Octreotide Reduces the Kinetic Index, Proliferating Cell Nuclear Antigen–Maximum Proliferative Index, in Patients with Colorectal Cancer

Graham J. Stewart, M.B.B.S., Jennie L. Connor, M.B., Ch.B., Jane A. Lawson, B.Sc., M.Sc., Angelo Preketes, M.B.B.S., Julie King, B.A., and David L. Morris, M.B., Ch.B., F.R.C.S., M.D., Ph.D., F.R.A.C.S.

Background. **Somatostatin has been shown to inhibit in vitro and xenograft growth of human colon cancer. The kinetic index, proliferating cell nuclear antigen (PCNA), has previously been used to measure the effects of manipulation of growth of normal rectal epithelium.**

Methods. **Twenty-five patients with distal colorectal cancer were considered for entry in a presurgical study of Sandostatin (Sandoz, East Hanover, NJ) 1 mg every 8 hours. Biopsies were performed pretreatment, during treatment (14 days), and day of surgical resection (2 days off treatment). A control series of 16 patients underwent endoscopic and subsequent surgical biopsy. A kinetic index was created called PCNA-maximum proliferative index (PCNA–MPI), which was reproducible within one biopsy and between two separate biopsies. Multiple biopsies were taken from the growing edge of tumors, the most cellular and best-stained fields selected, and the highest 6 of 10 separate counted fields were used to produce PCNA-MPI.**

Results. **A significant decline in PCNA-MPI was observed in 6 of the 10 treated patients for whom all three biopsies were available, followed by a significant elevation on withdrawal of treatment. Changes in PCNA-MPI in the control group were less frequent and smaller.**

Conclusions. **Sandostatin causes a reduction in PCNA-MPI in patients with human colorectal cancer.** ***Cancer*** **1995;76:572–8.**

Key words: colorectal cancer, proliferating cell nuclear antigen, somatostatin, clinical trial.

Colorectal cancer is a common and lethal disease, with few effective therapeutic options.

Somatostatin is a naturally occurring growth-regulating hormone that has been proven to inhibit the growth of human colorectal cancer cells, both in in vitro culture and as xenografts.[1] We previously reported a small clinical study in which short term administration of Octreotide (Sandoz, Basle, Switzerland) produced a reduction in the Ki67 cell index in 4 of 12 patients with rectal cancer.[2] The Ki67 index required the use of frozen tissues; our subsequent results with this index showed that it is dependent on storage time and that batch-to-batch variations were considerable. The concept of evaluating growth regulators in vivo by repeated tumor biopsy and measurement of kinetic index did, however, seem feasible, and this paper reports our efforts in producing a more reliable, and also practical, index. Others previously have used a similar approach to measure the effect of drugs and dietary change on normal epithelium.[3,4]

Growth-regulating treatments that reduce growth but do not cause tumor regression are unlikely to fare well in traditional types of early clinical trials designed to detect clinical, tumor marker, or radiologic response. The best type of study to detect the effect of such treatments would be randomized long term survival studies, in which an agent that produces a slowing of growth should influence survival. Such studies clearly take time and are expensive. Progression free survival may be a useful measure of efficacy of such growth-regulating agents but as a measure probably needs to include untreated controls and have definite measures of progression. This study is, we believe, a relatively novel at-

From the University of New South Wales Department of Surgery, The St. George Hospital, Kogarah, Sydney, Australia.

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Address for reprints: David L. Morris, M.B., Ch.B., F.R.C.S., M.D., Ph.D., F.R.A.C.S., UNSW Department of Surgery, The St. George Hospital, Kogarah, Sydney NSW 2217, Australia.

