Histochemical Journal 27, 482–486 (1995)

Endocrine cells of the human gastrointestinal tract have no proliferative capacity

P. BARRETT[1,2], R. C. HOBBS[2], P. J. COATES[3], R. A. RISDON[4], N. A. WRIGHT[5] and P. A. HALL[3]*

[1]*Army Histopathology Registry, Royal Army Medical College, London SW1P 4RJ, UK*
[2]*Division of Histopathology, UMDS, St Thomas's Campus, Lambeth Palace Road, London SE1 7EH*
[3]*Department of Pathology, Ninewells Hospital & Medical School, University of Dundee, Dundee, DD1 9SY*
[4]*Department of Histopathology, Great Ormond Street Hospital, London WC1N 3JH*
[5]*ICRF Histopathology Unit, ICRF Laboratories, Lincoln's Inn Fields, London WC2A 3PN & RPMS, Hammersmith Hospital, DuCane Road, London W12 0NN*

Summary

There is compelling evidence that the epithelial cell lineages of the gastrointestinal tract are derived from a common stem cell precursor, but the details of the subsequent cellular hierarchies remain uncertain. In this context, it is important to know the arrangement of cell proliferation that gives rise to the final cell populations. In rodents, a number of studies have been performed examining the possible proliferative capacity of endocrine cells, but a wide range of technical problems makes interpretation of these data difficult. Continuous labelling studies suggest that there is potential for proliferation in endocrine cells but flash labelling studies have not been conclusive. In man there are no data on this issue. We have taken advantage of the ability to perform double immunostaining for operational markers of proliferation (Ki67 antigen) and endocrine cell phenotype (chromogranin expression). We demonstrate that there are no double-labelled cells in the normal stomach, small intestine or colon of fetal, neonatal or adult humans. Moreover, no double-labelled cells are found in pathological states associated with endocrine cell hyperplasia (gastritis, ulcerative colitis). These data indicate that the normal endocrine cells of the human gut have no proliferative capacity and that, in this cell lineage, population expansion precedes differentiation.

Introduction

The vertebrate gastrointestinal tract is a highly organized structure with a carefully integrated arrangement of epithelial cells forming a continuous sheet lining the entire lumen. There is continuous turnover of this epithelial population and massive loss of cells with replacement of the entire cell mass every three days (Wright & Alison, 1984). Maintenance of this vital structure requires the production of differentiated cells from a stem cell zone with expansion of the population by a transit-amplifying compartment. The exact organization of these populations remains poorly understood (Hall & Watt, 1989; Potten & Loeffler, 1990). Indeed, the complexity is compounded by the existence of at least four discrete differentiated cell types; columnar, absorptive, Paneth and endocrine cells. For many years it was argued that this latter population derived from migration of neural crest cells. The notion that all these cells had a single origin (the Unitarian Hypothesis; Cheng & Leblond, 1974a, b) has, however, remained persuasive. Recently, direct evidence has been provided that the endocrine population is locally derived (Thompson *et al.*, 1990). What is much less clear is how this cell type (and the other differentiated cells of the gut) are derived from the putative stem cell compartment.

If we accept the Unitarian Hypothesis then it is necessary to consider the lineage structure that allows a single stem cell to give rise to all four differentiated cell types. Two extreme models might be proposed; there might be production of undifferentiated cells by cell division with subsequent differentiation, or alternatively, there may be some degree of differentiation *before* the final cell number of any given phenotype is defined. Evidence for one or other model would be provided by examining the proliferative capacity of differentiated cells with a given phenotype. In the case of the endocrine cells of the gastrointestinal tract there is conflicting evidence regarding their proliferative capacity. A number of studies have provided data suggesting that the endocrine cells of the rodent stomach, small intestine and colon do have some proliferative capacity (Deschner & Lipkin, 1966;

*To whom correspondence should be sent.

