Identification of 5-hydroxytryptamine-producing cells by detection of fluorescence in paraffin-embedded tissue sections

Y. Kaneko, 1, N. Onda, 1, Y. Watanabe, 1, 2, 3 M. Shibutani

1R&D Group, Olympus Corporation, Tokyo
2Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, Tokyo
3United Graduate School of Veterinary Sciences, Gifu University, Japan

Abstract

5-Hydroxytryptamine (5-HT) produced by enterochromaffin (EC) cells is an important enteric mucosal signaling ligand and has been implicated in several gastrointestinal diseases, including inflammatory bowel disease and functional disorders such as irritable bowel syndrome. The present study reports a new, simple and rapid visualization method of 5-HT-producing EC cells utilizing detection of fluorescence in paraffin-embedded tissue sections after formalin fixation. In human samples, there was a high incidence of fluorescence+ cells in the 5-HT+ cells in the pyloric, small intestinal and colonic glands, while co-localization was lacking between fluorescence+ and gastrin+ cells in the pyloric and small intestinal glands. Fluorescence+ EC cells were detected in the colon of mice and rats. Fluorescence+ cells were also observed in 5-HT+ β cells in the pancreatic islets of Langerhans in pregnant mice, while non-pregnant mouse pancreatic islet cells showed no 5-HT immunoreactivity or fluorescence. These results suggest that fluorescence+ cells are identical to 5-HT+ cells, and the source of fluorescence may be 5-HT itself or molecules related to its synthesis or degradation. This fluorescence signal detection method may be applicable for monitoring of inflammatory status of inflammatory bowel diseases in both the experimental and clinical settings.

Introduction

There are a variety of different types of enteroendocrine cell, each of which synthesizes different types of hormones, and some of them contain multiple hormones within one cell. 5 Enteroendocrine cells distributed dif-
After air-drying for 10 min, microscopic observation was performed with a fluorescence microscope (BX63; Olympus Corp., Tokyo, Japan) either with ultraviolet filter (Olympus Corp.; excitation 340-390 nm, photography 420-700 nm), blue filter (Olympus Corp.; excitation 460-495 nm, photography 510-700 nm), green filter (Olympus Corp.; excitation 530-550 nm, photography 570-700 nm), and microphotographs were taken with a DPC3 CCD camera (Olympus Corp.). After rinsing the slides for 5 min in water, immunostaining or hematoxylin and eosin (HE) staining was carried out.

In the heart and liver tissues of a common squirrel monkey, serially cut sections were either subjected to autofluorescence detection, HE staining or periodic acid-Schiff (PAS) staining.

Detection of fluorescence was similarly performed in acetone-fixed cryosections of human colonic tissues. Serially cut sections were either subjected to fluorescence detection or hematoxylin staining.

Measurement of fluorescence hyperspectrum in tissue sections

Fluorescence hyperspectrum in tissue sections was measured using hyperspectral camera (NH-7; EBA Japan, Tokyo, Japan) connected with a fluorescence microscope (BX63; Olympus corp.).

Immunohistochemistry

For immunohistochemistry, sections were pretreated with peroxidase-blocking solution (Dako, Glostrup, Denmark) for 5 min at room temperature to quench endogenous peroxidase. After several rinses in 1x phosphate-buffered saline (PBS; pH 7.4), tissue sections were incubated with 10 mM citrate buffer, pH 6.0, at 95°C for 20 min for antigen retrieval. After several rinses in PBS, the sections were blocked with 5% horse serum in PBS for 30 min at room temperature to eliminate nonspecific antibody binding. The sections were then incubated at room temperature with rabbit anti-5-HT antibody (Nichirei Biosciences Inc., Tokyo, Japan; ready-to-use) for 60 min, monoclonal anti-human 5-HT antibody (Dako; 1:50 dilution) for 30 min, monoclonal anti-human chromogranin A (CGA) antibody (Dako; 1:100 dilution) for 30 min, or rabbit anti-human gastrin antibody (Dako; ready-to-use) for 20 min. After several rinses in PBS, the sections were incubated with the second antibody: goat anti-mouse or anti-rabbit immunoglobulins conjugated to horseradish-peroxidase-labeled polymer (EnVision™ + Dual Link System-HRP; Dako) for 30 min at room temperature. After several rinses in PBS, immunodetection was carried out using Histofine DAB Substrate Kit (Nichirei Biosciences Inc., Tokyo, Japan; ready-to-use).

