Proliferative Characteristics of Differentiated Cells in Familial Adenomatous Polyposis-Associated Duodenal Adenomas

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The authors have previously shown that duodenal adenomas in familial adenomatous polyposis (FAP) patients typically reveal abundant cells with endocrine differentiation (ED), Paneth differentiation (PD), and goblet cell differentiation (GD). However, the biological significance and proliferative potential of these cells is unknown. To study the proliferative properties of cells with ED, PD, or GD in FAP-associated duodenal adenomas, the authors used a double-labeling immunohistochemical technique to detect simultaneously the presence of proliferating cell nuclear antigen (PCNA), and either chromogranin or lysozyme in individual neoplastic cells. Adenomatous cells with GD were identified morphologically and also evaluated for the degree of PCNA expression by immunohistochemistry. Duodenal adenomas and the adjacent nonadenomatous epithelium from 10 FAP patients were studied. Cells with ED, PD, and GD were present in all adenomas, and constituted 14.1%, 11.6%, and 17.7% of adenomatous cells, respectively. The overall proliferative index of nondifferentiated adenomatous cells was 33.3%, which was similar to the proliferative index obtained for adenomatous cells with GD (31.2%) and nonadenomatous crypt goblet cells (34.9%). In contrast, adenomatous cells with ED and PD showed a significant decrease in their proliferative potential ($P < .001$). Only 6.0% and 7.3% of cells with ED and PD, respectively, were proliferative. Nonadenomatous crypt endocrine and Paneth cells showed no proliferative potential (proliferative index 0%). These results suggest that, in the process of proliferation and differentiation, specific subpopulations of adenomatous cells attempt to recapitulate the biological characteristics of their normal counterparts in the small intestinal crypts. Adenomatous cells with ED and PD are hypoproliferative, a finding that is consistent with their differentiated phenotype and suggests that these cells may not participate as actively in the growth of these lesions. HUM PATHOL 27:63-69. Copyright © 1996 by W.B. Saunders Company

Key words: **familial adenomatous polyposis, Paneth cell, endocrine cell, goblet cell, proliferating cell nuclear antigen.**

Abbreviations: **FAP, familial adenomatous polyposis; ED, endocrine differentiation; PD, Paneth differentiation; GD, goblet cell differentiation; TBS, tris-buffered saline; Ad, adenoma; PCNA, proliferating cell nuclear antigen; PI, proliferative index.**

Familial adenomatous polyposis (FAP) is an autosomal dominant disease characterized by the development of multiple colonic adenomas and, ultimately, adenocarcinoma at a young age.[1-3] The most common type of malignant tumor that develops in FAP patients following prophylactic colectomy is duodenal adenocarcinoma.[4,5] There is evidence to suggest that duodenal carcinomas arise from adenomas in a manner analogous to the adenoma-carcinoma sequence described in the colon.[6] Although duodenal adenomas develop in 40% to 95% of FAP patients,[3,4,7-9] their natural history and malignant potential are poorly understood.

The authors have previously shown that most FAP-associated duodenal adenomas (> 95%) contain abundant cells with endocrine differentiation (ED), Paneth differentiation (PD), and goblet cell differentiation (GD).[10] These cells contain cytologically dysplastic nuclei, despite their differentiated phenotype. Furthermore, an inverse relationship exists between the proportion of cells with ED or PD and the classic histological features of aggressive behavior in these lesions, such as the degree of dysplasia, the extent of villous architecture, and size.[10] This finding suggested to us that adenomatous cells with either ED or PD may not participate as aggressively in the neoplastic growth of these lesions as adenomatous cells without differentiated features. It is similarly unclear whether adenomatous cells with GD are mitotically active, even though their mucin histochemical properties are similar to their normal nonneoplastic counterpart in the small intestine.[10] Therefore, using a double-label immunohistochemical technique, the authors undertook this study to evaluate the proliferative potential of adenomatous cells with ED, PD, and GD in FAP-associated duodenal adenomas.

