Persistent Clonal Areas and Clonal Expansion in Barrett’s Esophagus

Wendy H. Raskind, Thomas Norwood, Douglas S. Levine, Rodger C. Haggitt, Peter S. Rabinovitch, and Brian J. Reid

Departments of Medicine [W. H. R., D. S. L., R. C. H., B. J. R.] and Pathology [T. N., R. C. H., P. S. R.], University of Washington School of Medicine, Seattle, Washington 98195

ABSTRACT

Three patients with Barrett’s esophagus who had cytogenetic abnormalities detected in their metaplastic epithelium developed high-grade dysplasia or adenocarcinoma during prospective surveillance over a period of 1.5 to 6 years. In the 3 cases, cytogenetic abnormalities that were associated with the most advanced histological lesions were present in samples obtained 11, 25, and 48 months prior to the diagnosis of high-grade dysplasia or carcinoma. In a fourth patient, marker chromosomes found in a Barrett’s adenocarcinoma were also present in an esophageal region spatially removed from the tumor. In all 4 patients, clonal cytogenetic abnormalities were present in samples obtained at widespread locations in the Barrett’s segment. These observations suggest that in some patients with Barrett’s esophagus clonal proliferations arise in regions of benign histology and spread to involve large areas of Barrett’s mucosa. These clones persisted when the disease progressed to high-grade dysplasia or adenocarcinoma.

INTRODUCTION

Barrett’s esophagus is a condition in which the normal stratified squamous mucosa is replaced by a metaplastic columnar mucosa. It develops as a complication of chronic gastroesophageal reflux in 10–12% of patients and predisposes to the development of esophageal adenocarcinoma (1, 2). In the 1970s and 1980s, the incidence of Barrett’s associated adenocarcinoma, once rare, increased more rapidly than that of any other cancer in the United States (3, 4). Unfortunately, over 90% of esophageal adenocarcinomas are detected at an advanced, incurable stage (5–7).

Several studies have concluded that endoscopic surveillance of patients with Barrett’s esophagus is warranted for the early detection of adenocarcinoma (8, 9), but the factors responsible for neoplastic progression in this disease are poorly understood. However, it is clear that in Barrett’s esophagus evolution from the normal cell to the malignant one can be associated with large changes in DNA content (10–12). For example, four flow cytometric studies of Barrett’s esophagus have reported an increased prevalence of aneuploidy with increasing histological risk of malignancy (10, 13–15). Furthermore, each of these studies detected patients whose metaplastic epithelium had aneuploidy in the absence of high-grade dysplasia or adenocarcinoma, and one prospective study has shown that aneuploidy and increased G2/tetraploid populations identify a subset of patients at high risk for progression to high-grade dysplasia and adenocarcinoma (16).

DNA content flow cytometry cannot, however, identify with certainty clonal relationships that exist between different regions of the Barrett’s segment or that develop serially over time. Although clonal relationships can be recognized by cytogenetic investigation, evaluation of human solid tissue neoplasia is notoriously difficult and relatively few studies of premalignant tissues have been reported. Barrett’s esophagus represents an ideal condition in which to look for clonal changes that develop with progression because biopsies can be taken from the same patient during serial surveillance endoscopies. One previous study reported karyotypic abnormalities in biopsies of Barrett’s metaplasia that were cultured in vitro for prolonged periods of time (17). However, neither the spatial extent of the clone in the Barrett’s segment nor its temporal persistence was evaluated. Furthermore, because these biopsies were cultured for prolonged periods, it is possible that the observed abnormalities represented a minority population in the esophagus that had a selective growth advantage in vitro. To investigate the biology of Barrett’s esophagus in individual patients, serial flow cytometric, cytogenetic, and histological evaluations were performed as part of a prospective cancer surveillance program. We report findings in three patients whose Barrett’s esophagus had both cytogenetic and flow cytometric abnormalities and who progressed to high-grade dysplasia or adenocarcinoma during follow-up. We also report cytogenetic findings in a patient in whom adenocarcinoma was present at entry into the cohort.

MATERIALS AND METHODS

Patients. Case 1, a 72-year-old Caucasian male who presented with an upper gastrointestinal hemorrhage in November 1984 and was found at endoscopy to have an 8-cm length of Barrett’s esophagus (11); Case 2, a 58-year-old Caucasian male who presented in January 1985 with a long history of reflux esophagitis who had two episodes of upper gastrointestinal hemorrhage secondary to ulcers in a 9-cm segment of Barrett’s esophagus; Case 3, a 67-year-old Caucasian male who presented in April 1985 with a long history of reflux esophagitis and an 11-cm segment of Barrett’s esophagus who developed recurrent esophageal strictures after failure of a Nissen fundoplication (10); Case 4, a 68-year-old Caucasian male with a 20-year history of reflux esophagitis who presented in November 1985 with an adenocarcinoma arising in Barrett’s esophagus.