Figure 1. Fluorescence imaging of formalin-fixed paraffin-embedded human colon tissue sections. A-C) Arrows indicate fluorescence signals; scale bar: 50 μm. The same section was subjected to fluorescence imaging through UV filter (A), blue filter (B) and green filter (C) after deparaffinization, and then, the section was stained with hematoxylin and eosin (D).

Figure 2. Co-localization of fluorescence+ cells with neuroendocrine cell markers in the human colonic glands. Red arrows indicate identical cells. Scale bar: 50 μm.
Biosciences) as the chromogen. Sections were then counterstained using Mayer’s hematoxylin solution (Wako Pure Chemical Industries Ltd., Osaka, Japan).

**Distribution of fluorescence+ and immunolocalized cells**

In each of the pyloric, small intestinal and colonic glands, incidence of fluorescence+ cells within the population of immunoreactive cells for CGA, 5-HT and gastrin in the whole tissue area was estimated (n=3–5 in each tissue). In the pancreatic tissue, incidence of fluorescence+ cells within the population of 5-HT+ cells in the whole tissue area was estimated (n=1 of non-pregnant mouse and n=3 of pregnant mice).

**Results**

**Fluorescence imaging of human colon tissue sections**

Fluorescence signals were detected sparsely in epithelial cells of the colonic crypts (Figure 1 A-D). Fluorescence was detected under a visible wavelength range showing green in color through an ultraviolet filter (Figure 1A), yellow in color through a blue filter (Figure 1B), and red in color through a green filter (Figure 1C).

**Immunoreactivity of neuroendocrine cell markers in fluorescence+ cells**

Distribution of fluorescence+ digestive glandular cells was compared with that of neuroendocrine cell markers using identical sections of the colon in humans (Figure 2). Many, but not all, of fluorescence+ cells showed CGA immunoreactivity. In contrast, all of fluorescence+ cells were identical to 5-HT+ cells distributed in the pyloric glands, small intestinal glands, and the colonic glands. Conversely, gastrin+ cells were lacking in the colonic glands (Figure 2), while they were distributed mainly in the pyloric glands.

Distribution of fluorescence+ digestive glandular cells was compared with that of 5-HT using identical sections of the pyloric and small intestinal glands in humans (Figure 3). All of fluorescence+ cells were identical to 5-HT+ cells in these glands.

Incidence of CGA+ cells showing fluorescence+ cells showing fluorescence imaging of human colon tissue sections

**Figure 1.** Co-localization of fluorescence+ and 5-HT+ cells in the human pyloric and small intestinal glands. Red arrows indicate identical cells. Scale bars: 50 µm.

**Figure 2.** Incidence of CGA+, 5-HT+ or gastrin+ cells showing fluorescence in the human pyloric, small intestinal and colonic glands.

**Figure 3.** Incidence of CGA+, 5-HT+ or gastrin+ cells showing fluorescence in the human pyloric, small intestinal and colonic glands.

**Figure 4.** Incidence of CGA+, 5-HT+ or gastrin+ cells showing fluorescence in the human pyloric, small intestinal and colonic glands.

**Figure 5.** Co-localization of fluorescence+ and 5-HT+ cells in the colon across the species. A) Co-localization in the colon of a human, mouse and rat; red arrows indicate identical cells; scale bars: 50 µm. B) Incidence of 5-HT+ cells showing fluorescence.
cence was 31% in the pyloric glands, 73% in the small intestinal glands, and 70% in the colonic glands (Figure 4). Incidence of 5-HT⁺ cells showing fluorescence was 83% in the pyloric glands, 93% in the small intestinal glands, and 97% in the colonic glands. Incidence of gastrin⁺ cells showing fluorescence was 0% in all of the pyloric, small intestinal and colonic glands.

