution) in place of the incubation by non-absorbed primary antiserum. In immunohistochemical analysis of adjacent paired sections for simultaneous localization of two hormones, each antiserum of working solution containing excess counterpart antigen (more than 50 μg/ml working antiserum) was used to ensure the absence of possible cross-reaction with each other. Because of the presence of strong endogenous peroxidase in thyroid tissue, intensive pretreatment for 1 h by 0.1% hydrogen peroxide was often necessary. Nuclear staining was performed as needed by methyl green.

Results

Variable numbers of tumor cells were positively stained for GRP in 34 (81%) of 40 MCT cases. Most tumor cells were positive for GRP and CT in 5 tumors (Fig. 1). About half the number of tumor cells were positive for GRP in 9 tumors. Very few solitary positive cells were scattered in 20 tumors. The intensity of positive stains varied from cell to cell. Cytoplasmic processes were often intensely stained. Cells at tumor margins were, in general, more intensely stained. In one of these cases which displayed a clinical course of extensive metastases, cells in metastatic foci of the liver were stained for neither GRP nor CT, while cells in the primary focus were positive for both hormones. In 4 tumors negative for GRP, CT positive cells were rare and the cytoplasm of tumor cells was clear with vacuolation, indicating cytoplasmic degeneration. In two questionable cases of GRP reactivity in the primary focus, GRP was not recognized in metastatic liver foci. The number of cells positive for GRP seemed to parallel that of cells positive for CT. In paired adjacent sections of two tumors, identical cells were sometimes revealed to possess both hormones (Fig. 2). However, CT-positive cells were not always positive for GRP at the detection level of our immunohistochemistry.

In all three Sipple's cases, there were scattered microscopic MCT nodules and increased numbers of C cells in follicles, especially in the vicinity of visible tumors, which were shown by immunohistochemistry to be positive for CT. Some microscopic nodules and hyperplastic C cells in follicles stained for GRP and CT (Fig. 3) but others stained only for CT (Fig. 4). Some cells were confirmed to stain simultaneously for both hormones by immunostaining of two consecutive sections (Fig. 3).

In non-neoplastic thyroids of 2 hypercalcaemic patients, identical cells were simultaneously positive for both CT and GRP (Fig. 5). Non-neoplastic C cells were always strongly reactive to anti-CT. The intensity of immunostaining for GRP was generally weaker than that for CT, but some identical cells showed the same degree of reaction to both antisera (Fig. 5), under the same conditions in regard to incubation time, dilution of reagents and temperature.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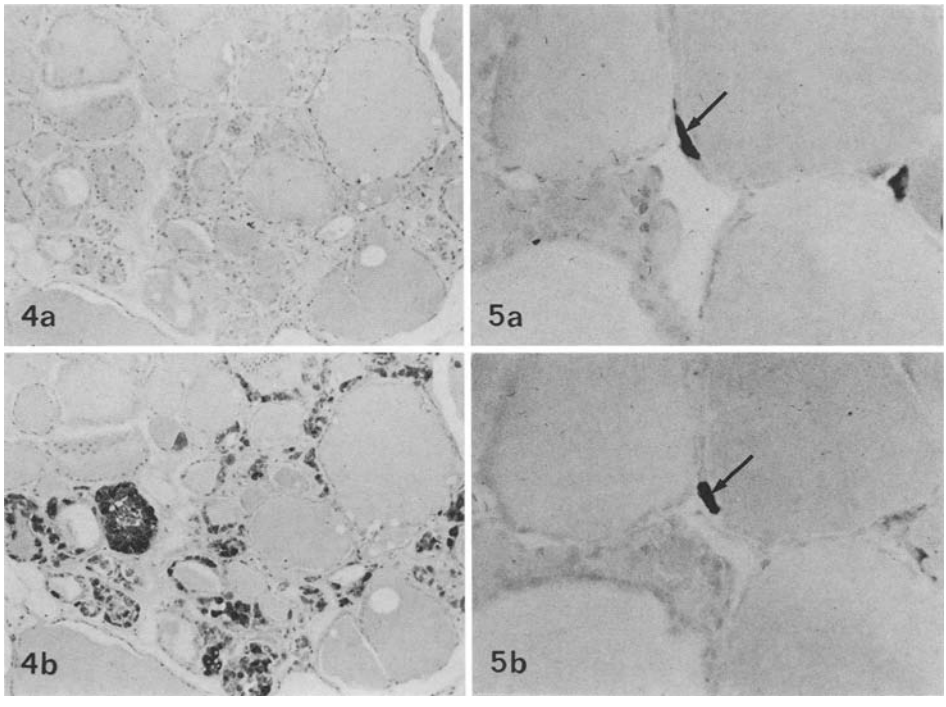

Fig. 1. MCT, where most tumor cells are positively immunostained for GRP, × 340

Fig. 2a, b. Two adjacent sections of an MCT immunostained for both GRP (**a**) and CT (**b**). Some cells are positive for both hormones. (*arrows*) × 340

Fig. 3a, b. Two adjacent sections of a microscopic MCT nodule with hyperplastic C-cells in follicles immunostained for GRP (**a**) and CT (**b**) in a Sipple's case. At least three cells (*arrows*) are positive for both hormones. × 430

Fig. 4a, b. Two adjacent sections of a microscopic nodule immunostained for GRP (**a**) and CT (**b**) in a Sipple's case. Note absence of IR-GRP. × 85

Fig. 5a, b. Two adjacent sections of a non-neoplastic C-cell strongly immunoreactive to both anti-GRP (**a**) and anti-CT (**b**) (*arrows*) in the thyroid of a malignancy-associated hypercalcemia patient, × 340

Antisera preabsorbed by the corresponding antigens did not stain all tissue sections, except follicular colloid and red cells in a few cases. Antisera supplemented with the other antigen, e.g. anti-CT sera supplemented with GRP or vice versa, did not change the intensity of immunostaining.

