S-100 PROTEIN-IMMUNOREACTIVE CELLS IN THE LAMINA PROPRIA AND CARCINOID TUMORS OF THE APPENDIX

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ABSTRACT

Appendiceal mucosa and carcinoid tumors were examined immunohistochemically by the immunoperoxidase method for S-100 protein. Light microscopic examination of serial sections showed sparse extraepithelial subglandular argyrophilic cells surrounded by S-100 protein-positive cells within the lamina propria of normal or slightly inflamed appendices. These pairs of cells closely resembled the ultrastructurally defined enterochromaffin cell-nerve fiber (ECC-NF) complexes in their location and topographical relationship, as well as in their cytological and biochemical properties. All four carcinoid tumors examined contained stellate cells giving a strong reaction for S-100 protein. In three tumors, these immunoreactive cells with long cytoplasmic processes were observed at the margin of tumor nests. They often enveloped smaller tumor nests. Conventional electron microscopy showed that the tumor nests were composed of neurosecretory cells containing enterochromaffin granules and polyaxonal unmyelinated Schwann cells located at the periphery of the nests. Electron immunoperoxidase staining for S-100 protein revealed that these immunoreactive cells were identifiable as Schwann cells. These findings show an intimate relation between appendiceal carcinoid tumors and nerve fibers, and suggest that these tumors develop from ECC-NF complexes.

Endocrine cells in the mucosa of the gastrointestinal tract, especially the appendix, are located not only in the glandular epithelium but also in the lamina propria separated from the epithelium. Recently, from ultrastructural observations, Auböck and Ratzenhofer (2) and Papadaki *et al.* (15) concluded that these extraepithelial endocrine cells of the appendix form a complex with polyaxonal unmyelinated nerve fibers, and that this complex, named the 'enterochromaffin cell-nerve fiber (ECC-NF) complex' by Auböck and Ratzenhofer (2), is separated from the surrounding stroma by a common basal lamina. Moreover, they suggested that some carcinoid tumors might have originated from these extraepithelial intraneural endocrine cells because on electron microscopic examination they found nerve fibers in the tumor nests (1, 18).

S-100 protein is considered to be a nerve-specific antigen, although it was recently found

in other kinds of cells besides Schwann cells, such as Langerhans cells, interdigitating cells, melanocytes, and lipocytes (12). On immunostaining for S-100 protein, the location and distribution of Schwann cells in nerve fibers can easily be determined from the intense immunoreactivity of these cells (3, 20). Therefore we used immunostaining for S-100 protein to study the location of the ECC-NF complex in human appendiceal mucosa and of nerve fibers in carcinoid tumors of the appendix.

MATERIALS AND METHODS

Fourteen normal or slightly inflamed appendices and four appendiceal carcinoid tumors were obtained at surgery. For light microscopy, the tissues were fixed in 10% neutralized buffered formalin, embedded in paraffin, cut into serial sections of 4 μ m thickness, and stained with

hematoxylin-eosin, silver staining by the Grime-lius procedure or immunostaining for S-100 protein. Immunostaining was performed by the avidin-biotin-peroxidase complex (ABC) method reported by Hsu *et al.* (7) with an ABC Kit (Vector Lab., Burlingame, CA, U.S.A.). Anti-bovine S-100 protein rabbit serum (13) (kindly provided by Professor T. Akagi and Dr K. Takahashi, Kochi Medical University) was diluted 1:8,000. The sections were treated with the antiserum for 30 min at room temperature and then incubated sequentially with biotinylated anti-rabbit IgG and avidin DH-biotin-peroxidase complex for 30 and 40 min, respectively. Finally they were treated with fresh 3,3'-diaminobenzidine- H_2O_2 mixture in Tris-HCl buffer (0.05 M, pH 7.6) for 5–10 min. Sections were counterstained with hematoxylin or methyl green. Control sections incubated with normal rabbit serum in place of the primary serum showed no staining.