Distribution of fluorescence⁺ colonic glandular cells was compared with that of 5-HT⁺ cells between humans, mice and rats (Figure 5A). Incidence of 5-HT⁺ cells showing fluorescence was 97% in humans, 93% in mice, and 93% in rats (Figure 5B).

Distribution of fluorescence⁺ islet cells was compared with that of 5-HT⁺ cells in the pancreas between pregnant and non-pregnant female mice (Figure 6A). While non-pregnant mice did not have any fluorescence⁺ and 5-HT⁺ cells in the pancreatic islets, pregnant mice showed that 95% of 5-HT⁺ cells were fluorescence⁺ (Figure 6B).

Detection of fluorescence in acetone-fixed cryosections

Acetone-fixed cryosections of human colonic tissues did not show fluorescence signals (Figure 7).

Lipofuscin fluorescence signals in lipofuscin-deposited heart and liver tissue sections

Fluorescence signals of lipofuscin in the heart and liver were detected under a visible wavelength range showing orange to yellow in color through an ultraviolet filter (Figure 8). PAS-positive lipofuscin deposits in myocardial fibers and liver cells showed mostly fluorescence signals.

Hyperspectral fluorescence in tissue sections

Shape of hyperspectral fluorescence curve was different between 5-HT⁺ cells of human colon and lipofuscin deposited in the heart and liver of a common squirrel monkey (Figure 9).

Discussion

In the present study, fluorescence signals were detected sparsely in the gastrointestinal tract cells, which are CGA⁺, and collectively constitute the gastrointestinal neuroendocrine tissue. CGA is co-stored and co-released with monoamines and peptide hormones of the adrenal medulla, pituitary gland, parathyroid, thyroid C-cells, pancreatic islets, endocrine cells of the gastrointestinal tract and sympathetic nerves. EC cells also express CGA, and therefore, fluorescence signals in subpopulation of CGA⁺ cells may be related to endocrine secretion system. In the present study, fluorescence⁺ cells showed the highest concordance with 5-HT⁺ cells in each of the pyloric, small intestinal and colonic glands, while fluorescence did not appear in any of the gastrin⁺ cells that are known to be mainly distributed in the pyloric antrum of the stomach to stimulate secretion of HCl by the parietal cells of the stomach. Furthermore, there were no species differences in the incidence of 5-HT⁺ cells in colonic glandular cells among humans, rats and mice, in the present study. The fluorescence⁺ cells were identically observed in 5-HT⁺ β cells in the pregnant mouse pancreatic islets of Langerhans, while non-pregnant mouse β cells lacked both fluorescence and 5-HT expression. These results suggest that fluorescence⁺ cells are identical to 5-HT⁺ cells. The source of fluorescence may be 5-HT itself or molecules related to its synthesis or degradation.

Synthesis of 5-HT by intestinal EC cells begins with the conversion of dietary tryptophan to hydroxy-L-tryptophan, which is catalyzed by tryptophan hydroxylase (Tph). Previous studies have identified two isoforms of the Tph enzyme, Tph1, which is present in mainly peripheral organs such as the gut, and Tph2, which is associated with the nervous system and present predominantly in the brain stem. The second step for 5-HT synthesis is catalyzed by L-amino acid decarboxylase, which is also present in EC cells, and converts 5-hydroxy-L-tryptophan to 5-HT. Degradation of 5-HT is done by monoamine oxidase A by...
conversion into 5-hydroxyindole acetaldehyde and then by aldehyde dehydrogenase for conversion into 5-hydroxyindoleacetic acid (5-HIAA). Until now, no studies have reported the presence of autofluorescence in Tph, monoamine oxidase A, 5-hydroxyindole acetaldehyde or l-amino acid decarboxylase. In contrast, autofluorescence was observed with 5-HT, tryptophan, 5-hydroxy-l-tryptophan, and 5-HIAA under the UV wavelength region from 344 to 352 nm. On the other hand, there are studies reporting that autofluorescence signals of intact form of 5-HT can be detected in the visible light wavelength region, when they are accumulated in high concentration, utilizing special equipment. Therefore, it is suggested that the detection method of 5-HT⁺ cells presented here has advantages in its easiness and sensitivity.