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tempt to measure the possible effect of a noncytotoxic putative growth-regulating drug on a tumor cell kinetic index designed to measure maximum proliferation, which we derived through a selective process on repeated tumor biopsies in patients with colorectal cancer undergoing a trial of Octreotide treatment before surgery. Proliferative cell nuclear antigen (PCNA) is an antigen specifically expressed during S-phase, and it already has been found to provide data similar to other methods of measuring the kinetic index including bromodeoxyuridine uptake and the Ki67 antigen.[5,6]

Method

In this prospective clinical trial, 25 consecutive patients with proven colorectal cancer of the left colon or rectum within the reach of a flexible sigmoidoscope, who were scheduled for elective resection and had an expected survival of more than 1 month, were considered for entry. Patients with symptoms of obstruction or an endoscopic appearance that indicated a high risk of obstruction were excluded. Other exclusion criteria included pregnancy, gross hepatic impairment, diabetes, myocardial infarction within 6 months, or the use of any investigational drug within the previous 4 weeks.

Tumor Biopsy

All patients underwent endoscopic tumor biopsy before treatment and rebiopsy after 14 days of Octreotide therapy, and the final tumor biopsy was obtained from the resected specimen; this was at least 48 hours after ceasing Octreotide treatment. Tumor specimens were taken whenever possible from the edge of a tumor rather than from its center. At least three biopsies were taken. These were bisected and half fixed in formalin; the remaining specimens were frozen at −70°C. Most biopsies were taken with rigid biopsy forceps through a sigmoidoscope. Flexible endoscopic biopsy forceps were used for more proximal tumors.

Drug Dosage

Octreotide was given subcutaneously. A 3-day dose-escalation protocol was used, starting with 200 μg, 300 mg, 500 μg, and then 1 mg, all administered three times daily by subcutaneous injection. Therapy was planned to continue for a total of 14 days. Therapy usually was given on an outpatient basis and either was self-administered or given by a surgical research fellow (G.S. and A.P.).

Safety Monitoring

Preentry, 7-, 14- and day-of-surgery blood samples were taken for analysis of full blood and differential counts, biochemical profile including urea, electrolytes, and liver function tests, and blood sugar and urine testing for sugar. Patients underwent clinical review on these days.

This protocol was approved by the Southern Sydney Area Health Service Ethics Committee. All patients gave informed consent.

Tumor Kinetic Cellular Measurement

Immunohistochemical staining of PCNA was used to evaluate tumor cell proliferation at Days 1, 14, and the day of surgery.

Proliferating Cell Nuclear Antigen Protocol

The biopsies were fixed in 10% formalin and embedded in paraffin, and 5-μm sections were mounted onto 0.01% poly-L-lysine–coated slides. After deparaffinization with Xylene (May & Baker, West Footscray, VIC, Australia) and rehydration with graded ethanol to 0.05 M Tris[hydroxymethyl]aminomethane hydrochloride (Tris/HCl) (pH 7.6), the slides were immersed in 10 mM citrate buffer (pH 6.0) and incubated in a 100°C water bath for 30 minutes. When cool, the slides were washed in Tris/HCl for 5 minutes. Endogenous peroxidase activity was blocked using 0.06% hydrogen peroxide in methanol at room temperature for 10 minutes, and after washing with Tris/HCl, nonspecific binding was blocked using a 30-minute incubation with normal rabbit serum (Dakopatts X902)(Dako Corporation, Carpenteria, CA). Excess serum was drained and the slides were incubated with mouse monoclonal antibody against PCNA (Novacastra Laboratories, Newcastle-upon-Tyne, UK) at a concentration of 1:100 for 60 minutes at room temperature. Nonspecific immunoglobulin G2A (1:25) (Silenius Laboratories, Hawthorne, VIC, Australia) was substituted for the primary antibody in negative controls. The sections then were incubated with biotinylated antimouse immunoglobulins (1:300 for 30 minutes, Dakopatts E354) (Dako Corporation), followed by incubation with avidin-biotin complex (30 minutes, Vectastain ABC kit PK4000) (Vector Laboratories Inc., Burlingame, CA). After each step, the sections were washed in Tris/HCl. The reaction product was visualized using a substrate solution containing 0.05% 3,3-diaminobenzidinetetrahydrochloride (Dako Corporation), hydrogen peroxide, and imidazole in Tris/HCl (pH 7.6) with a reaction time of 7 minutes. After washing, the slides were counterstained lightly

with hematoxylin, dehydrated, and mounted in Depex mounting medium (Sigma Chemical Co, St. Louis, MO).

