

REPORTS

Biologic Activity of Tamoxifen at Low Doses in Healthy Women

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Background: Results of a clinical trial recently completed in the United States indicate that administration of tamoxifen (20 mg/day) to women at risk can reduce breast cancer incidence by approximately 50% but is associated with an increased risk of developing endometrial cancer and venous thromboembolic events. Since these adverse effects may be dose related, we investigated the effect of tamoxifen on several biomarkers when the drug was given at doses lower than those currently in use. **Methods:** In two sequential experiments, 127 healthy hysterectomized women aged 35–70 years were randomly assigned to one of the following four treatment arms: placebo (n = 31) or tamoxifen at 20 mg/day (n = 30) (first experiment); or tamoxifen at 10 mg/day (n = 34) or tamoxifen at 10 mg/alternate days (n = 32) (second experiment). Baseline and 2-month measurements of the following parameters were compared: 1) total cholesterol (primary end point) and other surrogate markers of cardiovascular disease, e.g., low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, and lipoprotein(a); 2) blood cell count; 3) fibrinogen; 4) antithrombin III; 5) osteocalcin; and, 6) in a subgroup of 103 women, insulin-like growth factor-I (IGF-I), a possible surrogate marker for breast cancer. **Re-**

sults: After adjustment for the baseline values, there were reductions in circulating levels of total cholesterol and IGF-I of the same magnitude in all three tamoxifen treatment arms. A similar pattern was observed for most of the other parameters. In the placebo arm, fibrinogen level, which showed a decrease, was the only parameter exhibiting change. **Conclusions:** Up to a 75% reduction in the conventional dose of tamoxifen (i.e., 20 mg/day) does not affect the activity of the drug on a large number of biomarkers, most of which are surrogate markers of cardiovascular disease. This study was hypothesis generating, and larger studies are warranted to assess the efficacy of tamoxifen at low doses. [J Natl Cancer Inst 1998;90:1461–7]

Tamoxifen is an estrogen receptor modulator with proven efficacy in reducing breast cancer recurrence and mortality following surgery in patients with estrogen receptor-positive tumors (1). A significant reduction in contralateral breast cancer is observed in most trials of adjuvant treatment (1), which has further provided a rationale to assess the efficacy of tamoxifen as a primary preventive agent for women at risk of developing breast cancer (2).

An interim analysis of the U.S. prevention trial involving 13 388 participants led to the early closure of the study (3). It was shown that tamoxifen at a dose of 20 mg/day can reduce the incidence of breast cancer by approximately 50% and can decrease the incidence of osteoporotic bone fractures by approximately 35% (3). The study showed, however, that women aged 50 years or older receiving tamoxifen had a more than twofold increased risk of developing early stage endometrial cancer and a threefold increased risk of developing a pulmonary embolism compared with women receiving placebo (3). Altogether, these results underline the importance of strategies aimed at reducing toxic effects of tamoxifen while retaining its beneficial activity, particularly in postmenopausal women.

Given the consistent evidence from adjuvant therapy trials of a dose- and time-dependent risk of endometrial cancer during tamoxifen treatment (4–6) and taking into account the prolonged plasma half-life (4–11 days after the steady state) of this compound (7), a dose reduction and an intermittent administration appear to be plausible ways to improve the safety profile of tamoxifen. Quite surprisingly, the minimal active dose of tamoxifen is unknown, despite evidence from preclinical studies that the antitumor effect of the drug reaches a plateau above the concentration that saturates estrogen receptors (8,9). Consistent with this finding, an overview of the clinical trials worldwide of tamoxifen adjuvant therapy revealed an efficacy of 20 mg/day of tamoxifen equivalent to that of higher doses of the drug (i.e., 30–40 mg/day) (1).

To ascertain whether a reduction in the conventional dose of 20 mg/day was associated with a diminished biologic activity, we assessed the effects of different doses of the drug on a variety of estrogen receptor-regulated biomarkers known to reflect the activity of tamoxifen on different target tissues. These biomarkers include the blood lipid profile (10), blood cell count (11,12), levels of osteocalcin (13), levels of fibrinogen and antithrombin III (11,12), and levels of circulating insulin-like growth factor (IGF)-I (14,15).

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MATERIALS, SUBJECTS, AND METHODS

Subjects and Treatment

The search for the minimal active dose of tamoxifen was accomplished through two sequential experiments that formed part of an ongoing primary chemoprevention trial of tamoxifen. In this trial, where the primary end point is the reduction of breast cancer incidence, premenopausal and postmenopausal healthy women aged 35–70 years who had undergone a hysterectomy procedure previously for nonmalignant conditions and who were negative for breast cancer as determined by a bilateral mammography procedure were randomly assigned to placebo or to tamoxifen (20 mg/day) for 5 years. The main exclusion criteria were any of the following: previous diagnosis of malignant tumors; history of arterial or venous thromboembolic disease; or presence of severe hypertension, cardiac insufficiency, chronic atrial fibrillation, or severe renal or liver diseases. Women were considered postmenopausal if they were older than 50 years of age when one or both ovaries had been preserved during hysterectomy. As of December 31, 1997, when the recruitment was closed, a total of 5408 subjects were enrolled. A description of the trial and its preliminary results have recently been reported (16).

The first experiment used a double-blinded procedure. It was conducted during the period from January 1993 through July 1993 and included 61 consecutively enrolled eligible women in a 6-month study to determine the safety of tamoxifen by evaluating the effects on blood and lipid profiles important in cardiovascular health and on blood components in relation to clotting (11). After the tamoxifen intake was shown to be associated with an increased risk of endometrial cancer at the dose of 20 mg/day (4), a second experiment was initiated to study the effect of a reduced dose of tamoxifen given for 2 months on different biologic markers.