These four patients are among the 44 patients with Barrett’s esophagus evaluated by histology and flow cytometry at initial endoscopy as part of a cancer surveillance study at the University of Washington between November 1984 and March 1986 (10). Endoscopy and Biopsy. Experiments were approved by the Human Subjects Committee at the University of Washington. All patients participated in this study voluntarily and informed consent was obtained prior to each procedure. Endoscopies and biopsies were performed according to a previously published protocol (10). At each endoscopy, the length of the Barrett’s segment was recorded as the distance between the region of the lower esophageal sphincter and the squamocolumnar junction. The site from which each endoscopic biopsy was obtained was recorded as the distance in cm from the incisors. Four quadrant biopsies were taken at 2-cm levels in the Barrett’s segment; one biopsy at each level was divided in half for histology and flow cytometry. Samples for cytogenetic evaluation were taken from defined levels so that karyotypes could be related to histological and flow cytometric results. Esophagectomy specimens were mapped by coordinates on a grid as described previously (11). Endoscopic biopsies or tissue samples obtained at esophagectomy were placed in supplemented L-15 medium (18) for transport to the tissue culture laboratory. The samples were minced with scalpels and disaggregated by incubation with collagenase II (Sigma Chemical Co., St. Louis, MO) 0.4 mg/ml in Hanks’ balanced salt solution for 2 h at 37°C. Samples were cultured overnight in minimal essential medium (GIBCO, Grand Island, NY), supplemented with 15% fetal calf serum, l-glutamine (0.3 mg/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml) or in Chang medium and serum (10% v/v; Irvine Scientific, Santa Ana, CA), supplemented with insulin (5 μg/ml)/transferrin (5 μg/ml)/selenium (5 ng/ml) (Collaborative Research, Lexington, MA) and antibiotics as above.

Cytogenetics. Colcemid, 0.05 μg/ml, was added 4 to 12 h prior to

Received 12/10/91; accepted 3/1/92.

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1 This work was supported by NIH Grant PO1 DK32971 and Grant PDT316 from the American Cancer Society.

2 To whom requests for reprints should be addressed, at Department of Medicine RG20, University of Washington, Seattle, WA 98195.
harvest for cytogenetic evaluation. After incubation for 20-30 min in 0.075 M KCl, preparations were fixed 3 times in methanol/glacial acetic acid (3:1). Slides were prepared and banded by standard methods. Because the endoscopic biopsies from different levels of the Barrett’s esophagus were processed individually, only 3 or 4 slides could be prepared from each sample.

Flow Cytometry. This was performed as described previously (10-12, 19).

Histology. Biopsies were processed for histological evaluation as described previously (10). Barrett’s specialized metaplastic epithelium was graded negative for dysplasia, indefinite/low-grade dysplasia, high-grade dysplasia, or carcinoma. The categories of indefinite for dysplasia and low-grade dysplasia were combined because of the results of a study of observer variation in the diagnosis of dysplasia in Barrett’s esophagus (20).

RESULTS

The four cases described here were among the group reported previously (10). We performed cytogenetic studies on other patients in the University of Washington Barrett’s cohort, but we found that biopsies that contained only metaplastic tissue yielded either no metaphase cells or only cells with normal karyotypes. The exception, Case 3, was the only patient in our previous series with DNA content aneuploidy in metaplasia (Table 1; Ref. 10). A summary of the histological, flow cytometric, and cytogenetic evaluations performed on samples obtained simultaneously from the same level of the esophagus during endoscopic surveillance of the four cases is presented in Table 1.

Case 1. In November 1984, the patient’s first endoscopic biopsy evaluation revealed high-grade dysplasia extending over approximately 8 cm of Barrett’s esophagus and five aneuploid populations. Endoscopic samples obtained at multiple levels of the Barrett’s segment in April 1985 and November 1985 revealed high-grade dysplasia and residual foci of Barrett’s specialized metaplasia, but no intramucosal carcinoma. The two largest aneuploid populations in the earlier sample were again detected by flow cytometry and extended over 10 cm of esophageal mucosa. Biopsies taken in May 1986 documented intramucosal carcinoma. Flow cytometry was performed in July 1986 and the specimen contained 13 different aneuploid populations that could be resolved by flow cytometry (11).

Cytogenetic studies were done on biopsies taken in April 1985 and November 1985 and from the esophagectomy specimen in June 1987. The adenocarcinoma in the esophagectomy specimen was so small that it was not visible grossly and, for this reason, was not sampled for cytogenetic evaluation. However, 2 of the 5 samples submitted for cytogenetic study came from the Barrett’s segment on the wall opposite the tumor were found to have the same marker chromosomes. Two of the marker chromosomes had distinct configurations and could be identified in metaphase cells even though the exact nature of the rearrangements could not be determined. These markers are chromosomes documented in April 1985 and November 1985 in cells from endoscopically obtained biopsies from the Barrett’s segment and 15 months later in 4 regions of the surgical specimen.