The percentage of the nuclear area staining positively for PCNA in each tissue section was calculated by an image analyzer. A percentage nuclear stain analyzer program, developed by Donaldson Imaging Pty. Ltd. (Sydney, Australia) for the Bioscan Optimas Image Analysis System (Bioscan Inc., Washington, DC) was used. Images were transmitted to the system using a solid state video camera mounted to a light microscope, and a video monitor displayed a digitalized image with 256 gray scales plus color overlay to show thresholds. Tissue sections were examined with green filtered light (540/10-nM bandpass) using a 40× objective lens and a 10× eyepiece. After calibration and standardization of optical densities, 10 maximally staining fields were examined from each section. Total nuclear area and nuclear area that stained positive for PCNA each were mapped using gray value threshold settings selected by the user, and the percentage nuclear area stained automatically was calculated for each field. For each tumor section, the mean of the six highest percentages was recorded as the maximum proliferative index (MPI).

All sections were evaluated blind of treatment group or patient's name and by the same observer. For 10% of biopsies, the same section was evaluated blind a second time to assess reproducibility of the index. Sections that were histologically inadequate or showed only faint or no staining were excluded.

Proliferating Cell Nuclear Antigen–Maximum Proliferative Index Variation between Endoscopic and Operative Specimens: Control Series

We retrospectively identified a series of 16 patients with proven colorectal cancer who underwent resection at our hospital, treated by the same surgeons. Presurgical biopsy and surgical specimens for these patients, taken in the same way, were held in our pathology archives. These presurgical and surgical specimens then were examined for kinetic index by exactly the same method as above.

Results

Exclusions/Withdrawals

Two patients with diabetes were not entered in the study; another patient was excluded because of difficulty in rescheduling surgery at the planned end of the study period. The other 22 patients considered for this study were entered. One patient subsequently was found to have a squamous cell carcinoma on histology and was excluded. Three patients began receiving Octreotide therapy and were withdrawn because of pain (2) or rash (1). One patient was found to have a benign tumor (villous adenoma) but remained in the study. One patient failed to undergo surgery and was excluded. This left 17 patients with adenocarcinoma who received Octreotide treatment, and had all 3 biopsies. Treatment time for three of these patients was 12 days, and for a further patient was only 8 days rather than the 14-day protocol, due to difficulties in scheduling surgery. Data from these shorter treatments were included in our results.

The 17 study patients had a mean age of 66 years (range, 48–83 years).

Tolerance/Safety

Subcutaneous injection site pain was reported by almost all patients. Eight of 22 treated patients had abdominal pain that was usually colicky and sometimes severe. This pain settled within a few days. Diarrhea was noted in 14 of 22 patients. Stools were light colored, and some patients noted difficulty in flushing this stool, suggesting steatorrhea. In three patients, the planned build-up regime mistakenly was not used and 1 mg every 8 hours was used; all three had pain or diarrhea, and treatment was withdrawn in two. One patient developed a peripheral erythematous rash within the first 24 hours, and therapy was stopped. Another patient developed a febrile reaction but continued with treatment. There were no significant hematologic or biochemical changes noted during therapy.

Kinetic Index: Proliferating Cell Nuclear Antigen–Maximum Proliferative Index

Each patient should have had two endoscopic (pretreatment and Day 14) and one surgical biopsy assessed. Of these 51 biopsies, 7 endoscopic and 2 surgical biopsies were considered inadequate for evaluation because they did not show sufficient viable tissue, had inadequate PCNA staining, or did not contain tumor. Staining patterns of positive biopsies were heterogeneous within and between fields with intensity of nuclear staining ranging from faint to dark.