One specimen of appendiceal carcinoid that had been stored in 10% neutralized buffered formalin was also examined by conventional electron microscopy and immunoelectron microscopy for S-100 protein. For conventional electron microscopy, the tissue was postfixed in phosphate buffered 1% OsO_4 , embedded in Epon, and stained with uranyl acetate-lead citrate. For immunoelectron microscopy (14), cryostat frozen sections were treated with anti-S-100 protein serum and then with HRPO-labelled anti-rabbit F(ab) $_2$ goat Fab. After fixation in 1% glutaraldehyde, they were treated with 3,3'-diaminobenzidine, postfixed in 1% OsO_4 , and embedded in Epon. Ultrathin sections were observed after counterstaining with lead citrate.

RESULTS

Argyrophilic cells, demonstrated by the Grime-lius procedure, were observed not only in epithelial glands but also in the lamina propria of the mucosa of normal or slightly inflamed appendices. These intramucosal extraepithelial argyrophilic cells were seen as single cells or clusters of a few cells, and were located near the muscularis mucosae (Fig. 1), but they were few and were found in only seven of the appendices examined.

On immunostaining of the appendices for S-100 protein, numerous intensely immunoreactive fibers and cells indicating nerve fibers (Schwann cells) were seen forming a complicated network in the appendiceal wall. In the lamina

Fig. 1 Serial sections of normal appendiceal mucosa showing an extraepithelial argyrophilic cell surrounded by an S-100 protein-positive cell (arrow). A and C: S-100 protein-positive cells and fibers are seen in the lamina propria. Cytoplasmic processes of the immunoreactive cell surround a non-immunoreactive cell. ABC method for S-100 protein, counterstained with methyl green. $\times 360$. B: An extraepithelial argyrophilic cell (arrow) identical with the cell surrounded by S-100 protein-positive cell shown in A and C. Grimelius' stain. $\times 360$

propria of subglandular areas, S-100 protein-positive cells were occasionally found surrounding a non-immunoreactive cell that had a larger and more vesicular nucleus than neighbouring inflammatory cells. Most of these non-immunoreactive cells were shown to be argyrophilic by Grimelius staining of serial sections (Fig. 1). Some non-argyrophilic cells surrounded by S-100 protein-positive cells were also seen, which seemed to be ganglion cells from their morphology.

All four carcinoid tumors examined contained many polygonal argyrophilic tumor cells. In addition, strongly S-100 protein-positive stellate cells were observed in the tumor nests irrespective of whether the tumors were trabecular or alveolar. In three tumors, these S-100 protein-

Fig. 2 An appendiceal carcinoid tumor. Stellate, intensely S-100 protein-positive cells with long cytoplasmic processes are present at the margin of each nest. ABC method for S-100 protein, counterstained with hematoxylin. $\times 230$

positive cells were mainly located at the margins of the tumor nests, and thus completely surrounded some smaller nests (Fig. 2). These cells often had long cytoplasmic processes extending into the nests from their margin. The nucleus of these cells also gave a positive reaction for S-100 protein and was more irregularly shaped and more hyperchromatic than the round and vesicular nuclei of neighbouring tumor cells. The number of S-100 protein-positive nuclei in each tumor nest varied with the size of the nest, and about ten nuclei were observed in larger nests. In the fourth tumor, the S-100 protein-immunoreactive cells were mainly located in central regions of the nests, which were larger than those in the other three tumors, and their fine, arborescent processes extended into the intercellular spaces (Fig. 3). S-100 protein-positive fibers were also seen outside the tumor nests, and connections between fibers inside and outside of the nests were occasionally found.

The polygonal tumor cells were rarely S-100 protein-immunoreactive, and when they were, their reactivity was very weak compared with the intense reaction of the stellate cells.