The second experiment was conducted by the use of an unblinded procedure and included 66 eligible women randomly assigned during the period from March 1996 through June 1996 to either 10 mg/day of tamoxifen or to 10 mg of tamoxifen on alternate days for 2 months, i.e., a period presumably adequate to achieve drug steady-state levels even with the lowest dose (7). Blood samples were drawn at monthly intervals, and plasma fractions were separated by centrifugation at 1400g at room temperature for 10 minutes by use of an Heraeus Megafuge 2.0 (Heraeus Instruments, Hanau, Germany) and kept frozen at -70°C in 400- μL aliquots. Similarly, frozen aliquots of plasma specimens were available from the subjects of the first experiment given the placebo and 20 mg tamoxifen/day. Because plasma cholesterol is stable during prolonged storage (17), repeat enrollment of the placebo arm and 20-mg tamoxifen arm was considered unnecessary in the second experiment. Thus, the following biomarkers—i.e., total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, fibrinogen, antithrombin III, osteocalcin, and IGF-I—were simultaneously measured on frozen samples in a single laboratory from all 127 participants. The complete blood cell count was performed separately on fresh blood. Serum lipoprotein(a) [Lp(a)] levels were measured on

frozen samples stored at -70°C after completion of each experiment. Because of insufficient availability of specimens, plasma IGF-I concentrations were determined in a subgroup of 103 women ($n = 26, 27, 23,$ and 27 respectively, in the placebo arm, 10 mg/alternate days, 10 mg/day, and 20 mg/day tamoxifen arms). Because depletion of frozen plasma specimens occurred in a random way among the participants, the comparison of the IGF-I changes in subjects receiving different treatments was not expected to be affected.

Tamoxifen (20-mg) tablets and placebo tablets were supplied by Zeneca Pharmaceuticals (Macclesfield, U.K.), while the European Institute's pharmacy purchased the 10-mg tablets from the market (Zeneca, Basiglio, Italy). The difference in suppliers was not expected to have any effect, since the manufacturing process controls the amount of active ingredient to within 10% (18). Both experiments received approval from the Institutional Review Board, and all subjects gave their written informed consent. The disclosure of the blinded procedure in the first experiment was approved by the external Data Safety and Monitoring Committee after the completion of the laboratory analysis.

Blood Assays

Fasting blood samples were taken from all subjects between 8:00 and 10:00 AM at baseline and at 1 and 2 months of treatment. Except for blood cell count, which was determined on fresh samples, and Lp(a), which was measured after completion of each experiment to avoid possible degradation after prolonged storage (19), all other measurements from all four treatment groups were done simultaneously on frozen samples stored at -70°C . The samples from experiment 1 were frozen for 3 years and have been thawed only once for the analysis. Blood cell counts were determined by two different laboratories (the European Institute of Oncology and the "Angelo Bianchi Bonomi" Haemophilia and Thrombosis Centre) participating in national quality-control programs, one for each experiment by use of the same automatic instrument (Maxm; Coulter, Milan, Italy). Serum Lp(a) levels were measured in duplicate by an immunoturbidimetric method (20) by use of commercial kits purchased from Incstar Corp. (Stillwater, MN). The intra-assay and interassay coefficients of variation were below 4% and 7%, respectively. The sensitivity of the Lp(a) assay was 2 mg/dL, and the normal range is 0–30 mg/dL. Total cholesterol, HDL cholesterol, and triglycerides were measured by enzymatic methods with an Hitachi 911 (Boehringer, Mannheim, Germany). The intra-assay and interassay coefficients of variation for total cholesterol were below 1.15% and 3.0%, respectively, and the normal range is 125–200 mg/dL. HDL cholesterol was determined in the supernatant after precipitation with phosphotungstate and Mg^{2+} as per instructions from a kit (Boehringer, Mannheim, Germany); the intra-assay and interassay coefficients of variation for HDL cholesterol were below 1.3% and 5.0%, respectively, and the normal range is 38–82 mg/dL. The intra-assay and interassay coefficients of variation for triglycerides were below 1.5% and 3.0%, respectively, and the normal range is 50–200 mg/dL. LDL cholesterol was obtained according to the formula of Friedewald et al. (21): $\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL cholesterol} - (\text{triglycerides}/5)$. The normal range for LDL cholesterol

is 40–130 mg/dL. Comparison with fresh measurements of cholesterol and triglycerides obtained from each sample of both studies showed an average 3% loss (range, 1%–6%) in the frozen material, thus revealing no strong evidence of decay after prolonged storage of samples from the first experiment. Plasma fibrinogen was measured by use of a fibrin polymerization assay (Fibrinogen Reagenz; Boehringer). The intra-assay and interassay coefficients of variation for fibrinogen were below 5%, and the normal range is 160–375 mg/dL. Antithrombin III was assayed by a chromogenic method (Coamatic antithrombin; Chromogenix, Molndal, Sweden). The intra-assay and interassay coefficients of variation for antithrombin III were below 3.5%, and the normal range is 80%–120% of the normal activity obtained from a pool of 30 healthy subjects. Serum osteocalcin was measured by a double-antibody immunoradiometric assay as previously described (22) by use of a commercial kit purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA). The intra-assay and interassay coefficients of variation for osteocalcin were 5.1% and 6.6%, respectively. Sensitivity of the assay was 0.05 ng/mL. The normal range for osteocalcin is 2.4–10.0 ng/mL. Total plasma IGF-I was measured by radioimmunoassay by use of immunohistochemicals and [^{125}I]IGF-I provided by Medgenix Diagnostic (Fleurus, Belgium). The sensitivity of the assay was 0.02 nmol/L; the intra-assay and interassay coefficients of variation were 6% and 7.5%, respectively. To avoid interference from binding proteins, plasma samples were treated with acid ethanol according to the method of Daughaday et al. (23). The normal range for IGF-I in age-matched adult subjects is 13.1–45.9 nmol/L (100–350 ng/mL).

Study End Points and Sample Size

Serum cholesterol is a reliable biomarker of tamoxifen action as well as an established surrogate end-point biomarker of coronary heart disease (24). A previous observation (25) showed a full effect of 20 mg/day of tamoxifen on total cholesterol levels (i.e., a mean 10%–12% reduction from the baseline) as early as 2 weeks after starting the treatment. Therefore, the primary end point of the present study was the change (Δ) in total cholesterol from baseline after 2 months of tamoxifen treatment.