METHODS

Study Group

The study group consisted of 10 FAP patients (five male, five female; average age, 39 years) who had endoscopically and histologically confirmed duodenal adenomas. All individuals were part of an ongoing upper gastrointestinal tract surveillance program for patients with FAP at the Mt. Sinai Hospital, Toronto, Canada. All had a previous colectomy for diffuse colonic adenomatosis, and none had endoscopic or histological evidence of duodenal carcinoma. Mucosal biopsies of adenomas were obtained at the time of endoscopy with the use of a side-viewing flexible endoscope (Olympus Company, Burlington, MA).

Histological Analysis

All specimens were fixed overnight in 10% buffered formalin and embedded in paraffin. Serial 5-μm thick tissue sections were prepared. One section of each lesion was stained with hematoxylin-eosin and assessed for type (tubular, tubulovillous, and villous) and degree of dysplasia (mild, moderate, or severe) according to previously described methods.[10]

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Presented in part at the 83rd Annual Meeting of the United States and Canadian Academy of Pathology, San Francisco, CA, March 1994.

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0046-8177/96/2701-0012$5.00/0

Additional paraffin sections were stained immunohistochemically as described later.

Immunohistochemical Analysis

For each adenoma, two separate double-labeled immunostained sections were prepared: one for combined PCNA and chromogranin staining, and one for combined PCNA and lysozyme staining. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide (H_2O_2) for 15 minutes. After a wash in distilled water, nonspecific binding was blocked by incubation with 10% horse serum. Tissue sections were then incubated with anti-PCNA monoclonal antibody PC10 (Novocastra, Newcastle upon Tyne, England) at a dilution of 1:100 for 16 hours at room temperature and then washed with tris-buffered saline (TBS). The sections were then incubated with biotinylated horse antimouse IgG antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 for 45 minutes, washed, and then incubated with avidin-peroxidase complexes (Vectastain Elite ABC Kit, Vector Laboratories). Peroxidase activity was detected by incubation with .05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), .01% cobalt chloride, and .03% H_2O_2 in TBS.[11] The inclusion of cobalt chloride in the reaction solution resulted in a blue/black (rather than brown) reaction product.

Tissue sections were then secondarily stained for either chromogranin or lysozyme. To detect chromogranin, PCNA-stained tissue sections were incubated with a monoclonal antichromogranin A IgG antibody (Biomeda, Foster City, CA) for 1 hour at room temperature, washed, and incubated for 40 minutes with peroxidase-conjugated rabbit antimouse immunoglobulin (DAKO, Carpinteria, CA). Lysozyme was detected by incubation with polyclonal antilysozyme antibody (Dako) for 1 hour at room temperature followed by application of peroxidase-conjugated swine antirabbit immunoglobulin (DAKO) for 40 minutes at room temperature. Chromogranin-associated or lysozyme-associated peroxidase activity was detected by incubation with .05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and .03% H_2O_2 in TBS. This resulted in a brown reaction product. Tissue sections were mounted in Permount (Fisher Scientific, Fairlawn, NJ).

As negative controls, tissue sections were stained with irrelevant class and subclass matched monoclonal antibodies (for monoclonal PC10 or anti-chromogranin A antibodies) or preimmune rabbit serum (for antilysozyme antiserum). Additionally, the surrounding nonadenomatous epithelium (in each biopsy) served as an internal positive control.

Quantitative Analysis

For each specimen, both adenomatous and nonadenomatous epithelium was evaluated. In adenomas, at least 500 cells were examined for each double-labeled immunohistochemical preparation by selecting high power oil-immersion fields at all levels of each adenoma (base, midportion, and surface) and counting all cells in each field. Only cells with clearly identifiable nucleus and cytoplasm were considered adequate for evaluation. Although some variation in the intensity of the PCNA staining reaction was evident, only cells that showed a diffuse nuclear staining reaction darker than the background reaction seen in villous enterocytes were considered positive. Examples of cells scored as PCNA negative or PCNA positive are indicated by arrows and arrowheads, respectively, in Figs 2 and 3. The number of PCNA-positive cells, chromogranin or lysozyme-positive cells, cells with GD, PCNA-positive cells with GD, and double-labeled (PCNA-chromogranin or PCNA-lysozyme) cells were counted. The proliferative index of each adenoma (PI_{Ad}) was then calculated as the proportion of PCNA-positive cells among all of the adenomatous cells counted (including cells with ED, PD, and GD). The proliferative index of the cells with ED (PI_{ED}) was calculated as the proportion of double-labeled (combined