Electron microscopic study of a carcinoid tumor revealed that the tumor nests were composed of neurosecretory cells and peripherally located non-neurosecretory cells (Fig. 4). The

neurosecretory cells had round, euchromatic nuclei and numerous electron-dense, pleomorphic, membrane-bound granules of 125–390 nm, which resembled those of the enterochromaffin cells of the crypt epithelium. Non-neurosecretory cells always had long, thin cytoplasmic processes, which covered the neurosecretory cells and extended between them. These processes contained numerous intermediate filaments and occasionally encased axons. Therefore, these non-neurosecretory cells were identified as Schwann cells. Both components of the tumor nests were surrounded by a common and continuous basal lamina. Occasionally, very small nests were seen, composed of one neuroendocrine cell and one Schwann cell encasing axons (Fig. 5).

Immunoelectron microscopy also demonstrated that S-100 protein-positive cells were located in the periphery of tumor nests (Fig. 6). Reactive products were distributed weakly in the nucleus and diffusely in the cytoplasmic matrix, but not in the axons, which were encased by these cells. Even larger nests were covered almost completely by thin, compressed cytoplasmic processes containing S-100 protein. The identification of S-100 protein in the cells confirmed the conclusion from ultrastructural findings that these peripherally located cells were Schwann cells.

Fig. 3 Another carcinoid tumor of the appendix. S-100 protein-positive cells are seen in the inner portions of each nest and extend fine, arborescent processes between the polygonal tumor cells. ABC method for S-100 protein, counterstained with hematoxylin. $\times 200$

S-100 protein immunoreactivity could not be detected ultrastructurally in the neurosecretory tumor cells.

DISCUSSION

In this study we used a combination of argyrophilic silver staining (Grimelius) and S-100 protein immunostaining to examine serial sections of the appendix. In this way we clearly demonstrated by light microscopy the presence of complexes composed of extra-epithelial argyrophilic cells with surrounding S-100 protein-positive cell or cells in normal or slightly inflamed appendiceal mucosa. These complexes seemed to be identical with those named 'enterochromaffin cell-nerve fiber complexes (ECC-NF complexes)' (2), because of the argyrophilia of the enterochromaffin cells and immunoreactivity for S-100 protein of the Schwann cells, and also because of their resemblance to ECC-NF complex cells in location and

in topographical relation with each other (1, 2, 11, 15, 17, 18).

S-100 protein immunostaining has been useful in studies on the enteric nervous system (3, 20). Our combination method is useful for examining more extensive areas to find mucosal complexes, which are usually very sparsely distributed. Besides the complexes described above, we found other complexes composed of non-argyrophilic, S-100 protein-negative cells surrounded by S-100 protein-positive cells in the lamina propria. These non-argyrophilic cells resembled ganglion cells when stained with hematoxylin-eosin, but further examination is necessary to clarify their nature.

ECC-NF complexes were first found about 60 years ago (10), but they have only recently been examined by electron microscopy (1, 2, 11, 15). They were shown to consist of an enterochromaffin cell or cells and a polyaxonal unmyelinated Schwann cell or cells. Papadaki *et al.* (15) reported that they also contained nerve endings and occasionally neurons. In addition, the enterochromaffin cells in the complex contain immunoreactive serotonin, suggesting a relation to some functional role of these complexes (17). Papadaki *et al.* (15) concluded that these complexes were an integral part of the mucous nervous plexus and suggested that nerve fibers in these complexes might be peptidergic components of the enteric nervous system. In previous studies on the enteric nervous system, little attention was paid to the presence or functional role of these complexes within the lamina propria (4-6, 8, 16). They can now be regarded as 'a third ganglionated plexus' of the enteric nervous system from their morphological and, presumably, functional resemblance to the other two ganglionated plexuses (4, 5, 8, 16), the submucous and myenteric. However, they differ from the two latter plexuses in some ways, such as in the presence of argyrophilic cells and in their major cellular components, which are endocrine cells in the mucosal plexus but neurons in the submucous and myenteric plexuses. The characters of this mucosal complex require further study.

