BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Identification of Lineage-Uncommitted, Long-Lived, Label-Retaining Cells in Healthy Human Esophagus and Stomach, and in Metaplastic Esophagus

QIUWEI PAN,[1,*] ANNA M. NICHOLSON,[2,*] HUGH BARR,[3,*] LEA–ANNE HARRISON,[4,*] GEORGE D. WILSON,[5] JULIA BURKERT,[2] ROSEMARY JEFFERY,[2] MALCOLM R. ALISON,[2] LEENDERT LOOIJENGA,[6] WEY–RAN LIN,[2,7] STUART A. C. McDONALD,[2] NICHOLAS A. WRIGHT,[2] REBECCA HARRISON,[4] MAIKEL P. PEPPELENBOSCH,[1] and JANUSZ A. JANKOWSKI[2,4,8]

[1]Department of Gastroenterology and Hepatology, Rotterdam, the Netherlands; [2]Centre for Digestive Diseases, Queen Mary University of London, London, United Kingdom; [3]Department of Surgery, Gloucestershire Royal Hospital, Gloucester, United Kingdom; [4]Digestive Diseases Centre, Leicester Royal Infirmary, Leicester, United Kingdom; [5]Oakland University, Beaumont Health System, Rochester Hills, Michigan; [6]Josephine Nefkens Institute of Pathology, Erasmus MC-University Medical Center, Rotterdam, the Netherlands; [7]Department of Gastroenterology and Hepatology, Linkou Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Chang Gung, Taiwan; [8]Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth, United Kingdom

BACKGROUND & AIMS: The existence of slowly cycling, adult stem cells has been challenged by the identification of actively cycling cells. We investigated the existence of uncommitted, slowly cycling cells by tracking 5-iodo-2'-deoxyuridine (IdU) label-retaining cells (LRCs) in normal esophagus, Barrett's esophagus (BE), esophageal dysplasia, adenocarcinoma, and healthy stomach tissues from patients. **METHODS:** Four patients (3 undergoing esophagectomy, 1 undergoing esophageal endoscopic mucosal resection for dysplasia and an esophagectomy for esophageal adenocarcinoma) received intravenous infusion of IdU (200 mg/m^2 body surface area; maximum dose, 400 mg) over a 30-minute period; the IdU had a circulation half-life of 8 hours. Tissues were collected at 7, 11, 29, and 67 days after infusion, from regions of healthy esophagus, BE, dysplasia, adenocarcinoma, and healthy stomach; they were analyzed by in situ hybridization, flow cytometry, and immunohistochemical analyses. **RESULTS:** No LRCs were found in dysplasias or adenocarcinomas, but there were significant numbers of LRCs in the base of glands from BE tissue, in the papillae of the basal layer of the esophageal squamous epithelium, and in the neck/isthmus region of healthy stomach. These cells cycled slowly because IdU was retained for at least 67 days and co-labeling with Ki-67 was infrequent. In glands from BE tissues, most cells did not express defensin-5, Muc-2, or chromogranin A, indicating that they were not lineage committed. Some cells labeled for endocrine markers and IdU at 67 days; these cells represented a small population (<0.1%) of epithelial cells at this time point. The epithelial turnover time of the healthy esophageal mucosa was approximately 11 days (twice that of the intestine). **CONCLUSIONS: LRCs of human esophagus and stomach have many features of stem cells (long lived, slow cycling, uncommitted, and multipotent), and can be found in a recognized stem cell niche. Further analyses of these cells, in healthy and metaplastic epithelia, is required.**

Keywords: Adult Stem Cell; Gastrointestinal; Cancer; Tissue Regeneration.

The study of long-lived cells in the human gastrointestinal tract has been limited by experimental constraint in organized tissues.[1–4] Pulse-chase experiments use labeled nucleotides to identify label-retaining cells (LRCs)[1,5] and determine the turnover time of tissues by the administration of detectable nucleoside analogues for short periods (pulse) followed by detection of the label at a later period (chase). LRCs previously were assumed to be a population that may represent the stem cells of a tissue.[2] This assumption follows the theory that stem cells should seek ways of protecting their genome from mutations. One way is to divide less frequently than the transit-amplifying population, therefore limiting the opportunity for DNA replication errors to occur. Adult stem cells are a long-lived, multipotent population of cells responsible for the replacement of differentiated cells lost in systems with rapid cell turnover/loss and provide for regeneration after injury or insult.[6–10]

The area of stem cell biology is in major flux, competing and contradictory theories are being reported monthly. In addition, these workers use in vitro, animal, and, occa-

*Authors share co-first authorship.

Abbreviations used in this paper: ATPase, adenosine triphosphatase; BE, Barrett's esophagus; IBL, interpapillary basal layer; IdU, 5-iodo-2'-deoxyuridine; LGR5, leucine-rich repeat containing G-protein 5; LRC, label-retaining cell; mRNA, messenger RNA.