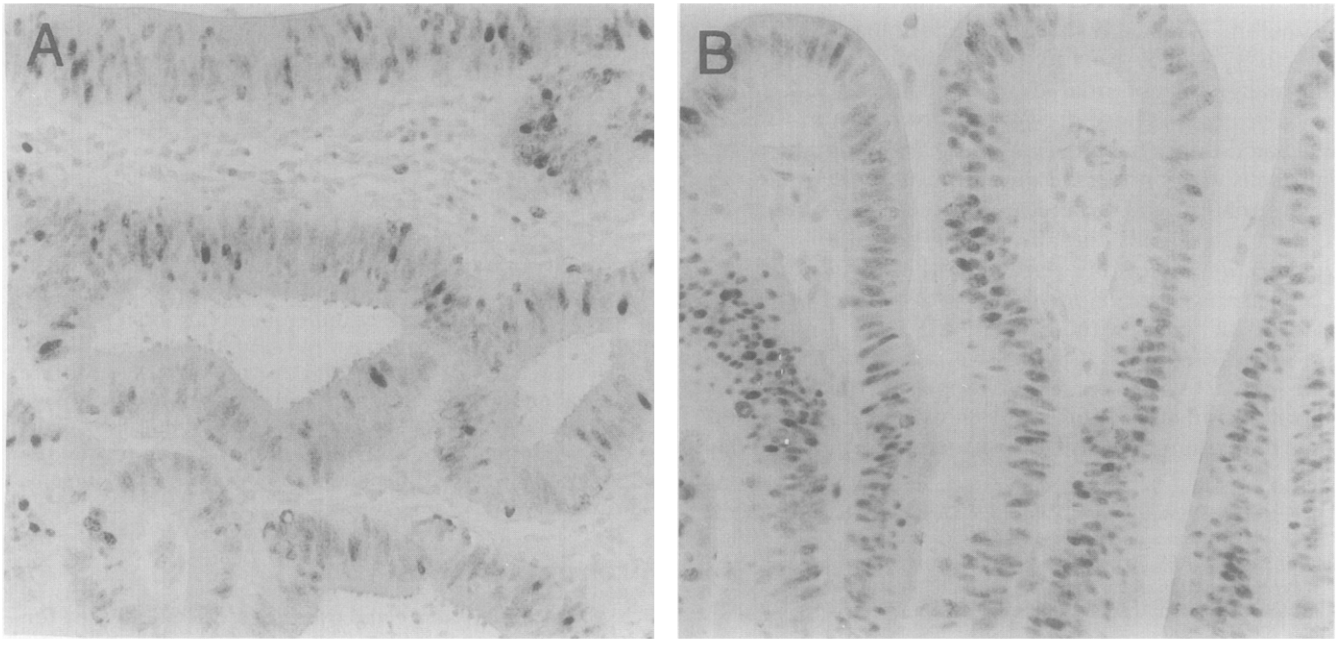

FIGURE 1. Distribution of PCNA, chromogranin, and lysozyme in FAP-associated duodenal adenomas. Low power view of a duodenal adenoma double-stained for PCNA and chromogranin (A) or PCNA and lysozyme (B). PCNA-positive cells (blue-black nuclear staining) are seen in the crypt and surface epithelium. Chromogranin- and lysozyme-positive cells (brown cytoplasmic staining) are also not restricted to the crypt epithelium but are present on the surface as well.