Conventional doses of tamoxifen decrease plasma IGF-I in breast cancer patients (14,15), and IGF-I can stimulate proliferation of normal breast epithelium (26) and can promote growth of breast cancer cells *in vitro* (27) and *in vivo* (28). Finally, higher circulating IGF-I levels have been found in breast cancer patients compared with healthy control subjects both in retrospective (29,30) and in prospective (31) studies. Because of these observations, a change in IGF-I levels was considered a potential surrogate biomarker of breast cancer prevention; consequently, we decided to use it as another main end point of the study.

The first experiment recruited 68 subjects, but only 61 subjects had available aliquots of frozen blood with sufficient volume to quantitate all the biomarkers. The second experiment was designed to be comparable to the first experiment. We anticipated a 12% reduction in mean total cholesterol with a tamoxifen dose of 20 mg/day. Assuming a correlation of .8 between the cholesterol measurements 2

months apart, this corresponds to a decrease of mean \pm standard deviation (SD) of 28 ± 22 mg/dL = $[230 \times 0.12 \pm 35 \times \sqrt{2 \times (1 - 0.8)}]$ from a baseline level 230 ± 35 mg/dL (mean \pm SD). In the second experiment, the sample size was calculated assuming a dose-response effect on total cholesterol, i.e., a 6% reduction from the baseline after 2 months of 10 mg/day of tamoxifen treatment and 3% with 10 mg/alternate days of tamoxifen. We estimated that, at 90% power and 5% significance, 20 subjects per group would be required for a one-way analysis-of-variance model with four groups. We planned to analyze the two experiments as one combined experiment, provided there were no substantial baseline differences. If there were substantial differences, a pooled analysis was not possible; then the second experiment had to have a 90% power to estimate the reduction in total cholesterol after a dose of 10 mg/day of tamoxifen such that the 95% confidence interval (CI) of the mean reduction did not contain 0. This required at least 28 subjects in the 10-mg/day treatment arm. We anticipated a loss of data in 15% of women and, therefore, planned to have 35 women per group in these low-dose arms. This meant that the second experiment was of a comparable size to the first, it could stand alone, and the main hypothesis of a difference among the four groups in the combined experiment would be tested at a power greater than 95%.

Statistical Analyses

The data for biomarker levels from two arms of each experiment were pooled and the differences between average baseline values of the two experiments and the differences in the biomarker levels among four treatment groups were analyzed by a one-way analysis-of-variance model. Because, at baseline, the distributions of all biochemical variables except platelet count and antithrombin III were not normal, square root or log transformations were made to achieve normality. All analyses of baseline values were performed on these transformed variables (square root transformation for total cholesterol, LDL cholesterol, HDL cholesterol, erythrocyte count, Lp(a), fibrinogen, and IGF-I; log transformation for triglycerides, leukocyte count, and osteocalcin). The principal analysis focused on the effects of the different tamoxifen doses on the change (2 months' value of a variable minus baseline value of that variable) in the outcome measures. For all variables except IGF-I, this response was approximately normally distributed. For IGF-I alone, a log transformation was required before the change in the score was calculated. Adjustment for baseline values was performed partly to control for differences between different experiments or different arms and also to assess if the decrease in cholesterol and other biomarkers was greater when the initial level was high. This adjustment was carried out through an analysis-of-covariance model with the baseline as the explanatory covariate and an interaction between treatment group and baseline (32). The occurrence of an interaction between the baseline biomarker level and treatment on the change in biomarker level over 2 months of treatment (i.e., a different effect of treatment depending on the baseline value) was assessed by a general linear model. If there was no interaction, only an adjustment for baseline was carried out. Data were expressed as either the mean \pm SD or the least square mean (32),

and the 95% CI was adjusted for baseline levels. Changes in IGF-I were expressed as percent changes in view of the log transformation required. To investigate any difference among the four treatment groups, we used three comparisons: placebo versus any dose of tamoxifen and linear and quadratic trends among the three tamoxifen groups. All calculations were performed by use of PROC GLM in SAS (33). All reported *P* values are two-sided.

RESULTS

The baseline characteristics of subjects are summarized in Table 1. Although ages of the subjects were similar, more premenopausal women were recruited in the second experiment as a result of a larger proportion of ovarian preservation (36.4% versus 19.7% in the first experiment). The percentage of women on hormone replacement therapy was also larger in the second experiment (16% versus 4.9% in the first experiment). No differences were observed in other characteristics, including age at menarche, age at first birth, waist-to-hip girth ratio, and first-degree family history of breast cancer (data not shown).

The baseline values of the biomarkers according to tamoxifen treatment assignment are shown in Table 2. There was evidence of differences between the two experiments particularly for white blood cell count and red blood cell count among fresh samples and for fibrinogen levels among frozen samples and also between the placebo and 20-mg/day arm for fibrinogen levels. Except for blood cell count, which can be attributed to differences between laboratories, these differences in baseline values between experiments essentially disappeared when premenopausal women were excluded from the analysis (e.g., the mean \pm SD baseline total cholesterol levels were 226.6 ± 26.9 mg/dL, 214.3 ± 40.9 mg/dL, 229.5 ± 44.0 mg/dL, and 223.9 ± 37.2 mg/dL in postmenopausal women in the

placebo arm and in the tamoxifen arms of 10 mg/alternate days, 10 mg/day and 20 mg/day, respectively).