The origin of the enterochromaffin cells in the complex is unknown, although intraepithelial enterochromaffin cells are now believed to be of endodermal origin (19). Auböck and Ratzenhofer (2) suggested that the extraepithelial enterochromaffin cells in the complex might be derived from neuroendocrine-programmed ectoblasts because they showed no relation with the glandular epithelium.

Fig. 4 Electron micrograph showing a tumor nest of a carcinoid tumor. A: The tumor nest is composed of neurosecretory cells (N) and a peripherally located non-neurosecretory cell (S) with long cytoplasmic processes. Both components are surrounded by a common basal lamina (arrows). B: The neurosecretory cells contain numerous electron-dense, pleomorphic, membrane-bound granules of 125–390 nm identical with those of enterochromaffin cells. C: The peripherally located cell occasionally encases axons (Δ). An arrow indicates the basal lamina.

The S-100 protein-immunoreactive cells seen by light microscopy in appendiceal carcinoid tumors seem to be Schwann cells, judging from their morphology and location as determined by electron microscopy in this and previous studies (1, 18). Electron immunocytochemistry also showed that S-100 protein-positive cells were identifiable as Schwann cells. Nakajima *et al.* (12) examined S-100 protein-positive cells in various tumors. They found one bronchial carcinoid containing many stellate cells with intense immunoreactivity for S-100 protein, which seemed to be very similar with ours, and they suggested that these cells were Schwann or satellite cells. There is only one previous report of S-100 protein immunoreactivity in an appendiceal carcinoid, but details of the morphology of the S-100 protein-positive cells were not described (9).

The polygonal tumor cells occasionally showed very weak S-100 protein immunoreactivity, as observed by Nakajima *et al.* (12). This weak immunoreactivity may be caused by a small

amount of antibody to the α -subunit of S-100 protein present in the anti-S-100 protein serum used in this study (personal communication). This anti- α -subunit antibody has been recently reported to react with neurons (ganglion cells) (21). Further investigation is required on this problem.

The S-100 protein-positive cells (Schwann cells) in three of four appendiceal carcinoid tumors were located at the margins of tumor nests, not among the non-reactive polygonal tumor cells. This suggests that the Schwann cells in tumor nests might be compressed to the periphery by proliferating tumor cells containing neurosecretory granules. Thus they may not be co-proliferating neoplastic components but remnants of pre-existing complexes of Schwann cells and other cells (enterochromaffin cells). Nakajima *et al.* (12) suggested that the presence of Schwann or satellite cells in a bronchial carcinoid tumor might be explained by the pluripotential nature of tumor cells. On the other hand, from ultrastructural observations,

Fig. 5 Electron micrograph showing a small nest composed of one neurosecretory cell (N) and one Schwann cell (S) encasing axons (Δ)

Auböck and Höfler (1) and Rode *et al.* (18) related the presence of Schwann cells or nerve fibers in the appendiceal carcinoid tumors to

the ECC-NF complex, and proposed that at least some gastrointestinal carcinoid tumors develop from intraneural endocrine cells. They considered intratumorous nerve fibers as morphological support for this idea (1). Their idea is also supported by our ultrastructural finding in a carcinoid tumor of very small tumor nests consisting of only one or two enterochromaffin cells and Schwann cells with features identical to those of ECC-NF complexes. This attractive hypothesis remains to be established by investigations on tumors at various stages of growth, including microcarcinoid tumors, and on various markers characterizing the function or nature of the cells forming the tumor.

We are grateful to Professor Tadaatsu Akagi and Dr Kiyoshi Takahashi for kindly providing anti-S-100 protein serum. We also thank Mrs N. Amo for excellent technical assistance. This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

Received for publication 3 February 1984

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Fig. 6 Immunoelectron micrograph of a small tumor nest. S-100 protein is present in peripherally located Schwann cells (S), but not in axons (Δ). No reactive products could be detected in the neurosecretory cells (N).

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