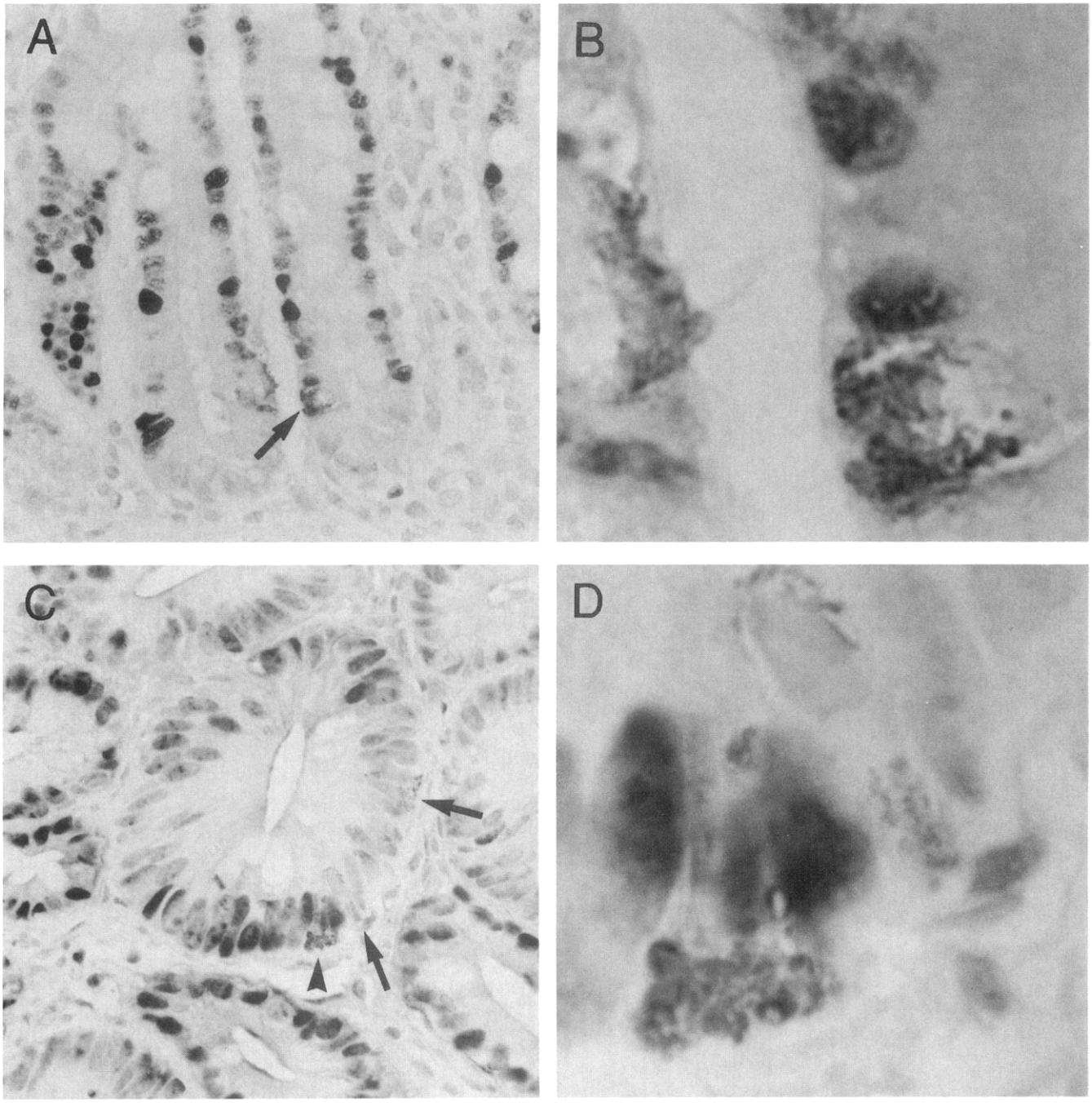

FIGURE 2. Double-label immunohistochemistry for PCNA and chromogranin in nonadenomatous duodenal epithelium (A and B) and FAP-associated duodenal adenomas (C and D). Intermediate power (A and C) and corresponding high power (B and D) views are shown. In normal nonadenomatous epithelium, PCNA-positive cells (blue-black nuclear staining) are present mainly in the lower two thirds of the crypt epithelium and are separate from cells that stain positively for chromogranin (arrow, brown cytoplasmic staining). In contrast, adenomatous epithelium contains cells that are double-labeled for both nuclear PCNA and cytoplasmic chromogranin (arrowhead). However, even in adenomas, not all chromogranin-positive cells are PCNA positive (arrows). Both PCNA-positive and PCNA-negative nonadenomatous goblet cells and adenomatous cells with GD are easily found.

PCNA-chromogranin positive) cells among the total number of chromogranin-positive cells counted. Similarly, the proliferative index of the cells with PD (PI_{PD}) was calculated as the proportion of double-labeled (combined PCNA-lysozyme positive) cells among the total number of lysozyme-positive cells counted. The proliferative index of the cells with GD (PI_{GD}) was calculated as the proportion of PCNA-positive cells with GD among the total number of cells with GD counted.