The changes in biomarker levels after 2 months of treatment by study arm are reported in Table 3. If we consider the second experiment only, the mean change (95% CI) reduction in total cholesterol was -27.8 (-38.2 to -17.3) with tamoxifen at a dose of 10 mg/alternate days and -25.3 (-36.5 to -14.1) with tamoxifen at a dose of 10 mg/day, indicating a significant reduction at a greater level than anticipated. In the combined analysis, after adjustment for the baseline values, there were no significant differences in the changes in the vast majority of the biomarkers, including IGF-I, among the three tamoxifen arms. Specifically, while levels of all biomarkers except the level for fibrinogen were unchanged after 2 months of placebo, all levels of biomarkers except the level for triglycerides showed a significant decline during tamoxifen treatment, irrespective of dose. Results were similar after stratifying for menopausal status. For example, for postmenopausal women, the least square mean (and 95% CI) change in total cholesterol (mg/dL) was: 8.8 (-1.6 to 19.2) for placebo; -32.7 (-44.6 to -20.9) for tamoxifen at 10 mg/alternate days; -24.9 (-37.7 to -12.0) for tamoxifen at 10 mg/day; and -22.5 (-32.3 to -12.7) for tamoxifen at 20 mg/day. For premenopausal women, the least square mean (and 95% CI) change in total cholesterol was, respectively, 1.6 (-25.8 to 29.1), -15.3 (-37.6 to -7.1), -19.7 (-43.4 to -3.9), and 27.5 (-8.3 to 63.2). For cholesterol measures, triglycerides, and blood cell count, 1-month measurements were also available. Overall, the changes induced by tamoxifen treatment were already evident at that time (data not shown).

For all biomarkers except HDL chole-

Table 1. Main characteristics of subjects according to tamoxifen treatment assignment

Characteristic	Placebo (n = 31)	Tamoxifen		
		10 mg/alternate days (n = 32)	10 mg/day (n = 34)	20 mg/day (n = 30)
Age, y*	52.7 \pm 4.7	52.7 \pm 7.3	51.4 \pm 7.6	51.7 \pm 4.2
Body mass index, kg/m ² *	24.0 \pm 3.2	25.4 \pm 3.7	26.6 \pm 4.8	24.2 \pm 3.9
Age at menopause, y*	43.4 \pm 5.0	47.2 \pm 4.4	48.5 \pm 4.7	43.5 \pm 5.6
Premenopausal/postmenopausal	8/23	11/21	13/21	4/26
Hormone replacement therapy, no/yes	29/2	25/7	25/9	29/1
Smoking, never/current/former	15/7/9	20/7/5	19/14/1	19/8/3

*Values = means \pm standard deviation.

Table 2. Baseline biomarker levels* according to tamoxifen treatment assignment

Variable	Placebo	Tamoxifen			P value	
		10 mg/alternate days	10 mg/day	20 mg/day	Among arms†	Between trials‡
Total cholesterol, mg/dL	229 ± 30	211 ± 45	212 ± 44	219 ± 38	.22	.07
LDL-C,‡ mg/dL	142 ± 31	129 ± 39	127 ± 36	138 ± 40	.28	.06
HDL-C,§ mg/dL	62 ± 14	65 ± 17	63 ± 17	60 ± 19	.69	.57
Triglycerides, mg/dL	115 ± 50	106 ± 54	112 ± 42	101 ± 45	.51	.86
Lipoprotein(a), mg/dL	23 ± 15	33 ± 25	22 ± 19	26 ± 27	.32	.37
Erythrocytes, 10 ⁶ /mm ³	4.5 ± 0.3	4.8 ± 0.4	4.7 ± 0.4	4.4 ± 0.2	.0001	.0001
Leukocytes, 10 ³ /mm ³	5.7 ± 1.0	6.3 ± 1.6	6.5 ± 1.6	5.8 ± 1.4	.13	.03
Platelets, 10 ³ /mm ³	236 ± 35	244 ± 51	265 ± 51	240 ± 47	.07	.06
Fibrinogen, mg/dL	369 ± 82	272 ± 72	266 ± 54	305 ± 67	.0001	.0001
Antithrombin III, % normal	100 ± 8	100 ± 13	103 ± 12	97 ± 15	.28	.15
Osteocalcin, ng/mL	7.5 ± 2.0	6.3 ± 2.0	6.1 ± 1.6	6.5 ± 2.1	.07	.07
IGF-I, nmol/L	18.6 ± 7.6	20.9 ± 6.4	20.5 ± 6.2	19.3 ± 8.7	.65	.22

*Values = means ± standard deviation.

†Experiment 1 compares tamoxifen at a dose of 20 mg/day with placebo. Experiment 2 compares tamoxifen at a dose of 10 mg/day with tamoxifen at a dose of 10 mg/alternate days. The P value for the comparison among the four arms tests the equality of the four means in columns 2–5 by use of an F test. The P value for the comparison between the two trials tests the equality of the means in columns 2 and 5 with those in columns 3 and 4 by use of an F test.

‡Low-density lipoprotein cholesterol.

§High-density lipoprotein cholesterol.

||Insulin-like growth factor-I.

terol, erythrocyte count, and fibrinogen, there was evidence of an interaction between baseline and treatment on the change in biomarker level, indicating a

different effect among arms, depending on the baseline value of the biomarker (Table 3); in most cases, comparison of each tamoxifen arm with the placebo arm

revealed the interaction. For instance, the higher the baseline value was, the greater the reduction was in total cholesterol levels after taking tamoxifen (any dose), whereas no effect was observed in the placebo group (Fig. 1). A similar trend was observed in the changes in platelet count and osteocalcin; in contrast, for triglycerides and Lp(a), the trend was more complex but did not reflect a dose–response relationship among the three tamoxifen treatment arms (data not shown).