Case 2. This patient presented in January 1985 with 9 cm of Barrett’s metaplasia containing abnormalities in the indefinite/low-grade dysplasia range and increased S phase and G2 fractions. Serial endoscopic biopsies revealed progression to high-grade dysplasia (January 1987) and intramucosal adenocarcinoma (June 1987) (Table 1). Flow cytometry performed on successive endoscopic biopsies between January 1985 and June 1987 showed persistently increased S and G2 fractions; in some samples the DNA content histogram suggested the presence of a hypodiploid aneuploidy that was not consistently resolved.

Esophagectomy was performed in June 1987.

Cytogenetic studies were performed on endoscopic biopsies taken in May 1985, December 1985, June 1986, and June 1987 and in samples from the esophagectomy specimen in June 1987. A hypodiploid population with a modal number of 41-42 was evident in all samples studied and several structurally rearranged marker chromosomes defining a clone were present in the samples that were technically evaluable (Fig. 1). The clone was identified at the first evaluation in May 1985, was detected at 7 levels of the Barrett’s metaplasia, spanning at least 4 cm, and persisted for the next 25 months. The adenocarcinoma was not grossly visible in the surgical specimen and was entirely contained within the portion sent for histological examination. Therefore, it was not sampled for cytogenetics or flow cytometry. However, the hypodiploid clone persisted and was present in samples taken from two widely separated regions of the esophagectomy specimen (approximately 5 cm apart).

Case 3. At endoscopy in April 1985, this patient had 12 cm of Barrett’s metaplasia negative for dysplasia, but flow cytometric evaluation detected a major aneuploid population of 2.3 N (47-76% of cells). A cell with the karyotype 48,XY,+3,+8 was detected in a biopsy taken at the same endoscopy. In March 1986, flow cytometry again documented an aneuploid population with a DNA content of approximately 2.2 N at multiple levels of the Barrett’s segment. Biopsies submitted for histological evaluation showed Barrett’s metaplasia with focal abnormalities in extremely-low-grade dysplasia range. At the same endoscopy, a clone with trisomies for chromosomes 3 and 8 was identified and was found at multiple levels of the Barrett’s segment. The 2.2 N aneuploidy and the cytogenetically abnormal clone were both detected in some biopsies from subsequent endoscopies. During the interval between 1985 and 1991, the area covered by Barrett’s metaplasia partially receded and squamous islands appeared distal to the ora serrata.

Case 4. At entry into the Barrett’s cohort this patient was found to have an esophageal adenocarcinoma. Samples were taken from the esophagectomy specimen for cytogenetic analysis. Metaphase cells from the Barrett’s segment on the wall opposite the tumor were found to have the same marker chromosomes seen in the cancer. Flow cytometry showed a major aneuploid population with a DNA content of 3.4 N.

DISCUSSION

In general, studies of the pathogenesis of human solid tumors are hampered by problems in identifying patients at high risk of developing malignancy, infeasibility of obtaining serial samples, and difficulty in recovering adequate cytogenetic preparations. Because the development of malignancy in Barrett’s esophagus follows a progression of metaplasia to dysplasia to carcinoma and endoscopic surveillance allows sequential sampling of epithelium that is at risk of neoplastic progression, this disease provides an excellent system for studying the events involved in this process.

It is not known whether Barrett’s specialized metaplasia develops as a clonal proliferation or results from polyclonal regeneration of the acid-injured mucosa. Aneuploid cell populations have been documented by flow cytometry in endoscopic biopsies from patients with Barrett’s dysplasia and metaplasia without dysplasia (10, 13-15). Although DNA content aneuploidy suggests the presence of a clonal proliferation, proof of clonality requires a more specific marker. There is only one previous report of cytogenetic results in Barrett’s specialized metaplasia without high-grade dysplasia or adenocarcinoma (17). The investigators reported that cultured Barrett’s epithelial cells often exhibited loss of the Y chromosome. In addition, one culture contained extra copies of chromosome 7 and another had a translocation involving chromosomes 3 and 6. These findings suggest that clonal genomic alterations can be
present in the mucosa of some patients with Barrett's esophagus who do not have dysplasia or cancer. However, multiple regions were not sampled from a single patient; therefore the extent of the abnormalities could not be determined, nor were patients reevaluated to document the persistence of the cytogenetic abnormalities. To investigate whether development and progression of Barrett's esophagus involve a persistent clonal process we repeatedly evaluated patients enrolled in a surveillance program. To decrease the possible artifact of in vitro selection of majority populations, we harvested samples for cytogenetic analysis after overnight incubation.