As previously mentioned, 5-HT can be changed to an intensely fluorescent derivative by the Falck–Hillarp method or its modification, suggesting that fluorescence can be detected in formaldehyde-fixed tissues. In the present study, we observed that acetone-fixed cryosections of human colonic tissues did not show fluorescence signals as observed in formalin-fixed paraffin-embedded colonic tissues. 5-HT produced by EC cells is an important enteric mucosal signaling molecule and has been implicated in several gastrointestinal diseases, including inflammatory bowel disease and functional disorders such as irritable bowel syndrome. On the other hand, colonic preneoplastic proliferative lesions in humans increases autofluorescence signals as compared with the normal or hyperplastic colonic epithelia due to increase of lysosomal lipofuscin granules. There is also a pathological condition, named as melanosis coli, characterized by lipofuscin deposition in macrophages of the lamina propria of intestinal mucosa. In the present study, fluorescence signal of 5-HT⁺ cells in formalin-fixed tissue sections was detected under a visible wavelength range showing green/yellow in color through an ultraviolet filter, in contrast to the orange to yellow in lipofuscin granules. We also found that shape of hyperspectral fluorescence curve was different between 5-HT⁺ cells and lipofuscin deposits. Therefore, autofluorescence signals derived from lipofuscin deposits can be distinguished from 5-HT⁺ cell-derived fluorescence signals. In the present study, we report a new visualization method of 5-HT-producing cells utilizing detection of fluorescence in paraffin-embedded tissue sections after formalin fixation. By this method, EC cells of the gastrointestinal tract can easily be detected in humans, rats and mice, and therefore it may be applicable for monitoring of inflammatory status of inflammatory bowel diseases in both the experimental and clinical settings.

Figure 8. Fluorescence imaging of formalin-fixed paraffin-embedded heart and liver sections of a common squirrel monkey. Scale bars: 50 µm.

Figure 9. Hyperspectral fluorescence curve of 5-HT⁺ cells in a human colon and lipofuscin deposits in the heart and liver of a common squirrel monkey.
References

1. Cho HJ, Robinson ES, Rivera LR, McMillan PJ, Testro A, Nikfarjam M, et al. Glucagon-like peptide 1 and peptide YY are in separate storage organelles in enteroendocrine cells. Cell Tissue Res 2014;357:63-9.

2. Scott RV, Tan TM, Bloom SR. Can Bayliss and Starling gut hormones cure a worldwide pandemic? J Physiol 2014;592:5153-67.

3. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007;132:397-414.

4. Côté F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J. Maternal serotonin is crucial for murine embryonic development. Proc Natl Acad Sci USA 2007;104:329-34.

5. Karsenty G, Gershon MD. The importance of the gastrointestinal tract in the control of bone mass accrual. Gastroenterology 2011;141:439-42.

6. Sumara G, Sumara O, Kim JK, Karsenty G. Gut-derived serotonin is a multifunctional determinant to fasting adaptation. Cell Metab 2012;16:588-600.

7. Kim H, Toyofuku Y, Lynn FC, Chak E, Uchida T, Mizukami H, et al. Serotonin regulates pancreatic beta cell mass during pregnancy. Nat Med 2010;16:804-8.

8. Keating DJ, Spencer NJ. Release of 5-hydroxytryptamine from the mucosa is not required for the generation or propagation of colonic migrating motor complexes. Gastroenterology 2010;138:659-70.e2.

9. Spencer NJ, Nicholas SJ, Robinson L, Kyoh M, Flack N, Brooks SJ, et al. Mechanisms underlying distension-evoked peristalsis in guinea pig distal colon: is there a role for enterochromaffin cells? Am J Physiol Gastrointest Liver Physiol 2011;301:G19-27.

10. Kidd M, Gustafsson BI, Drozdov I, Modlin I, IL1β- and LPS-induced serotonin secretion is increased in EC cells derived from Crohn’s disease. Neurogastroenterol Motil 2009;21:439-50.

11. Bertrand PP, Barajas-Espinosa A, Neshat S, Bertrand RL, Lonax AE. Analysis of real-time serotonin (5-HT) availability during experimental colitis in mouse. Am J Physiol Gastrointest Liver Physiol 2010;298:G446-55.