Investigation of the treatment comparisons for the main end points revealed that the differences among the four treatment groups could generally be explained by the comparison of any tamoxifen group with placebo. Any dose of tamoxifen was associated with a 10.8% ± 2.9% (mean ± SD) reduction in total cholesterol, and there was no evidence of any difference among the three tamoxifen groups (P = .31). There was an 18.7% ± 6.6% (mean ± SD) decrease in IGF-I with any tamoxifen compared with placebo, with no evidence of any difference among the three tamoxifen groups (P = .73). Similar results

Table 3. Changes in biomarker levels after 2 months of tamoxifen treatment

Variable	Placebo*	Tamoxifen			P value for treatment effect†
		10 mg/alternate day*	10 mg/day*	20 mg/day*	
Total cholesterol, mg/dL	3.4 (-7.2 to 14.0)	-27.8 (-38.2 to -17.3)	-25.3 (-36.5 to -14.1)	-15.3 (-25.3 to -5.3)	.01‡
LDL-C,§ mg/dL	10.1 (-0.8 to 21.0)	-19.6 (-30.2 to -9.0)	-25.8 (-37.1 to -14.5)	-13.7 (-23.8 to -3.7)	.08‡
HDL-C, mg/dL	-1.5 (-4.8 to 1.7)	-6.3 (-9.4 to -3.2)	-3.4 (-6.7 to -0.2)	-3.8 (-6.8 to -0.8)	.21
Triglycerides, mg/dL	1.6 (-12.4 to 15.6)	-3.9 (-18.6 to 10.7)	16.0 (0.4 to 31.6)	12.9 (-1.5 to 27.2)	.009‡
Lipoprotein(a), mg/dL	1.1 (-1.6 to 3.8)	-2.2 (-5.1 to 0.7)	1.6 (-1.9 to 5.0)	-5.7 (-8.4 to -3.0)	.001‡
Erythrocytes, 10 ⁶ /mm ³	-0.05 (-0.12 to 0.02)	-0.16 (-0.24 to -0.09)	-0.12 (-0.19 to -0.05)	-0.12 (-0.21 to -0.05)	.21
Leukocytes, 10 ³ /mm ³	0.24 (-0.14 to 0.62)	-0.62 (-0.99 to -0.25)	0.23 (-0.15 to 0.61)	0.05 (-0.33 to 0.43)	.005‡
Platelets, 10 ³ /mm ³	-3.5 (-12.7 to 5.6)	-23.7 (-32.8 to -14.6)	-8.1 (-17.8 to 1.5)	-9.4 (-18.7 to -0.2)	.002‡
Fibrinogen, mg/dL	-39.6 (-56.8 to -22.5)	-52.0 (-68.2 to -35.8)	-48.6 (-65.4 to -31.7)	-58.6 (-74.1 to -43.1)	.43
Antithrombin III, % normal	1.3 (-1.4 to 4.1)	-10.7 (-13.7 to -7.8)	-12.6 (-15.8 to -9.4)	-6.4 (-9.1 to -3.5)	.0001
Osteocalcin, ng/mL	0.14 (-0.2 to 0.5)	-0.6 (-0.9 to -0.2)	-1.1 (-1.5 to -0.7)	-1.1 (-1.4 to -0.7)	.01‡
IGF-I,¶ % change	-0.1 (-10.9 to 12.1)	-17.8 (-26.6 to -8.0)	-15.9 (-25.6 to -5.0)	-23.5 (-31.8 to -14.1)	.01

*Levels are expressed as least square means or adjusted means (95% confidence interval) and have been adjusted for baseline values by including the baseline values and the interaction between tamoxifen group and baseline as covariates.

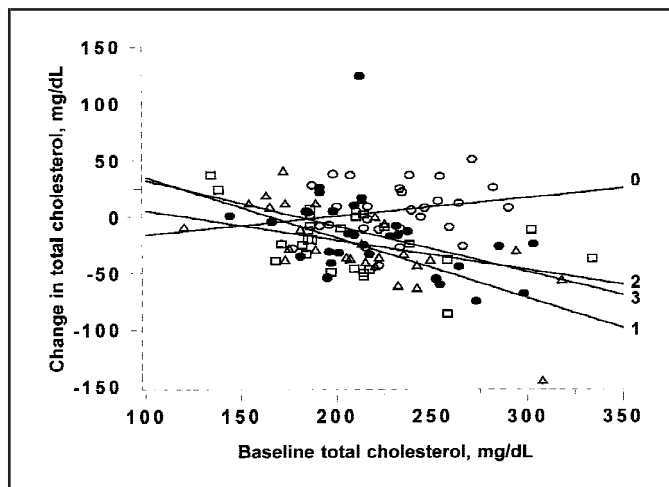
†The P value is for the F test of the main effect of treatment (to show any difference among the four groups) when the slopes of the relationships between the change and the baseline are the same in all four groups. If the slopes are different (denoted ‡), then the P value is for the F test of equality of the slopes because the treatment effect depends on the baseline.

§Low-density lipoprotein cholesterol.

||High-density lipoprotein cholesterol.

¶Insulin-like growth factor-I.

Fig. 1. Effect of administration of different doses of tamoxifen and placebo on the change in total cholesterol level according to baseline cholesterol (0 = open circles, placebo; 1 = triangles, tamoxifen 10 mg/alternate days; 2 = squares, 10 mg/day; 3 = solid circles, 20 mg/day). The slope of the relationship between the change in total cholesterol and cholesterol baseline is significantly different in the placebo group compared with the tamoxifen groups ($P = .01$; F test [two-sided] for the equality of the slopes [analysis of covariance]).



were observed in all biomarkers with a significant treatment effect except for triglycerides (P value for quadratic trend = .002) and leukocyte count (P value for linear trend = .03), where significant differences among the three tamoxifen groups were observed (Table 3). The figures provided represent the average \pm SD generated from individual values. Table 3 provides least square means that have been adjusted for baseline values. For these reasons, the figures provided here do not exactly correspond to those one can extrapolate from the table.

DISCUSSION

While pursuit of the highest tolerated dose in order to increase cell killing is an intrinsic pharmacologic principle of cancer cytotoxic chemotherapy, the search for the minimal active dose underpins strategies of cancer chemoprevention, where noncytotoxic agents with a selective mechanism of action are being investigated in the attempt to inhibit carcinogenesis at the preinvasive stage (34,35). Tamoxifen, a prototype of these agents, exerts its antitumor effect by binding to the estrogen receptor with a plateau of activity above the concentration that saturates the receptor (8,9). This mechanism likely explains the lack of difference in efficacy between tamoxifen given at 20 mg per day and tamoxifen given at higher doses observed in clinical trials of adjuvant treatment of breast cancer (1).