Odartchenko *et al.*, 1970; Lehy & Willems, 1975, 1976; Yeoman & Trier, 1976; Fujimoto *et al.*, 1979). In contrast other studies indicated that, although there was labelling in continuous labelling studies, no flash labelling with tritiated thymidine could be observed (Messier & Leblond, 1960; Chang & Leblond, 1971; Ferriera & Leblond, 1971; Cheng & Leblond, 1974a, b; Tsubouchi & Leblond, 1979). Methodological problems make interpretation of much of these data difficult and at present no information is available with regard to the endocrine cells of the human gut. The availability of antibodies that allow the operational definition of endocrine cells in formalin-fixed, wax-embedded material, coupled with the recent development of antibodies that recognize the Ki67 antigen (a well defined marker of proliferation: Gerdes *et al.*, 1984; McCormick *et al.*, 1993a; Schluter *et al.*, 1993) now allows re-examination of this issue in human gastrointestinal epithelium. These studies provide further evidence that population expansion precedes differentiation of endocrine cells in the human gastrointestinal epithelium.

Materials and methods

Formalin-fixed, wax-embedded blocks (n = 53) representing a wide range of normal and pathological conditions were retrieved from the archives of the Department of Pathology, UMDS and Great Ormond Street Hospitals, London. Samples included normal stomach (n = 3), normal small intestine (n = 6), normal colon (n = 3), gastritis (n = 3), gastric adenocarcinoma (n = 13), inflammatory (n = 6) and neoplastic (n = 2) small intestinal disease, and inflammatory (n = 6) and neoplastic (n = 11) colon. Examples of gastric, small and large intestinal neoplasms all had adjacent non-neoplastic mucosa. Histological material from fetuses (n = 6, estimated gestational ages 13 to 28 weeks) and neonates (n = 6, ages three to 36 months) were also examined.

Sections (4 μm thick) were cut from all cases and Haemotoxylin and Eosin as well as immunostained sections were examined. Immunostaining was performed for chromogranin and Ki67 both singly and as a sequential double stain. In these experiments double labelling was facilitated by the availability of polyclonal anti-Ki67 (Dakopatts A047) and monoclonal anti-chromogranin A (Dakopatts M869). For immunohistochemistry, sections placed on silane-coated slides were dewaxed and taken to phosphate-buffered saline (PBS). Anti-chromogranin A was incubated at a dilution of 1:50 for a period of 1 h at room temperature. After extensive washes in TRIS buffer, this was detected by the alkaline phosphatase method using a Fast Red coupler and substrate. After blocking endogenous peroxidase with hydrogen peroxide in methanol for 30 min, the sections were immersed in approximately 500 ml 0.01 M sodium citrate, pH 6.0, and microwaved for 20 min at maximum power in an 850 W Panasonic microwave oven. After washing in PBS, the sections were incubated in polyclonal Ki67 at a dilution of 1:50 for 1 h at room temperature. After washing in PBS, the polyclonal antibody was detected using the streptavidin-ABC method with nickel–cobalt enhancement. A light Haematoxylin counterstain was then used and the section mounted.

Quantification of both chromogranin-positive endocrine cells and Ki67-positive cells was performed by examining stained sections under a ×40 objective and counting the number of chromogranin-positive endocrine cells per crypt. The number of cells that showed cytoplasmic chromogranin and also nuclear Ki67 immunoreactivity were also assessed. In each case, at least 20 crypts or glands were examined (mean number ±SD of crypts or glands = 85.3 ±25.8, range 20–154). In tumours, at least 3000 epithelial cells were examined (typically in a minimum of 10 fields). Rigorous interpretation was employed to avoid terming a cell double-labelled incorrectly. A cell was deemed to be double-labelled only if the brown-black nuclear immunoreactivity (Ki67 antigen) was surrounded by red cytoplasmic immunoreactivity (chromogranin A). Cells in which there were Ki67-positive nuclei and in which the adjacent cytoplasm showed chromogranin labelling were deemed not to be double-labelled due to the difficulty in objectively distinguishing this from staining of contiguous cells. This approach is reasonable since endocrine cells have centrally placed nuclei and there is no evidence that neuroendocrine granules are asymmetrically arranged within the cytoplasm (Ham, 1974).