Our findings suggest that neoplastic progression in Barrett's esophagus involves the development of abnormal clones that can expand to occupy large regions of esophageal mucosa and persist for prolonged periods. The presence of abnormal clones was suspected in three of the four patients (Cases 1, 3, and 4) in whom aneuploid populations were identified by flow cytometry. In three patients who had or developed adenocarcinoma (Cases 1, 2, and 4), cytogenetically abnormal clones were documented to exist in regions of the Barrett's segment that were repeated evaluated patients enrolled in a surveillance program. To decrease the possible artifact of in vitro selection of minority populations, we harvested samples for cytogenetic evaluation. In three patients who had or developed adenocarcinoma (Cases 1, 2, and 4), cytogenetically abnormal clones were documented to exist in regions of the Barrett's segment that were.
Fig. 1. R-banded karyotypes from endoscopic biopsies from Case 2. Arrows, recurrent marker chromosomes. All analyzable abnormal metaphase cells at all dates were lacking the Y chromosome. a, obtained May 8, 1985: 41 chromosomes with trisomy 16, der(2)t(2;?)(q35;?), del(5)(q14), der(8)t(8;?)(q24;?), rob(14;21), der(15)t(9;15)(q11;p13), and small pale metacentric marker. The 2q+ (3 of 10 cells) and trisomy 16 (5 of 10 cells) were not identified in later samples. b, obtained December 4, 1985: 38 chromosomes with der(6)t(6;?)(qter;?), der(8)t(8;?)(q24;?), rob(14;21), der(15)t(9;15)(q11;p13), and del(17)(p11). The 17p abnormality was seen only in this cell.
distinct from the adenocarcinoma. In these 3 patients as well as in Case 2, in whom the DNA content was diploid, cytogenetic evaluations documented the presence of a clone. Furthermore, three of the patients developed karyotypic abnormalities prior to the detection of high-grade dysplasia (Case 3), adenocarcinoma (Case 1), or both high-grade dysplasia and cancer (Case 2).

Persistence of a cytogenetically abnormal clone was found in Cases 2 and 3. In Case 2, prospective endoscopic biopsy surveillance documented histological progression from Barrett’s metaplasia with indefinite/low-grade dysplasia abnormalities to high-grade dysplasia. A hypodiploid clone was initially detected in regions that had metaplasia or abnormalities in the indefinite/low-grade dysplasia range and was subsequently detected in a region of high-grade dysplasia that developed during surveillance. The cancer eventually developed within the high-grade dysplasia. During this time, multiple subclonal karyotypic changes were detected within the predominant hypodiploid clone (Fig. 1).

The presence of an aneuploid clone may not predict inevitable progression of disease and development of cancer. Despite the persistence of a hyperdiploid clone for at least 6 years and the development of high-grade dysplasia, the extent of Barrett’s metaplasia in Case 3 has been observed to decrease. Continued surveillance will be required to determine whether or not this patient develops cancer in the future. These cases demonstrate the complementary nature of cytogenetics and flow cytometry. DNA content flow cytometry can determine the relative proportions of populations of different ploidies but may not detect near-diploid aneuploidies or minority populations. Cytogenetics, on the other hand, is not quantitative. This method is able to identify a clone, even when it comprises a small number of cells, but cannot estimate its prevalence. Cytogenetics can prove that an aneuploidy detected by flow cytometry represents a clone and can identify subclonal relationships.

One patient (Case 4) was found to have adenocarcinoma on entry to our study. An abnormal clone was detected by cytogenetics in the noncancerous mucosa as well as in the adenocarcinoma. A theoretical possibility in this case is that the abnormal metaphase cells recovered from the region of the Barrett’s segment that was spatially separate from the cancer were contributed by an unidentified microscopic focus of carcinoma, because these specimens were obtained simultaneously. However, it is unlikely that this explanation accounts for the abnormalities in Cases 1–3, which persisted for several years before high-grade dysplasia (Case 3) or cancer (Cases 1 and 2) was diagnosed. The possibility that the abnormal metaphase cells in Cases 1–3 came from undetected regions of high-grade dysplasia or adenocarcinoma is made even less likely because of the intensive endoscopic biopsy surveillance in these patients (10). An average of 6 endoscopies (range, 5–8) and 173 total biopsies (range, 154–199) were performed per patient.

We have provided evidence that Barrett’s metaplasia can contain cytogenetically abnormal clones that occupy extensive regions of the Barrett’s segment, persist for several years, and progress to high-grade dysplasia and adenocarcinoma. Taken together, these findings are consistent with Nowell’s hypothesis (21) and suggest that neoplastic progression in Barrett’s esophagus occurs by a multistep pathway of clonal evolution.

**ACKNOWLEDGMENTS**

We are grateful to Steven Forbes, Susan Irvine, Catherine Morgan, Carissa Sanchez, and John Wolff for excellent technical assistance and to Dr. Harinder Garewal for helpful suggestions.

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