In contrast to its antitumor effect, the carcinogenic effect of tamoxifen on susceptible target tissues appears to follow a dose-response relationship (4-6,36). In

the rat, tamoxifen-induced liver carcinogenesis is directly associated with DNA adduct formation in a dose-dependent manner (36); in the human endometrium, the dose-response effect observed in clinical trials might be the result of an interaction between the genotoxic effect and the estrogen agonistic effect of tamoxifen (37,38). These considerations endorse dose reduction as a plausible attempt to decrease tamoxifen toxicity while retaining its activity.

Our results show that both 10 mg/day of tamoxifen and 10 mg/alternate days of tamoxifen are comparable to 20 mg/day of tamoxifen in modulating a wide array of estrogen receptor-regulated biomarkers, including established or putative surrogate end points of cardiovascular disease such as cholesterol (24), fibrinogen (39), Lp(a) (19), platelet count (40), and blood cell count (41), as well as promising surrogate end points of breast cancer such as circulating IGF-I (31,42). Although the study was designed to assess a dose-response effect of tamoxifen, so that the power to detect equivalence among the doses is more limited, our findings clearly indicate that the conventional dose of 20 mg/day largely exceeds the dose required for the full biologic activity of tamoxifen. Given the exploratory nature of our study, however, our conclusions are important for hypothesis generation about the effect of tamoxifen at low doses.

Importantly, modulation of these biomarkers by tamoxifen is, at least in part, known to be due to estrogen receptor binding (43-48). Consequently, our results suggest also that the maintained po-

tency of the lower doses reflects the saturation of the estrogen receptor, which, in turn, might result in a comparable preventive activity on breast cancer formation. However, since all the biomarkers studied except IGF-I are not closely related to breast carcinogenesis, this supposition could not be tested in the present study. Thus, the clinical activity and the toxicity of the lower doses of tamoxifen should be compared with the conventional dose in future clinical studies. It is interesting that the full biologic activity of the every-other-day administration, which, given the prolonged half-life of tamoxifen (7), may be assumed to be equivalent to a daily dose of 5 mg, suggests that further dose reductions and/or a more prolonged interval between drug intakes might be worth testing, particularly in a preventive setting.

Admittedly, there are some limitations to our study: The trial used the data of two experiments conducted 3 years apart, and a trend toward a difference in several baseline measures was observed between the two experiments, although statistical modeling allowed adjustment for these differences. These baseline differences did not materially affect the results of our study, however, inasmuch as no dose-response effect was observed among the three tamoxifen treatment arms, even in the cases where the biomarker changes were modified by the baseline levels. The comparable effect among the three doses also observed in postmenopausal women, where baseline measures were similar, provides further evidence for the absence of a bias in our study.

Although most biomarkers from all four treatment groups were simultaneously determined on frozen samples, the analysis of the data as a single trial may be criticized in view of the time elapsed between the two parts of the study. We have, however, taken steps in the analysis to justify that this approach is reasonable; and even if the two parts of the trial are analyzed separately and compared, the same conclusions are evident. In the first experiment, the percentage decrease in total cholesterol with a daily dose of 20 mg of tamoxifen was $7.6\% \pm 3.2\%$ (mean \pm SD), while an increase in total cholesterol of $2.1\% \pm 1.8\%$ was observed in the placebo arm, thus demonstrating the significant effect of this dose of tamoxifen on reducing total cholesterol

level compared with placebo. In the second experiment, the percentage decreases in total cholesterol with the 10-mg/alternate days dose and the 10-mg/day dose of tamoxifen were $9.9\% \pm 2.8\%$ and $10.0\% \pm 2.7\%$, respectively, thus evincing that there is no difference between the reduction in total cholesterol levels following different doses of tamoxifen. The percentage numbers given here represent the average computed from individual changes. Table 3 provides least square means or mean change in a biomarker with adjustment for its baseline value. Thus, the figures (given as percents) above will not be identical to those that one can extrapolate from values given in Tables 2 and 3. Our results on the percentage decrease in total cholesterol with 20 mg tamoxifen concur with findings of previous studies (an approximately 10% reduction in total cholesterol (10,25,49,50) as do the percentage reductions in total cholesterol with the 10-mg/alternate days dose and the 10-mg/day dose of tamoxifen. Thus, the 10-mg/alternate days dose and the 10-mg/day dose of tamoxifen are associated with the same reduction in total cholesterol as is the 20-mg/day dose of the drug. The similarity in the reductions in total cholesterol with the lower doses of tamoxifen lends support to the supposition that a dose-response relationship does not exist between tamoxifen doses we evaluated and the cholesterol change. Furthermore, if a dose-response relationship did exist and if the 20-mg arm had been repeated in the second experiment, given the 10% reduction observed with 10 mg/day of tamoxifen, we would have had to anticipate a 20% reduction in total cholesterol with a dose doubling. Such a reduction is not observed in any trials [reviewed in (50)] and has not been reported in the literature [reviewed in (50)]—thus further upholding the claim that there is no dose-response relationship between total cholesterol change and tamoxifen treatment in the dose range studied. Likewise, the 20% reduction of IGF-I induced by all tamoxifen doses is consistent with previous reports in healthy subjects (51) or patients with metastatic breast cancer (52) given tamoxifen at a dose of 20 mg/day.

Because our results highlight a more effective means to exploit tamoxifen pharmacology, the implications for future preventive and therapeutic strategies are

important. Indeed, the maintained biologic activity of the lower doses of tamoxifen strongly supports the implementation of trials with clinical end points to compare the efficacy and the toxicity of the lower doses with the conventional dose of tamoxifen in both preventive and therapeutic settings. It is interesting that similar conclusions regarding the efficacy of a lower dose of tamoxifen emerged from experiments in animal tumor models (53–55). For instance, Maltoni et al. (53) have reported that tamoxifen at a daily dose of 0.1 mg/kg body weight in the rat, which, assuming an equivalent interspecies drug metabolism, corresponds to a daily dose of tamoxifen of at most 6 mg per day in a woman weighing 60 kg, can achieve full inhibition of cancer formation in a spontaneous mammary tumor model. Apart from the effect on the endometria in postmenopausal women, it will be important to evaluate whether a dose reduction is associated with a lower ovarian stimulation in premenopausal women (56) and with a lower incidence and severity of other adverse effects of tamoxifen, including deep venous thrombotic events and vasomotor and genitourinary symptoms (57,58), which have greater impact in a primary prevention context (49). Finally, comparison of low-dose tamoxifen with novel selective estrogen receptor modulators with potentially improved safety profiles (2,59) could provide important clues for the choice of safe and effective preventive approaches for a wide range of estrogen-related diseases.