Results

The number of chromogranin-positive endocrine cells identified in the stomach, small intestine, and colon are shown in Table 1. The number of identifiable chromogranin-positive cells was the same in single label as in double label experiments. Table 1 indicates that, with the exception of carcinoid tumours, in all tissues examined no chromogranin and Ki67 double-labelled

Table 1. Absolute number of chromogranin-labelled cells in single-label and double-label experiments, and the number of chromogranin/Ki67 double-labelled cells per crypt or gland

	Chromogranin (single label)	*Chromogranin (double label)*	*Chromogranin Ki67*
Stomach	10.5 (6.98)[a]	11.9 (10.03)[a]	0.0
Small intestine	2.21 (1.18)[b]	1.74 (0.9)[b]	0.0
Large intestine	–	5.6	0.0
Adenocarcinomas	–	–	0.0

[a,b] There is no signficant difference between the number of chromogranin-labelled cells in single-label and double-label experiments.

cells were identified. This represents no double-labelled cells out of more than 6000 chromogranin-positive cells, in a total estimated population of 200 000 cells examined. In contrast, in carcinoid tumours occasional double-labelled cells were identified, although these were typically less than 1% of the population. It is also of note that in fetal and neonatal gut epithelium no double-labelled cells were identified. Similarly, no double-labelled cells were seen in the context of inflammatory conditions of the gut associated with endocrine cell hyperplasia (gastritis, ulcerative colitis and Crohn's disease).

Discussion

The detailed kinetic organization of the mammalian gut remains an enigma. Although there is excellent evidence that a common stem cell gives rise to all epithelial lineages, the detailed organization of these populations is not well understood. Furthermore, it is important to recognize that, even within mammals, there may be important differences in the detailed histology and kinetic organization of this tissue. Despite this, several points appear to be generally accepted. First, that in mice, goblet cells share a common progenitor with columnar cells. Second, that on the basis of both experimental and modelling studies, the murine goblet cell lineage differentiates from the transit population two or three generations *before* the end of the lineage (i.e. population expansion accompanies differentiation; Paulus *et al.*, 1993). The generality of this view is difficult to define, since in rodents it has been proposed that there are functionally distinct sub-populations of goblet cell (Wright & Alison, 1984). Similar arguments pertain to the columnar lineage (Wright & Alison, 1984; Paulus *et al.*, 1993). Finally, that the situation in the Paneth cell compartment is unclear, but that the weight of evidence points to this population being an end cell derived directly from the adjacent stem cell compartment (Wright & Alison, 1984). This leaves the endocrine cell lineage where the available data from rodents are conflicting, and where no data have been previously reported from human gastrointestinal tissues.

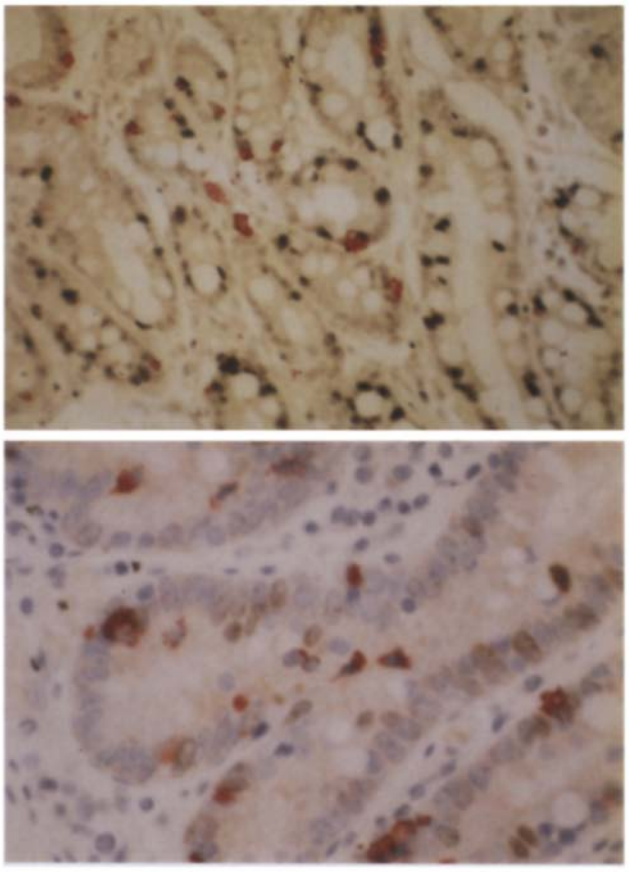

Fig. 1. Chromogranin A immunoreactivity (red) within the cytoplasm of epithelial cells in the gastrointestinal tract: (a) stomach and (b) colon. Nuclear Ki67 immunoreactivity (brown or black) is present in many cells but in no case is there co-localization of Ki67 and chromogranin within the same cell.

