Isolation and Characterization of a Putative Intestinal Stem Cell Fraction From Mouse Jejunum

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Background & Aims: **Although there have been many recent advances regarding the biology of intestinal stem cells, the field has been hampered significantly by the lack of a method to isolate these cells. Therefore, the aim of this study was to explore the hypothesis that viable intestinal stem cells can be isolated as a side population (SP) by fluorescence-activated cell sorting after staining with the DNA-binding dye Hoechst 33342.** ***Methods:*** **Preparations of individual cells from either whole mucosa or epithelium of mouse jejunum were stained with Hoechst 33342 and propidium iodide and then sorted using fluorescence-activated cell sorting. Cells were characterized using fluorochrome-labeled antibodies to surface markers, intracellular markers, and annexin V to detect early apoptosis. Total RNA was isolated from sorted fractions and used for quantitative real-time reverse-transcription polymerase chain reaction to evaluate the expression of cell lineage markers and the intestinal stem-cell marker, Musashi-1.** ***Results:*** **Adult and neonatal jejunum contain a viable population of cells that shows the SP phenotype and is sensitive to verapamil. This population of cells (from both mucosal and epithelial preparations) includes a CD45-negative fraction corresponding to nonhematopoietic cells, which shows minimal expression of surface markers typically found on stem cells from other tissues and of intracellular markers found in mesenchymal cells. Additionally, these cells were enriched for Musashi-1 and β1-integrin, were cytokeratin positive, and survived in culture for up to 14 days.** ***Conclusions:*** **The CD45-negative SP fraction, although not pure, represents the successful isolation of a viable population significantly enriched in small intestinal epithelial stem cells.**

Among the mammalian tissues that show continuous renewal (bone marrow, skin, and gastrointestinal epithelium), the epithelium of the small intestine has by far the highest rate of turnover. In both rodents and humans, the majority of cells within the epithelium are replaced every 3–4 days.[1] Three principal cell lineages are found on the villi, namely: (1) absorptive cells (also called *enterocytes*), which are by far the dominant lineage, making up more than 90% of total cells; (2) goblet cells (also called *mucus cells*), comprising 8%–10% of the villus population; and (3) enteroendocrine cells (a diverse group), comprising approximately 1% of the epithelium.[2,3] A fourth lineage, namely the Paneth cells, arise from downward migration and are found at the very base of the crypts.[2,3]

Pioneering studies by Cheng and Leblond[2] in the early 1970s suggested that undifferentiated cells (which they called *crypt-base columnar cells*) located in the intestinal crypts just above the Paneth cells may serve as multipotent stem cells responsible for the generation of all 4 lineages of the small intestinal epithelium. These studies relied on the observation that at early times after administration of ^{3}H-thymidine, cells damaged by the local radiation were phagocytosed only by the crypt-base columnar cells. Subsequently, phagocytic fragments could be detected in cells of all 4 lineages.[2] Although there has been some debate as to the alternate origin of enteroendocrine cells,[3] the *Unitarian Hypothesis*, as it was called,[2] has stood the test of time.

As reviewed in detail by Gordon et al,[4] in the mid-1980s supporting evidence came from studies of mice chimeras in which the parental origin of intestinal epithelial cells could be identified by lectin staining. More recently, unequivocal confirmation of the hypothesis has been forthcoming from studies in which adult mice with intestinal epithelium that normally stains negative for the lectin *Dolichos biflorus* agglutinin are subjected to mutagenesis.[5] This leads to the appearance of lectin-positive ribbons of cells, some of which persist for very long periods of time (>150 days) and thus presumably

Abbreviations used in this paper: FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HBSS, Hank's buffered saline solution; Msi-1, Musashi-1; PE, phycoerythrin; PI, propidium iodide; PISC, putative intestinal stem cell; SP, side population.

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0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.08.011

reflect mutagenesis of a crypt stem cell. The lectin-positive ribbons were found to include all 4 lineages, thus showing conclusively that all arise from a common stem cell.[5] Understanding the biology of these stem cells is central to the development of effective rejuvenative therapies for intestinal failure and short-gut syndrome. Moreover, in situations in which the stem cells themselves are damaged, transplantation of healthy stem cells may afford a novel and effective therapy.