In the adjacent nonadenomatous epithelium, at least 500 endocrine, Paneth, and goblet cells from the entire length of the crypts were evaluated. A quantitative analysis of absorptive enterocytes was not performed. For the occasional goblet cell that contained particularly voluminous cytoplasm, the nucleus that was present directly adjacent to, and in contact with, the basal portion of the cytoplasmic mucin vacuole was considered to be the nucleus of that particular cell. For particularly difficult cells, evaluation of thin (5-μm) serial levels was able to resolve the issue in all instances. The number of dou-

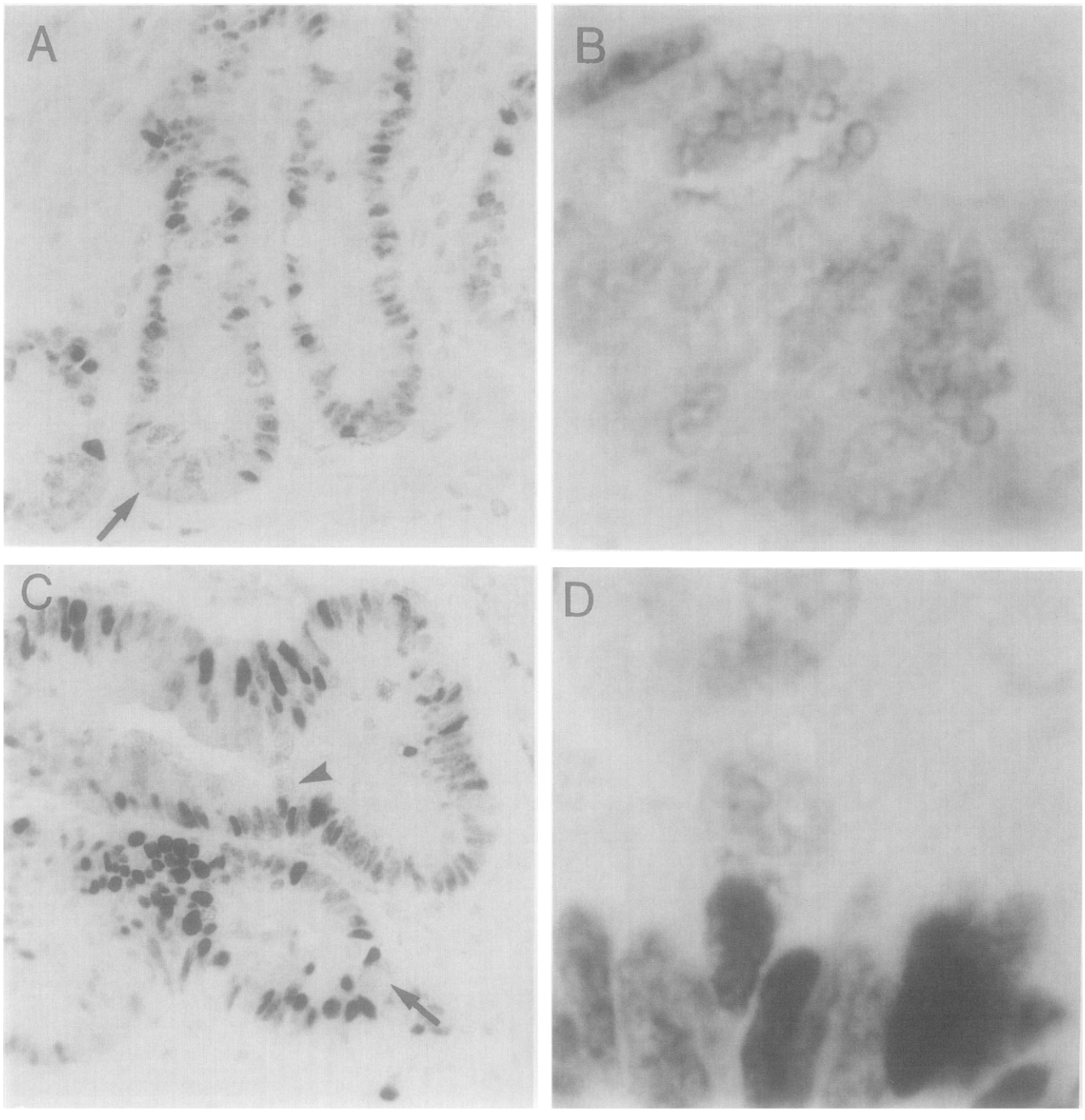

FIGURE 3. Double-label immunohistochemistry for PCNA and lysozyme in nonadenomatous duodenal epithelium (A and B) and FAP-associated duodenal adenomas (C and D). Intermediate power (A and C) and corresponding high power (B and D) views are shown. In normal nonadenomatous epithelium, PCNA-positive cells (blue-black nuclear staining) are present mainly in the lower two thirds of the crypt epithelium and are not seen in cells that stain positively for lysozyme staining (arrow, brown cytoplasmic staining). In contrast, adenomatous epithelium contains cells that are double-labeled for both nuclear PCNA and cytoplasmic lysozyme (arrowhead). However, even in adenomas, not all lysozyme-positive cells are PCNA positive (arrow). Both PCNA-positive and PCNA-negative nonadenomatous goblet cells and adenomatous cells with GD are easily found.

ble-labeled (PCNA-chromogranin or PCNA-lysozyme) and PCNA-positive goblet cells were counted and expressed as a fraction of the total number of chromogranin-positive, lysozyme-positive, or goblet cells counted.

Statistical Analysis

Statistical comparisons were evaluated using Student's *t*-test.

RESULTS

Six adenomas were tubular, two were tubulovillous, and two were villous. Four adenomas exhibited mild dysplasia, four had moderate dysplasia, and two had severe dysplasia. Seven adenomas were less than 1 cm in size and three were ≥ 1 cm. All adenomas (100%)