The studies reported here can only be accepted if the antigens detected are good operational markers of cell proliferation and endocrine cell differentiation. With regard to Ki67 there are abundant data that expression of this molecule is tightly associated with cell proliferation. Specifically, the spatial and temporal pattern of expression is closely linked to the cell cycle (Gerdes *et al.*, 1984; McCormick *et al.*, 1993a), and quantification of expression closely reflects the experimentally determined growth fraction in *in vitro* and *in vivo* assays (McCormick *et al.*, 1993b). Consequently, the weight of evidence points to Ki67 expression being an excellent marker of proliferation. Similarly, there is a large body of evidence in support of the concept that expression of chromogranin A is a good marker of endocrine cell differentiation (Wilson & Lloyd, 1984; Varndell *et al.*, 1985). The chromogranin A protein is a component of the secretory granule in which the hormone products of endocrine cells are packaged (Varndell *et al.*, 1985). Given that the presence of neurosecretory granules is the *sine qua non* of an endocrine cell, it follows that expression of chromogranin A is a reasonable operational definition of an endocrine cell. Indeed, the expression of chromogranins occurs early during the development of neuroendocrine cells in the gastrointestinal tract, before the appearance of individual hormones (Facer *et al.*, 1989). One caveat must be that if neurosecretory granules are sparse then the levels of labelling with antibodies to chromogranin A will be low. This can be countered by using sensitive detection methods as we have done in this study.

Given that chromogranin A and Ki67 represent

robust markers of endocrine cell differentiation and proliferation, then a simple conclusion can be drawn from the immunohistological studies presented here: the endocrine cells of the human gut are a terminally differentiated population with no proliferative capacity. However, we concede that very early committed endocrine cells *may* be proliferative since they may fall below the threshold of detection by chromogranin A immunostaining. Our data provide another component of the information required to define the lineage relationships within the gastrointestinal tract epithelium and to issues relating to the control of cell number in this tissue. However, it does lead to an important conclusion relating to endocrine cell hyperplasia as seen in a range of conditions including exposure to omeprazole. If the endocrine cell is terminally differentiated and post-mitotic, then the increased production of endocrine cells in pathological and physiological states must relate to alterations in the stem cell population itself. This is of particular relevance to an understanding of the controversial topic of endocrine cell hyperplasia and neoplasia in the context of hypergastrinaemia (Dayal, 1992). Our data suggest that the effect of hypergastrinaemia acts at the stem cell level rather than on endocrine cells themselves. As with most issues in gastrointestinal epithelial cell biology, we are again led to the conclusion that understanding the behaviour of stem cells is absolutely required in order to have any real insight into how gut epithelial cell populations are controlled.

Acknowledgements

We thank the RAMC (AMRSE Project 483) and Dako Ltd for generous financial support. PAH is supported by the Cancer Research Campaign.

References

CHANG, W. W. L. & LEBLOND, C. P. (1971) Renewal of the epithelium in the descending colon of the mouse. II Renewal of argentaffin cells. *Am. J. Anat.* **131,** 141–69.

CHENG, H. & LEBLOND, C. P. (1974a) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. *Am. J. Anat.* **141,** 537–62.

CHENG, H. & LEBLOND, C. P. (1974b) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. III. Enteroendocrine cells. *Am. J. Anat.* **141;** 503–20.

DAYAL, Y. (1992) Hyperplastic conditions of the ECL cells. *Yale J. Biol. Med.* **65,** 805–25.

DESCHNER, E. E. & LIPKIN, N. (1966) An autoradiographic study of the renewal of argentaffin cells in the human rectal mucosa. *Expl. Cell Res.* **43,** 661–5.

FACER, P., BISHOP, A. E., COLE, G. A. *et al.* (1989) Developmental profile of chromogranin, hormonal peptides, and 5-hydroxytryptamine in gastrointestinal endocrine cells. *Gastroenterology* **97,** 48–57.