Of the 10 patients with technically satisfactory biopsies at all three time points, 6 had a significant decrease in PCNA–MPI (Table 1, Fig. 1) (by 66%, 46%, 39%, 60%, 37%, and 51% of the pretreatment PCNA–MPI) (paired t test, $P < 0.05$). Two of the remaining patients showed no change, whereas the other two showed a significant increase in PCNA–MPI (37% and 24%). Of the six responders, five had a subsequent significant rise in PCNA–MPI from Day 14 to the day-of-surgery specimen. If all 12 patients with Day 14 and

Table 1. Effect of In Vivo Sandostatin on PCNA-MPI (±SD) of Colorectal Cancer Biopsies

Biopsy no.	Pre-SMS (A)	Day 14 (B)	Surgery (C)	A vs. B	%A-B	B vs. C	%B-C*
1	45.56 ± 3.52	15.60 ± 1.85	31.80 ± 7.46	†	−65.8	†	+98.0
2	20.00 ± 4.35	19.49 ± 2.59	29.49 ± 5.31	N/S	−2.6	‡	+51.8
3	51.82 ± 6.82	33.40 ± 3.08	42.93 ± 7.09	†	−46.0	NS	+28.5
4	21.43 ± 2.59	13.14 ± 1.13	21.98 ± 1.32	†	−38.8	†	+67.3
5	14.83 ± 2.41	13.55 ± 1.30	19.08 ± 3.55	NS	−8.6	§	+40.8
6	31.66 ± 6.36	43.45 ± 3.50	36.42 ± 7.72	§	+37.0	NS	−16.2
7	47.60 ± 10.72	19.23 ± 2.18	26.67 ± 2.27	†	−59.6	NS	+38.7
8	33.48 ± 4.42	41.77 ± 4.29	49.33 ± 5.20	§	+24.0	NS	+18.1
9	26.86 ± 6.07	16.77 ± 1.65	32.22 ± 5.54	§	−37.6	†	+92.2
10	56.29 ± 6.69	28.00 ± 1.44	25.58 ± 2.00	†	−50.8	NS	−8.6
11	9.20 ± 4.99	16.47 ± 2.51	F	†	+79.0		
12	27.70 ± 8.71	F	11.23 ± 0.73				
13	28.30 ± 2.70	F	9.56 ± 2.56				
14	20.81 ± 4.32	F	23.18 ± 2.79				
15	F	12.72 ± 1.38	23.38 ± 2.49			†	+83.8
16	N	14.45 ± 3.51	29.77 ± 3.50			†	+106.0
17	I	I	I				

PCNA-MPI: proliferating cell nuclear antigen-maximum proliferative index; SD: standard deviation; NS: not significant; F: inadequate PCNA staining; I: inadequate biopsy specimen, too few cells; N: necrotic; Pre-SMS: pre-Sandostatin treatment.

* For example, +10.2 = surgical biopsy PCNA-MPI is 10.2% greater than the day 14 biopsy PCNA-MPI.

† $P < 0.001$, using paired Student's t test.

‡ $P < 0.01$, using paired Student's t test.

§ $P < 0.05$, using paired Student's t test.

day-of-surgery specimens were studied (including those who did not have data for all three time points), 7 of 12 had a significant rise in tumor PCNA-MPI from Day 14 (with Octreotide administration on the day of surgery [48 hours off Octreotide specimen]). No patient had a significant fall in PCNA-MPI from Day 14 to the day of surgery.

The paired pretreatment and Day 14 PCNA-MPI values were compared as a group for the 10 patients with both values. This just failed to achieve statistical significance ($P < 0.06$) (paired Student's t test). However, Day-14 and day-of-surgery PCNA-MPI values for the 12 patients when both values were available were significantly different ($P < 0.01$) (paired Student's t test), with an increase from Day 14 to the day of surgery. There was no difference between pretreatment and day-of-surgery groups ($P = 0.40$).

A second blind evaluation of 10% of the sections showed no significant difference from the first index. Proliferating cell nuclear antigen-maximum proliferative index values were remarkably consistent. There was no significant difference between the two groups ($P = 0.66$) or between specimens of individual patients. The paired data for the five patients for whom the kinetic index was measured by PCNA-MPI on two separate occasions were: 26.58:27.82, 16.77:16.20, 32.22:30.59, 30.31:30.36, and 15.04:14.84.