TABLE 1. Proliferative Index of Specific Cell Populations in FAP-Associated Duodenal Adenomas

Specimen and Cell Type	Proportion of Cells in Adenoma (% ± SD)	Proliferative Index (% ± SD)
Adenoma	NA	33.3% ± 7.9%
Nondifferentiated cells		
Cells with ED	14.1% ± 5.9%	6.0% ± 5.4%*
Cells with PD	11.6% ± 2.1	7.3% ± 6.5%†
Cells with GD	17.7% ± 4.9	31.2% ± 13.8%†
Nonadenomatous crypt epithelium		
Endocrine cells	NA	0%
Paneth cells	NA	0%
Goblet cells	NA	34.9% ± 3.9%

Abbreviations: NA, not applicable; ED, endocrine differentiation; PD, paneth differentiation; GD, goblet differentiation; FAP, familial adenomatous polyposis.

* $P < .001$.

† $P < .001$.

contained cells with cytological features of ED, PD, or GD. Adenomatous cells with ED, PD, or GD were distributed both superficially and deep within the adenomatous epithelium. Cytologically, each of these differentiated cell types had elongated hyperchromatic nuclei similar in appearance to the surrounding nondifferentiated adenomatous cells. Adenomatous cells with ED contained small basally located cytoplasmic granules similar in appearance to those of nonneoplastic endocrine cells. Adenomatous cells with PD contained large apically located eosinophilic granules typical of nonneoplastic Paneth cells. Adenomatous cells with GD typically had a single large apical mucin vacuole that distended the cytoplasm.

In nonadenomatous duodenal epithelium, PCNA-positive cells were limited to the lower two thirds of the crypts. Endocrine and Paneth cells were predominantly located in the lower one third of the crypts. Goblet cells were present throughout the crypt and villous epithelium. No double-labeled (PCNA-chromogranin or PCNA-lysozyme) cells were identified in nonadenomatous duodenal epithelium. PCNA-positive goblet cells were present in the lower two thirds of the crypts, but not in the upper one third of the crypts or in the villous epithelium (Table 1).