12. Bischoff SC, Maller R, Pabst O, Weier G, Siedlik W, Li Z, et al. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. Am J Physiol Gastrointest Liver Physiol 2009;296:G685-95.

13. Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, et al. Serotonin has a key role in pathogenesis of experimental colitis. Gastroenterology 2009;137:1649-60.

14. Haub S, Ritzé Y, Berghelm I, Pabst O, Gershon MD, Bischoff SC. Enhancement of intestinal inflammation in mice lacking interleukin 10 by deletion of the serotonin reuptake transporter. Neurogastroenterol Motil 2010;22:826-34, e229.

15. Falck B, Torp A. New evidence for the localiz-ation of noradrenalin in the adrenergic nerve terminals. Med Exp Int J Exp Med 1962;6:169-72.

16. Falck B. Observation on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta Physiol Scand 1962;56(Suppl. 197):1-25.

17. Lindvall O, Bjorklund A. The glialmodic method: a detailed account of the methodology for the visualization of central catecholamine neurons. Histochemistry 1974;39:97-127.

18. Taupenot L, Harper KL, O’Connor DT. The chromogranin-secretogranin family. N Engl J Med 2003;348:1134-49.

19. Wiedenmann B, Huttnner WB. Synaptophysin and chromogranins/secre-togranins–widespread constituents of distinc-tive types of neuroendocrine vesicles and new tools in tumor diagnosis. Virchows Arch B Cell Pathol Incl Mol Pathol 1989;58:95-121.

20. Giovannozzo F, Schimmack S, Svejda B, Alaimo D, Pfargner R, Modlin I, et al. Chromogranin A and its fragments as reg-u-lators of small intestinal neuroendocrine neoplasms proliferation. PLoS One 2013;8:e81111.

21. Sachs G, Prinz C, Loo D, Bamberg K, Besancon M, Shin JM. Gastric acid secre-tion: activation and inhibition. Yale J Biol Med 1994;67:81-95.

22. Takaishi S, Shibata W, Tomita H, Jin G, Yang X, Erickson R, et al. In vivo analysis of mouse gastric gene regulation in enhanced GFP-BAC transgenic mice. Am J Physiol Gastrointest Liver Physiol 2011;300:G334-44.

23. Walther DJ, Peter Ju, Bashamaksh S, Hörtagl H, Voits M, Fink H, et al. Synthesis of serotonin by a second trypto-phan hydroxylase isofrome. Science 2003;299:76.

24. Walther DJ, Bader M. A unique central tryptophan hydroxylase isofrome. Biochem Pharmacol 2003;66:1673-80.

25. Håkansson R, Owman C, Sjöberg NO, Sporrong B. Amine mechanisms in enterochromaffin and enterochromaffin-like cells of gastric mucosa in various mammals. Histochemie 1976;21:189-220.

26. Manocha M, Khan WI. Serotonin and GI Disorders: An update on clinical and experimental studies. Clin Transl Gastroenterol 2012;3:e13.

27. Kato T, Tokuhashi Y, Hashidzume Y, Miyachi K, Mori Yama K, Morimoto S, et al. [Simple and highly sensitive fluorometric determina-tion of serotonin using propylene glycol]. Bunseki Kagaku 2011;60:685-9 [In Japanese].

28. Crespi F, Croce AC, Fiorani S, Masala B, Heidbreder C, Bottiroli G. Autofluo-rescence spectrofluorometry of central nervous system (CNS) neuromediators. Lasers Surg Med 2004;34:39-47.

29. Balaji J, Desai R, Kaushalya SK, Eaton MJ, Maiti S. Quantitative measurement of serotonin synthesis and sequestration in individual live neuronal cells. J Neurochem 2005;95:1217-26.

30. DaCosta RS, Andersson H, Cirocco M, Marcon NE, Wilson BC. Autofluor-escence spectrofluorometry of central nervous system (CNS) neuromediators. Lasers Surg Med 2004;34:39-47.

31. Freeman HJ. “Melanosis” in the small and large intestine. World J Gastroenterol 2008;14:4296-9.