FERRIERA, M. N. & LEBLOND, C. P. (1971) Argentaffin and other endocrine cells of the small intestine of the adult mouse. II. Renewal. *Am. J. Anat.* **131,** 331–42.

FUJIMOTO, S., KAWAI, K., HATTORI, J. & FUJITA, S. (1979) Tritiated thymidine autoradiographic study on origin and renewal of gastrin cells in pyloric area of hamsters. *Gastroenterology* **78,** 1136–46.

GERDES, J., LEMKE, H., BAISCH, H. *et al.* (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki67. *J. Immunol.* **133,** 1710–15.

HALL, P. A., WATT, F. M. (1989) Stem cells and the generation and maintenance of cellular diversity. *Development* **106,** 119–33.

HAM, A. W. (1974) *Histology,* 7th edn. Toronto: J. B. Lippincott Company.

LEHY, T. & WILLEMS, G. (1975) Combined use of immunocytochemical and autoradiographic techniques for studying the renewal of gastrin cells. *Biomed. Exp.* **23,** 443–6.

LEHY, T. & WILLEMS, G. (1976) Populations dynamics of antral gastrin cells in the mouse. *Gastroenterology* **71,** 614–9.

McCORMICK, D., CHONG, H., HOBBS, C., DATTA, C. & HALL, P. A. (1993a) Detection of the Ki67 antigen in fixed and wax embedded sections with the monoclonal antibody MIB1. *Histopathology* **22,** 355–60.

McCORMICK, D., YU, C., HOBBS, C. & HALL, P. A. (1993b) The relevance of antibody concentration to the immunohistological quantification of cell proliferation associated antigens. *Histopathology* **22,** 543–8.

MESSIER, B. & LEBLOND, C. P. (1960) Cell proliferation and migration as revealed by autoradiography after injection of thymidine-^{3}H into male rats and mice. *Am. J. Anat.* **106,** 247–85.

ODARTCHENKO, N., HEDINGER, C., RUZICKA, J. & WEBER, E. (1970) Cytokinetics of argentaffin cells in the mouse intestinal mucosa. *Virchows Arch. Cell. Path.* **6,** 132–9.

PAULUS, U., LOEFFLER, M., ZEIDER, J., OWEN, G. & POTTEN, C. S. (1993) The differentiation and lineage development of goblet cells in the murine small intestinal crypt: experimental and modelling studies. *J. Cell Sci.* **106,** 473–84.

POTTEN, C. S., LOEFFLER, M. (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110,** 1001–20.

SCHLUTER, C., DUCHROW, M., BECKER, M., KEY, G. & GERDES, J. (1993) Molecular cloning of the cell proliferation antigen associated antigen defined by the antibody Ki67. *J. Cell Biol.* **123,** 513–22.

THOMPSON, E. M., FLEMING, K. A., EVANS, D. J., FUNDELE, R., SURANI, M. A. & WRIGHT, N. A. (1990) Gastric endocrine cells share a clonal origin with other gut cell lineages. *Development* **110,** 477–81.

TSUBOUCHI, S. & LEBLOND, C. P. (1979) Migration and turnover of enteroendocrine and caveolated cells in the epithelium of the descending colon, as shown by radioautography after continuous infusions of ^{3}H-thymidine in mice. *Am. J. Anat.* **156,** 431–51.

VARNDELL, I. M., LLOYD, R. V., WILSON, B. S. & POLAK, J. M. (1985) Ultrastructural localization of chromogranin: a potential marker for the electron microscopical recognition of endocrine secretory granules. *Histochem. J.* **17,** 981–92.

WILSON, B. S. & LLOYD, R. V. (1984) Detection of chromogranin in neuroendocrine cells with a monoclonal antibody. *Am. J. Pathol.* **115,** 458–68.

WRIGHT, N. A. & ALISON, M. (1984) *The Biology of Epithelial Cell Populations.* Oxford: Oxford University Press.

YEOMAN, N. D. & TRIER, J. S. (1976) Epithelial cell proliferation and migration in developing gastric mucosa. *Develop. Biol.* **53,** 206–10.