The evaluation of biopsies from the 16 untreated control patients showed no significant difference in PCNA-MPI (Table 2, Fig. 2) between endoscopic and surgical biopsies when compared using a paired Student's t test ($P = 0.23$). There was a statistically significant variation in individual PCNA-MPI values for the endoscopic and surgical biopsies in 11 of the 16 control patients. This was an increase of PCNA-MPI values in four and a decrease in seven, in marked contrast to the Octreotide-treated patients, for whom all seven significant alterations from Day 14 to the date the surgical specimen that were noted were an increase in PCNA-MPI values. The size of variation between samples in the control series was also different. Only one of the control series differed by more than 37%, whereas the PCNA-MPI values of all seven tumors that were significantly inhibited by Octreotide differed by more than this. If the control and treated groups were compared, 7 of 10 treated patients had greater than a 37% variation in PCNA-MPI compared with only 1 of 16 control subjects whose PCNA-MPI values were altered by this amount; this was also a change in the opposite direction ($P < 0.01$) (chi-square test with Yates' correction).

Discussion

Two useful observations emerged from this study: that Octreotide inhibited tumor kinetic index in 6 of 10 pa-

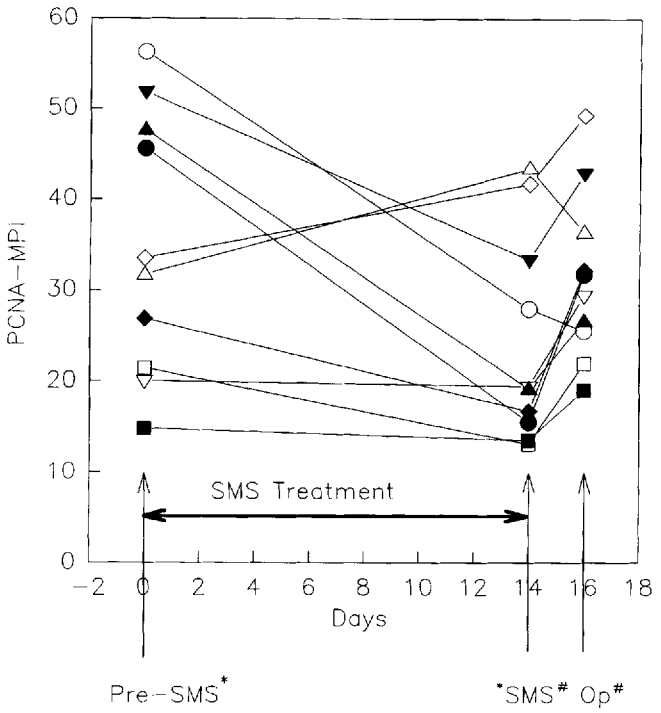

Figure 1. Proliferating cell nuclear antigen–maximum proliferative index (PCNA–MPI) before, during and after Octreotide (Sandoz, Basle, Switzerland) treatment of 10 patients with colorectal cancer. *Pre-SMS versus Day 14 PCNA-MPI, $P = 0.06$ using a Paired Student's t test. #Day 14 versus surgical PCNA–MPI, $P < 0.01$ using a Paired Student's t test

tients with colorectal cancer and that serial measurements of tumor kinetic index by our PCNA–MPI technique can be used to evaluate putative growth-regulating therapy.

Octreotide previously has been shown to inhibit in vitro and in vivo growth of colon cancer,[1,7] cause a decrease in carcinoembryonic antigen secretion in in vitro culture, and reduce serum carcinoembryonic antigen in treated animals with xenografts of human colorectal cancer.[8] Recently, a randomized trial of Ocreotide in 107 patients with gastrointestinal cancer was reported.[9] Although no objective response was observed, there was a significant survival advantage in all treated patients (20 vs. 11 weeks, $P < 0.0001$) and in the gastric, pancreatic, and colorectal subgroups.