The overall average proliferative index of the adenomas (PI_{Ad}) was 33.3% ± 7.9% (mean ± SD) with a range of 21.6% to 48.2%. PCNA-positive adenomatous cells were distributed evenly throughout adenomas (Fig 1), both superficially and at the base, in contrast to their distribution in nonadenomatous epithelium. Cells with ED constituted 14.1% ± 5.9% (range, 6.4% to 26.2%); cells with PD formed 11.6% ± 2.1% (range, 7.4% to 15.6%); and cells with GD constituted 17.7% ± 4.9% (range, 12.0% to 24.0%) of adenomatous cells, respectively (Table 1). The proliferative index of the adenomatous cells with GD (PI_{GD}) was 31.2% ± 13.8% (range, 13.3% to 47.1%) and was similar to the PI_{Ad} (33.3% ± 7.9%). In contrast to nondifferentiated adenomatous cells and adenomatous cells with GD, only a small proportion of adenomatous cells with ED and PD showed proliferative activity (Figs 2 and 3). The PCNA-positive adenomatous cells with ED or PD were present in the superficial and basal portions of the adenomas, and did not appear to have any preferential localization within these tumors. The proliferative index of cells with ED (PI_{ED}) and of cells with PD (PI_{PD}) was 6.0% ± 5.4% (range, 0% to 17.5%) and 7.3% ± 6.5% (range, 0% to 20.3%), respectively. The PI_{ED} and PI_{PD} were significantly lower than the PI_{Ad} ($P < .001$). A positive correlation was noted between the PI_{ED} and the PI_{Ad} ($R^2 = .78$) and also between the PI_{GD} and the PI_{Ad} ($R^2 = .98$). However, a similar association was not present between the PI_{PD} and the PI_{Ad} ($R^2 = .25$). No significant correlation was noted between the PI_{ED}, PI_{PD}, or PI_{GD} of the adenomas and any of the histological features including the degree of dysplasia.

DISCUSSION

The authors have previously shown that FAP-associated duodenal adenomas typically contain a significant proportion of cells with ED and PD, and that the proportion of these differentiated cells in adenomas is inversely related to histological features of aggressive behavior.[10] Although this suggests that the presence of numerous cells with ED or PD in adenomas may serve as a marker of less aggressive malignant potential, the biological properties of these differentiated-appearing cells remains unknown. These tumors also show abundant cells with GD; although their mucin histochemical properties are similar to normal small intestinal goblet cells, the proliferative potential of these cells is similarly unknown.

Therefore, the purpose of this study was to evaluate the proliferative potential of cells with ED, PD, or GD in FAP-associated duodenal adenomas to determine if adenomatous cells with these features express the proliferative phenotype of their nonneoplastic counterparts or the hyperproliferative properties typical of adenomatous epithelium. The authors used a double-label immunohistochemical technique that enabled us to detect nuclear staining simultaneously with PCNA, and either cytoplasmic chromogranin or cytoplasmic lysozyme, in individual adenomatous cells. PCNA is a nuclear antigen that accumulates during the late G1 and S phases of the cell cycle, and is present in actively proliferating cells.[12] Chromogranin is a component of endocrine cell granules and is a sensitive marker of cells with endocrine differentiation.[10,13,14] Lysozyme is an enzyme component of Paneth cell granules.[10,15-17] Using this immunohistochemical technique, the authors were able to evaluate the proliferative activity of subpopulations of adenomatous cells with either ED or PD. These results showed that adenomatous cells with either ED or PD are hypoproliferative relative to both the nondifferentiated adenomatous epithelium and the cells with GD. However, in contrast to normal nonadenomatous crypt endocrine and Paneth cells, which did not possess any proliferative potential, adenomatous cells with ED and PD did show a slight degree of proliferative activity. This finding suggests that these cells do not represent

entrapped nonneoplastic crypt cells but are neoplastic, a theory supported by the dysplastic morphological appearance of their nuclei and by the fact that these cells were present in a dispersed pattern throughout the adenomas. The decreased mitotic activity of these cells in comparison with nondifferentiated adenomatous cells is likely a reflection of their specific differentiated phenotypes and may indicate that they do not participate as actively in the process of neoplastic growth as nondifferentiated neoplastic cells. In contrast, the authors showed that adenomatous cells with GD are as mitotically active as nondifferentiated adenomatous cells and also proliferate at a rate comparable with nonneoplastic crypt goblet cells. It seems that as adenomatous cells differentiate, they acquire not only the morphological but also the proliferative characteristics of their normal nonneoplastic counterparts in the small intestinal crypts. Perhaps adenomatous cells with features of ED, PD, or GD represent an attempt by neoplastic cells to recapitulate the normal pathways of gastrointestinal stem cell differentiation.