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NOTES

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Relationship Between Serum Concentrations of the Growth Factor Pleiotrophin and Pleiotrophin-Positive Tumors

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Background: Growth factors produced by tumor cells are essential for tumor expansion and may be useful in monitoring tumor progression or therapeutic efficacy if the factors are released into the circulation. In this study, we measured serum levels of pleiotrophin, a secreted heparin-binding growth and angiogenesis factor, in mice bearing human tumor xenografts to determine whether these levels reflected overall tumor burden, and we examined the relationship between tumor expression of pleiotrophin and serum levels of this factor in patients with cancer. **Methods:** Pleiotrophin in serum from mice and humans was measured by use of a highly sensitive enzyme-linked immunosorbent assay. For the clinical studies, serum specimens were obtained from 193 patients with various cancers of the gastrointestinal tract and from 28 healthy control subjects. In a subset of 64 cancer patients, serum levels of pleiotrophin were measured at the time of surgery, and tumor expression of this factor was detected immunohistochemically. All *P* values are two-sided. **Results:** In mice, serum pleiotrophin levels were found to increase as a function of tumor size. In humans, elevated serum pleiotrophin levels were found in patients with pancreatic cancer ($n = 41$; $P < .0001$) and colon cancer ($n = 65$; $P = .0079$) but not in patients with stomach cancer ($n = 87$; $P = .42$). A statistically significant positive association was found between elevated levels of pleiotrophin in serum drawn at the time of surgery and expression of this factor by tumors ($P < .0001$). In both mice and humans, serum pleiotrophin levels dropped after successful tumor removal. **Conclusions:** Elevated serum

pleiotrophin levels can indicate the presence of tumors expressing this factor. Monitoring serum levels of pleiotrophin may prove useful in determining the pharmacologic efficacy of cytotoxic or anti-pleiotrophin therapy. [J Natl Cancer Inst 1998;90:1468-73]

Tumor expansion and metastasis is dependent on growth factors produced by the tumor cells and/or the stroma (1). In particular, one would expect that angiogenic factors targeting sprouting blood vessels can be shed into the circulation and, hence, may provide a direct measure of tumor progression. With respect to the significance of growth factor expression and release into the circulation, several clinical studies found elevated levels of basic fibroblast growth factor in the serum of patients with cancer of the prostate (2), breast (3), cervix uteri (4), and kidneys (5) as well as in the urine of patients with a wide spectrum of different cancers (6). These clinical findings and studies in animals (7) suggest that in principle angiogenic factors released from tumors can enter the circulation and may serve as useful indicators of tumor progression or as surrogate end points of therapeutic efficacy [reviewed in (8)].

We report the development of a highly sensitive enzyme-linked immunosorbent assay (ELISA) for the secreted heparin-binding growth factor pleiotrophin (9). Pleiotrophin belongs to a family of growth factors that includes one other member, midkine (10), and pleiotrophin is involved in growth and differentiation processes that are tightly regulated during development (11). Furthermore, pleiotrophin is a mitogen for fibroblasts (12), epithelial cells (12,13), and endothelial cells (12-15). It stimulates plasminogen-activator production (16), can induce tube formation of endothelial cells *in vitro* (15), and thus can serve as a tumor angiogenesis factor *in vivo*. Pleiotrophin is expressed in a variety of tumor cell lines and tumor samples (12) and was found to be oncogenic when overexpressed in NIH 3T3 cells (17) and SW-13 human adrenal carcinoma cells (12). Furthermore, the growth, angiogenesis, and metastasis of pleiotrophin-positive melanoma (18,19) and the invasion and angiogenesis of choriocarcinoma (20) were reverted by depleting the tumor cells of their endog-

enous pleiotrophin with specific ribozymes.

In this study, we analyzed serum levels of pleiotrophin during the growth of human tumor xenografts in experimental animals and showed that serum levels of this factor reflect the overall tumor load of pleiotrophin-positive tumors in the animals. In clinical studies, we observed elevated serum pleiotrophin levels in patients with various cancers of the gastrointestinal tract relative to healthy subjects, and we demonstrated a direct relationship between pleiotrophin expression in the tumors and elevated serum levels of the growth factor at the time of surgery.

MATERIALS AND METHODS

Growth factors, antibodies, and cell lines. Recombinant human pleiotrophin was produced in SF9 cells by using the baculovirus system (12) and purified by chromatography of heparin-Sepharose and Mono S columns. The amount of pleiotrophin protein was determined on the basis of silver staining, which had been standardized previously (12) by N-terminal sequencing of the recombinantly produced protein. Recombinant human midkine and affinity-purified goat anti-pleiotrophin polyclonal antibodies conjugated and nonconjugated to biotin were obtained from R&D Systems, Inc. (Minneapolis, MN). Basic fibroblast growth factor was purchased from Collaborative Research, Inc., Bedford, MA. Streptavidin-alkaline phosphatase conjugate was obtained from Pierce Chemical Co. (Rockford, IL). The mouse 4B7 monoclonal anti-pleiotrophin antibody, which was raised against chicken pleiotrophin that was prepared as described (21), is a purified immunoglobulin G1 and cross-reacts with mouse and human pleiotrophin. Cell lines 1205LU (19), MDA-MB231 (12), and MCF-7/FGF-4 (22), were cultured as reported (12,19). Tumor xenografts in athymic nude mice were established and monitored as described (19).