The mode of action of somatostatin is complex and multifactorial, but it inhibits the effects of second messengers to the tyrosine kinase family of receptors,[10] which are important receptors for growth factors.[11]

Our data indicate that Octreotide significantly inhibited PCNA–MPI values in 6 of 10 patients with rectal cancer. However, in two patients, the index actually rose during treatment, although by a smaller amount. The pretreatment and Day 14 group comparison almost achieved statistical significance ($P = 0.06$), and the PCNA–MPI groups' Day 14 data were significantly lower than at surgery. That is, there was a significant increase in PCNA–MPI values when the Octreotide administration was stopped. Reproducibility of our analytical technique was good. The five randomly chosen repeat specimens produced similar figures; therefore, variation in the PCNA–MPI technique seems an unlikely explanation for this difference. Another possible explanation is that there could be an effect of surgery, anesthesia, or, perhaps, even bowel preparation on the PCNA–MPI to explain the rise in index after stopping Octreotide. Our control study determining the kinetic indices of endoscopic and surgical specimens from 16 patients who did not receive Octreotide did not show any significant difference in the groups and provides assurance that PCNA–MPI data derived from presurgical endoscopic and surgical biopsies are comparable. In fact, they were again remarkably similar. The control series also produced a mean day-of-surgery PCNA–MPI of 31.29%, which was similar to that of the post-treatment value in the Octreotide-treated patients (27.81%), suggesting that the day-of-surgery PCNA–MPI in our treatment group was normal, and it is the Day 14 data (with Octreotide treatment) that were altered by therapy. The size of difference in PCNA–MPI in the Octreotide-treated patients was also greater. Al-

Table 2. Control Series: Comparison of PCNA-MPI (±SD) Derived From Endoscopic and Surgical Colorectal Cancer Biopsies

Biopsy label	Endoscopic biopsy (A)	Surgical specimen (B)	A vs. B	%A-B*
a	32.56 ± 4.49	30.05 ± 5.19	NS	−7.7
b	41.97 ± 1.12	40.57 ± 5.05	NS	−3.3
c	30.94 ± 1.16	24.86 ± 1.07	†	−19.7
d	31.24 ± 2.44	34.62 ± 2.66	‡	+10.8
e	30.43 ± 3.96	33.52 ± 1.33	NS	+10.2
f	29.42 ± 2.58	26.56 ± 0.87	‡	−9.8
g	33.61 ± 3.99	30.76 ± 1.30	NS	−8.5
h	39.31 ± 3.48	33.03 ± 4.96	‡	−16.0
i	25.13 ± 3.66	33.96 ± 3.50	†	+35.1
j	34.22 ± 1.55	30.50 ± 3.21	‡	−10.9
k	30.20 ± 1.83	28.97 ± 1.47	NS	−4.1
l	21.00 ± 4.39	34.99 ± 2.23	†	+66.6
m	35.14 ± 4.52	22.41 ± 1.62	†	−36.2
n	37.59 ± 1.09	33.99 ± 2.42	†	−9.5
o	38.56 ± 4.24	28.67 ± 3.71	†	−15.6
p	27.86 ± 1.61	33.32 ± 4.11	‡	+19.6

NS: not significant; F: inadequate PCNA staining; I: inadequate biopsy specimen, too few cells; N: necrotic.

* For example, +10.2 = surgical PCNA-MPI is 10.2% greater than endoscopic PCNA-MPI.

† $P < 0.001$, using paired Student's t test.

‡ $P < 0.05$, using paired Student's t test.

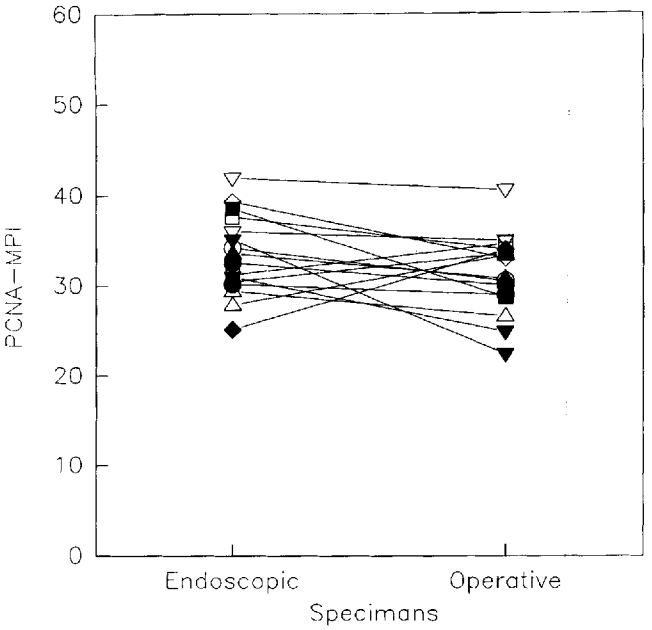

Figure 2. Comparison of proliferating cell nuclear antigen–maximum proliferative index (PCNA–MPI) of 16 untreated control endoscopic and surgical colorectal cancer biopsies. Endoscopic PCNA–MPI versus surgical PCNA–MPI, $P = 0.51$ using a Paired Student's t test.