Other than the present study, only one report has examined the PCNA-staining properties of differentiated cells within gastrointestinal neoplasms. Ooi et al[18] reported that chromogranin-positive gastric carcinoma cells are not PCNA-immunoreactive and, therefore, not proliferative. There are several potential explanations for the discrepancy between our results and those of Ooi et al. For example, Ooi et al used anti-PCNA monoclonal antibody clone 19A2, whereas the authors used clone PC10. The proportion of PC10-positive cells correlates with other methods of analyzing cell proliferation, such as bromodeoxyuridine and ^{3}H-thymidine labeling indices.[19-23] Additionally, PC10 has been reported to be more sensitive than 19A2 in formalin-fixed paraffin-embedded tissue.[20] Also, it is possible that a true biological difference exists between cells with ED in gastric carcinomas[24] and those in FAP-associated duodenal adenomas. Finally, it is possible that the some of the chromogranin-positive cells identified in association with gastric carcinomas in their study represent entrapped nonneoplastic cells. Interestingly, a recent study that evaluated endocrine/paracrine cells in benign and neoplastic prostate tissue showed that these cells are postmitotic by PCNA immunoanalysis.[25]

Because neoplastic cells with ED or PD in FAP-associated duodenal adenomas are hypoproliferative, one might expect that adenomas with an abundance of these cells may behave in a biologically less aggressive manner. Furthermore, the presence of numerous differentiated cells may be interpreted as a feature of a better differentiated, and thus less aggressive, adenoma. Although this has yet to be proved in duodenal adenomas, several studies that have documented the presence of endocrine or Paneth cells in adenomas and adenocarcinomas of the stomach, small intestine, and colon failed to reveal any significant relationship to prognosis.[6,10,13,14,16,17,24,26-28] Bonar and Sweeney[13] examined 100 gastric adenocarcinomas, of which 26 contained endocrine cells, and concluded that gastric adenocarcinomas containing endocrine cells are as biologically aggressive as those without endocrine cells. Smith and Haggitt[28] compared survival in colorectal carcinomas that contained endocrine cells and found no significant effect on survival relative to controls without endocrine cells. In contrast, both de Bruine et al.[29] and Hamada et al.[30] examined chromogranin-positive cells in colorectal neoplasms and found that tumors with greater numbers of these cells tended to be more aggressive than chromogranin-negative tumors.[31] Thus, although some previous studies suggest that the presence of endocrine-differentiated cells in malignant gastrointestinal lesions may be prognostically useful, they did not address the properties of these cells in premalignant neoplastic lesions, as in this study. The authors did not detect a significant correlation between any of the histological features of the adenomas and the PI_{Ad}, PI_{ED}, PI_{PD}, or PI_{GD}. However, one possible explanation may be related to the small sample size used in this study.

Small series of gastrointestinal neoplasms with Paneth cells have been reported including examples of Paneth cell-rich adenocarcinomas, which continue to show Paneth cell differentiation at metastatic sites.[32] However, no information on the prognostic significance of these cells in gastrointestinal neoplasms is available.

There are no data available regarding the biological or prognostic significance of goblet cell differentiation in benign premalignant lesions of the gastrointestinal tract, even though most gastrointestinal tract adenomas contain a significant proportion of cells with GD.[33] Although most investigators think that mucins have limited value in assessing the risk of malignant change in the gastrointestinal tract,[34] the authors have previously shown that the proportion of adenomatous goblet cells with sulphomucin expression in FAP-associated duodenal adenomas is directly related to large size of these lesions,[10] a feature more often associated with malignant degeneration.

In summary, by using a double-label immunohistochemical technique, the authors have shown that cytologically dysplastic cells in FAP-associated duodenal adenomas with ED or PD are hypoproliferative relative to nondifferentiated adenomatous cells and adenomatous cells with GD. Whether the presence of these subtypes in FAP-associated duodenal adenomas is a useful indicator of prognosis remains to be determined.

Acknowledgment. The authors thank Drs D. Antonioli and J. Madara for their helpful comments in preparation of this manuscript and Bernadette Fallon for her secretarial assistance.

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