Discussion

The present study revealed that not only 81% of MCTs but also non-neoplastic thyroids contained cells positively immunostained for GRP. In 4 negative and 2 questionably positive cases, there were three possibilities;

1) there was no storage of GRP in tumor cells, 2) the intracellular storage of GRP was too low to be detected by immunohistochemical examination of routinely processed tissue sections (Timson et al. 1979), and 3) the tumor cells were severely degenerated. The second possibility is suggested by the fact that the GRP content of one of these immunohistochemically negative tumors was very low (below 10^2 ng/g wet tissue by RIA) and that GRP was not detected by RIA in a metastatic focus of the liver, where tumor cells were negatively immunostained. In our experience, IR-GRP below 10^2 ng/g wet weight of lung cancer tissues were very rarely recognized by immunohistochemical examination of routinely processed tissue sections (Kameya, unpublished data). The third possibility is suggested by clear vacuolation of tumor cells and paucity of CT-positive tumor cells. These findings suggest that almost all MCTs might be capable of GRP production, even if the immunohistochemical findings are negative.

Demonstration of simultaneous localization of IR-GRP and IR-CT by the consecutive section method suggests that both hormones are produced by the same cells. This suggestion is also supported by RIA data that a significant correlation existed between the amounts of IR-CT and IR-GRP in individual tumors (Yamaguchi et al., submitted for publication). However, there is no evidence yet that all MCT cells can produce or store both IR-CT and IR-GRP at the same time and it is evident that the degree of hormone production or storage by MCT cells greatly varies from cell to cell in individual tumors. While simultaneous localization of IR-ACTH and IR-β-lipotropin in the same cells can be explained now by the theory of a common precursor molecule of these peptides (Nakanishi et al. 1979), the probability that IR-CT and IR-GRP have a common precursor is low, because the structure of the rat CT gene of the thyroid, in which 2 predicted precursor molecules are encoded, has been elucidated. These precursors did not contain an amino acid sequence corresponding to porcine GRP or bombesin (Amara et al. 1982). An analogy has been found between ACTH and CT in MCTs (Goltzman et al. 1979) and between somatostatin and CT in normal rat C cells (Noorden et al. 1977) and human MCTs (O'Briain et al. 1980; Sano et al. 1981).

Bilaterality, multiplicity and C cell hyperplasia in the vicinity of macroscopically visible and microscopic tumor nodules are well known in Sipple's or familial cases of MCT (Wolfe et al. 1973; DeLellis et al. 1977). The present study revealed that some of these lesions, but not all, contained cells positively immunostained for both GRP and CT.

Non-neoplastic C cells with CT production were also occasionally demonstrated to contain IR-GRP. This implies that not only neoplastic but also non-neoplastic C cells are capable of GRP production and that therefore this does not represent a phenomenon occurring uniquely with neoplastic tranformation. The situation seems to be similar to that in case of ACTH and somatostatin production, although no report has been found on the presence of IR-ACTH in non-neoplastic C cells, and many authors have reported on ACTH production by MCTs (Williams et al. 1968; Melvin

et al. 1970; Bussolati et al. 1973; Birkenhäger et al. 1976; Capella et al. 1978; Kameya et al. 1977; Goltzman et al. 1979).

The weaker reactivity of non-neoplastic C cells to anti-GRP than to anti-CT could be interpreted in several ways. The first interpretation is that the GRP antigenic site is more liable to be affected by tissue specimen processing including fixation (Timson et al. 1979). The second is that the storage of GRP in C cells is lower than that of CT. The third is that the anti-GRP serum used has a low titer. The second possibility is greatest, because all 8 primary MCTs examined by our RIA system always contained higher amounts of IR-CT than IR-GRP (Yamaguchi et al., under consideration for publication). These findings indicate that the storage of IR-CT by the C-cell system is, in general, higher than that of IR-GRP.

Bombesin-like or GRP-like peptide has recently been shown to be produced by lung cancers, especially by small cell carcinomas and bronchial carcinoids (Wood et al.; Moody et al. 1981; Gazdar et al. 1981; Erisman et al. 1982; Tamai et al. in press). Recent data indicate that the same cells in the bronchial epithelium of the fetal and adult lungs may (Cutz et al. 1981) or do (Tsutsumi et al. personal communication) contain both IR-CT and IR-GRP. These and the present data pose a question on the relationship between thyroid C cells and pulmonary K cells, as the concept of the neural crest origin of pulmonary K cells has been severely challenged from the data on cell origin of the gastroenteropancreatic endocrine system (Pictet et al. 1976; Andrew 1974; Chen and Leblond 1974). Regardless of cell origin, the two peptides seem to be closely associated in some endocrine system, although no data on GRP gene analysis in relation to the CT gene is available.

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