A critical issue in the study of intestinal epithelial stem cells has been the lack of markers for these cells. Numerous genes are expressed in the crypt compartment but not in the villus[6] and some, such as EphB2,[7] CD44,[8] and Hes1,[9] display a gradient of increased expression toward the stem-cell zone. However, until very recently, no protein or messenger RNA (mRNA) had been observed to be expressed exclusively in intestinal epithelial stem cells. Although gene expression profiling by microarray analysis, as reported by Stappenbeck et al,[10] provides a promising approach, to date a definitive stem-cell marker has not emerged from these studies. Others have used a candidate gene approach and in early 2003 this proved very fruitful with simultaneous publications by Kayahara et al[9] and Potten et al.[11] These groups reported, by immunohistochemistry and in situ hybridization, that expression of the RNA-binding protein Musashi-1 (Msi-1) is confined to the stem-cell zone of the intestinal epithelium.[9,11] This protein, which is known to play a key role in asymmetric cell division by neural stem cells,[12] thus has become a marker for intestinal epithelial stem cells. More recent studies have shown that within the murine intestine members of the BMP and Wnt signaling pathways, phospho-PTEN and phosphor-AKT, also localize to intestinal stem cells.[13,14] These findings represent significant advances in the understanding of intestinal stem-cell biology. Unfortunately, because of the nature of these proteins, they are of limited use for identifying isolated intestinal stem cells.

Despite extensive studies on the kinetics of intestinal epithelial stem cells, in both physiologic and pathologic states, efforts to isolate these cells have met with little success. For the colonic epithelium, fluorescence-activated cell sorting (FACS) based on expression of β1-integrin yields a viable population but affords only 3-fold enrichment of clonogenic activity over unsorted cells.[15,16] Although a combination of surface markers is likely to be more effective, as is the case with bone marrow stem cells,[17] to date the lack of identification of an appropriate set of such markers has hindered this classic cell-sorting approach. Thus, we chose to explore an alternate method based on recent studies that have shown that stem cells from bone marrow[18] and other various tissues can be isolated as a side population (SP) by FACS after staining with the DNA-binding dye Hoechst 33342.[19–25] By using this technique, we identified mouse intestinal SP cells and showed that these cells are distinct from hematopoietic stem cells and their progeny based on the absence of surface markers for CD45, c-kit, and CD34. Moreover, RNA prepared from CD45-negative intestinal SP cells was found to be enriched for Msi-1, leading us to conclude that the cells of this fraction are putative intestinal stem cells (PISCs).

Material and Methods

Mucosal Preparations

All mice were housed in our animal facility under a 12-hour light/dark cycle and were allowed unlimited access to rodent Lab Chow #5001 (PMI Nutrition International, Brentwood, MO) and acidified tap water. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Mucosal cells were prepared from jejunum of adult male and neonatal (postnatal days 7–14) C57Bl/6J mice by a modification of the protocol outlined by Evans et al.[26] Briefly, jejunum was removed and flushed with Hank's buffered saline solution (HBSS) and split open lengthwise. The jejunum then was cut into pieces approximately 3 mm in length and washed 6 times on an orbital shaker in 20 mL of HBSS (80 rpm). Next, the pieces were cut into 1-mm pieces and shaken (80 rpm) in HBSS containing 15 U/mL type III collagenase (Sigma, St. Louis, MO) and 0.3 U/mL dispase (Invitrogen, Carlsbad, CA) at room temperature for 1 hour. The digested tissue was pipetted up and down for 15 minutes and transferred to a conical tube, after which fetal bovine serum (FBS) was added to 5% to inhibit collagenase/dispase activity. The dissociated tissue was allowed to sediment under gravity for 1 minute and then the supernatant was removed into a new tube and sedimentation was repeated twice. The resulting supernatant was centrifuged at 300 rpm for 3 minutes and the pellet was collected. This was repeated once. The cell pellets were combined in HBSS containing 5% FBS and passed through a 70-μm filter. This method is not specific for the epithelium, but rather digests both the epithelium and some of the underlying stromal tissue (but not muscle layers). Thus, throughout the text we refer to these digests as *mucosal preparations*.

Epithelial Preparations

Intestinal epithelial cells were prepared from jejunum of adult male C57Bl/6J mice by a modification of the protocol outlined by Bjerknes and Cheng.[27] Briefly, jejunum was removed, flushed with phosphate buffered saline, and everted on a glass rod. The glass rod was immersed and vibrated in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 30 mmol/L ethylenediaminetetraacetic acid (EDTA) for 20–25 minutes to yield individual crypts and villi. These were pelleted at 500 rpm for 5 minutes. The pellet was resuspended in

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