though 7 of 10 in the Octreotide-treated group had a reduction of PCNA–MPI, only 1 control patient's PCNA-MPI varied by as large an amount ($P < 0.01$), which was an increase; no control patient had a decrease in PCNA–MPI values of this size.

We did not study differences between the three biopsy specimens taken on each occasion, only the difference between the pretreatment, during therapy, and posttreatment data sets.

The practical significance of the observation that Octreotide inhibits tumor cell PCNA–MPI is not at all clear. This is at best an interim measure of efficacy and would need to be confirmed in large studies with clinical endpoints. However, if the inhibition of PCNA–MPI is maintained and is not just a short term phenomenon, then a considerable effect on the progression of at least some cancers could be expected. The effect of somatostatin on tumors would appear to be receptor-dependent.[12] We did not measure receptor status in the patients in this small study but hope to do so in the future.

The morbidity produced by Octreotide in this study (diarrhea and abdominal cramping) was not insignificant, and the current cost of the compound would also probably make its use, at least at the doses used in this study, impractical.

Other more practical applications of Octreotide in colon cancer may include short term treatment at the time of surgery, which may reduce the development of liver metastases,[13] and it also may be a useful modulator of the commonly used cytotoxic agent, 5-fluorouracil.[14]

The ethical aspect of studies such as ours deserves some thought. We delayed resection of primary colorectal tumors in this study, although the presurgical workup was accomplished in the 14 treatment and 2 washout days. We believe that enough is known of the natural history of colorectal cancer to suggest that this length of delay is unlikely to be of any disadvantage to the patient other than that of risking local complications, principally obstruction. We excluded patients thought to be at risk of obstruction, and when scheduling of cases would have required waiting another week for an available surgery list, we ceased therapy prematurely, as was detailed in the text.

The use of the repeated measurement of tumor kinetic index as a measure of effect of a putative growth inhibitor in humans is, we believe, novel and exciting. There are other noncytotoxic compounds that appear to be growth regulators in colonic cancer that may be more effective, particularly gastrin and histamine inhibitors,[15–20] but these compounds have not yet been evaluated by PCNA–MPI in patients.

Our method of producing an index that we called PCNA–MPI is also novel and was designed to select the most actively growing parts of a tumor in three ways: by avoiding central ulceration at the time of biopsy; by selecting the most cellular and best stained areas of the slides, avoiding necrotic fields to count on a slide; and, finally, by only including the highest 6 of 10 PCNA percentage observations in creating the index. We chose to use 6 of 10 fields in our index on a fairly arbitrary basis, and it may well be that other proportions of fields in creating this index could be better. This PCNA–MPI technique however, produced reproducible results as judged by the low standard deviation associated with our PCNA–MPI values and also the reproducible data produced by a second blind measurement of 10% of our specimens. There was a considerable variation in PCNA–MPI between individual patients that was greater in the treated than in the control groups. The repeated biopsy design of our study was designed to allow comparison within individual patients and to avoid the problem of considerable variation of kinetic index that is observed between different tumors. Proliferating cell nuclear antigen–maximum proliferative index tumor kinetic index measurement will need to be validated against other interim measures of outcome and finally against survival, but it may well provide a useful, relatively inexpensive, and rapid way of assessing growth-regulating drugs rather than relying on expensive and lengthy Phase III–type studies. Although measurement of response rate is appropriate for cytotoxic compounds, we do not expect to see response with

growth regulators (unless an important autocrine loop is involved), and, therefore, large and long term survival data studies are required to measure the rate of tumor growth.

We believe that these results are most encouraging and justify further such work with other growth inhibitors.

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