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sionally, human model systems. These confusing data are particularly troublesome in the case of the esophagus and the common premalignant lesion Barrett's esophagus (BE). First, there have been reports of fast cycling cells with stem cell capacity in animal models and some of them indicate a residual stem cell population is needed whereas others do not.[11–15] Second, some additional reports have implicated endodermal (including epithelial) embryonic markers in human beings but their relevance to human stem cells remains unclear.[16] Third, other markers also have been implicated in the epithelial mesenchymal transition of BE cells as they progress to cancer.[17] It remains unclear therefore whether these markers actually label stem cells or relate more to cancer stem cells. Fourth, markers such as the homeobox gene *cdx2* appear to promote intestinalization rather than label specific stem cell populations per se.[18] Fifth, even the relatively simple question of the location of stem cells in the normal stomach and esophagus in human beings have not been proposed previously, although data are available in animal models. Despite these problems, other techniques that trace lineage by genetic analyses strongly favor the likelihood of common stem cells between squamous and columnar esophageal mucosa.[19–24] The site of stem cells in BE has been more problematic[10–15] as well as the availability of true biomarkers of stem cells.[16–18] Furthermore, it has been shown that cancer stem cells may divide quickly after recovery from chemotherapy, thereby allowing re-expansion of resistant clones.[11] Therefore, assessing whether stem cell turnover is different between benign, premalignant, and neoplastic lesions in the same patient would be informative.

Identification of stem cells would help in understanding the pathophysiology of the gastroesophageal junction. Specifically, the frequency and location of stem cells may enable their isolation for study. This could improve the diagnosis, prognostication, and even the targeting of new therapies for BE.

Our aims therefore were to show that on either side of the esophagogastric junction there are long-lived undifferentiated cells (>11 days) (true LRCs) that can periodically cycle and also still can commit to mature cell types. The availability of dysplastic and neoplastic lesions in these tissues also allowed us to study the presence of label-retaining cells in various stages of disease including cancer. Therefore, we aimed to assess whether LRCs also could be found in the metaplastic, dysplastic, and neoplastic esophagus, as well as normal mucosa.

Materials and Methods

Clinical Protocol

The Stem cell Assessment In Neoplastic Tissues trial was approved by the Leicestershire Ethics Board, reference number: 09122; Medicines Health Regulatory Authority, number: CTA 21275; and the Research Ethics Committee, number: 7213; in 2002 (this followed an earlier approval by the University of Birmingham Hospitals 1998). The trial sponsor was the University Hospitals of Leicester Trust and chief investigator Janusz Jankowski. Two sites were used for tissue acquisition: Gloucestershire Royal Hospital and Leicester Royal Infirmary, both in England. Four patients were recruited to the study. After informed consent was obtained, an intravenous infusion of 5-iodo-2'-deoxyuridine (IdU) (gift from George Wilson) at a dose of 200 mg/m^2 body surface area (maximum dose, 400 mg) was given over a 30-minute period and had a circulation half-life (t1/2) of 8 hours. Labeled cells therefore represent only a small proportion of the total number of cells dividing in even 24 hours. Each 200-mg vial was reconstituted with 10 mL of water and the resultant solution then was added to 250 mL of 0.9% sodium chloride to generate the infusion solution. After the infusion the vital signs were recorded and patients were monitored every 30 minutes for a further 3 hours.

Patients' Summary

Patients 1 and 2 underwent esophagectomy 7 days after infusion. Patient 3 was infused 11 days before surgery, and patient 4 was infused 29 days before an esophageal endoscopic mucosal resection for dysplasia and an esophagectomy for esophageal adenocarcinoma, 67 days after infusion (Supplementary Figure 1). None of the patients underwent preoperative chemoradiotherapy. Tissues were obtained from normal esophagus, any areas of BE, dysplasia, or adenocarcinoma, and from normal stomach within the resection margins.

In Situ Hybridization

Specific localization of human leucine-rich repeat containing G-protein 5 (*LGR5*) messenger RNA (mRNA) was accomplished by in situ hybridization using an antisense riboprobe synthesized with SP6 RNA polymerase using ^{35}S-uridine triphosphate and appropriate tissue and experimental controls as previously described.[25]

Tissue Fixation, Immunohistochemistry, Immunofluorescence, and Flow Cytometry

Tissue was fixed and processed into paraffin blocks as per standard procedures.[24] We cut between 500 and 1000 sections (including intervening spares) from each block and we had approximately 10–15 blocks per patient resection and 5 for endoscopic resection. Because we had 4 patients for esophagectomy and 1 patient with endoscopic resection, we had more than 50,000 sections. Antibodies used were as follows: IdU (IdU/bromodeoxyuridine) (18.8 μg/mL; Dako UK Ltd, Cambridgeshire, UK), sheep anti-IdU/bromodeoxyuridine (10 ug/mL; Abcam, Burlingame, CA) anti–Ki-67 (0.5 g/μmL; Dako UK Ltd), anti-Pan cytokeratin (9.2 μg/mL; Dako UK Ltd), anti–Mucin-2 (40 μg/mL; Abcam), anti–chromogranin A (0.42 μg/mL; Dako UK Ltd), adenosine triphosphatase (ATPase) (0.25 μg/mL; Dako UK Ltd), anti–carbonic anhydrase II (1 μg/mL; Abcam), and anti–defensin 5 (5 μg/mL; Abcam). Other antibodies to stem cell markers were used, as follows: rabbit anti–musashi-1 (1 μg/mL; Millipore, Billerica, MA), LGR5 (0.25 μg/mL; Abgent Inc, San Diego, CA), CD133 (1 μg/mL; Biorbyt, Cambridgeshire, UK), DCAMKL1 (0.5 μg/mL; Abcam), and CDX2 (prediluted; Abcam). Immunohistochemistry, immunofluorescence, and flow cytometry were performed as previously described using appropriate experimental/antibodies and tissue-positive and tissue-negative controls including normal stomach, intestine, skin, colon, and pancreas; esophageal, gastric, and colonic cancer; as well as various epithelial cell lines from the squamous (OE21), columnar esophagus (OE33), as well as colorectal cancer cell lines (HCA-7, CACO-2).[9,19–24]