Case and control subjects. Patients from the Department of Surgery, Christian Albrechts University, Kiel, Germany, participated in this study. The age range was 38-90 years for patients with colon cancer, 37-78 years for patients with pancreatic cancer,

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and 29–86 years for patients with stomach cancer. The percentage of male patients in each group was 52%, 46%, and 68%, respectively. Approval of the institutional review board and informed consent of the patients were obtained before the study. Serum samples were drawn and stored frozen at -20°C for up to 4 years before processing. Serum samples from randomly selected blood donors served as controls.

Processing of blood samples. Blood samples from mice were collected via the retro-orbital sinus with a Pasteur pipet. Blood was allowed to clot overnight at room temperature and centrifuged at 12 000 rpm in a minicentrifuge at 4°C to isolate serum. After informed consent, serum samples from patients were drawn preoperatively and samples from normal subjects (blood donors) were provided by the blood bank of the Christian Albrechts University. Serum was diluted 1:1 with $2\times$ TBST ($1\times$ TBST is 50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.5% Tween 20) for the ELISA assay.

ELISA procedure. A mouse anti-pleiotrophin monoclonal antibody (4B7) was diluted to $1\ \mu\text{g}/\text{mL}$ in Tris-buffered saline (TBS). Aliquots ($100\ \mu\text{L}$) of the diluted antibody were incubated in 96-well plates (Corning, Inc., Corning, NY) at 4°C overnight. The wells were washed three times with TBST. The remaining free binding sites in the wells then were blocked with $200\ \mu\text{L}$ of blocking solution (TBST containing 1% bovine serum albumin) for 2 hours at 4°C , and the wells were washed three times with TBST. Samples were diluted in $2\times$ TBST, and $100\text{-}\mu\text{L}$ aliquots were added to the wells and incubated at room temperature for 1 hour. The wells were washed three times with TBST, and the second antibody, a biotinylated affinity-purified anti-human pleiotrophin goat immunoglobulin G (IgG) (R&D Systems, Inc.), was added at a concentration of 500 ng/mL and incubated at room temperature for 1 hour. After washing three times with TBST, $100\ \mu\text{L}$ of streptavidin-conjugated alkaline phosphatase (50 ng/mL) was added to each well, and the plate was incubated for 1 hour at room temperature. The plate then was washed three times with TBST and incubated with $100\ \mu\text{L}$ of a *p*-nitrophenyl phosphate substrate (Pierce Chemical Co.) in the dark at 4°C for 18 hours or at room temperature for 2 hours. Absor-

bance was measured with a microplate reader at 405 nm.

Immunohistochemistry of tumor samples. Cryostat sections were prepared and fixed in acetone, and endogenous peroxidase was blocked by incubation with a 1:100 dilution of H_2O_2 in methanol. Sections were washed in phosphate-buffered saline (PBS) and then incubated with the primary anti-pleiotrophin antibody (which was used in the ELISA [goat anti-human pleiotrophin]) diluted to $5\ \mu\text{g}/\text{mL}$ (or a 1:20 dilution) in PBS containing 1% bovine serum albumin at room temperature (for 2 hours) or at 4°C (overnight). After subsequent washing (three 2-minute washes) in PBS, the secondary antibody was added (peroxidase-coupled rabbit anti-goat IgG [Dianova, Hamburg, Germany]) in 10% type ABO human serum and incubated for 1 hour at room temperature. After a further wash in PBS, peroxidase activity was localized by staining with diaminobenzidine as a substrate (Vector Laboratories, Burlingame, CA). The sample then was rinsed in water (two 2-minute washes), and nuclei were stained with hematoxylin (30–60 seconds). After a final rinse in water, the samples were dried and covered.

Statistical analysis. Analyses were carried out with the PRIZM/Graphpad (San Diego) software. Analysis for normal distribution, Wilcoxon's signed rank, Mann-Whitney *U* test, and Student's *t* test, as well as χ^2 analysis with Fisher's exact test, were used as indicated in the text. All *P* values are from two-sided tests. Only *P* values less than .05 were considered statistically significant.

RESULTS

Sensitivity, Specificity, and Interference of Serum With the Pleiotrophin ELISA

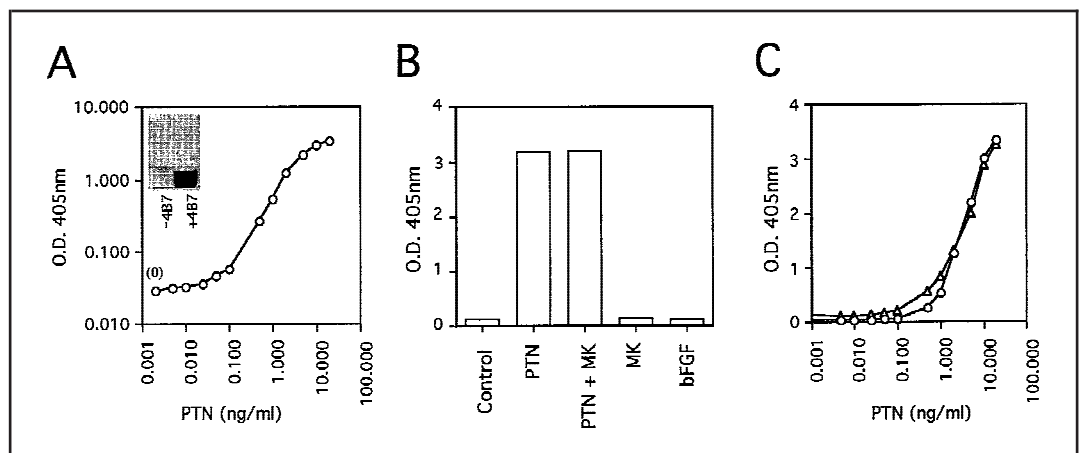
A sandwich ELISA for pleiotrophin was developed that used an anti-pleiotrophin mouse monoclonal antibody (4B7) that captures pleiotrophin (Fig. 1, A, inset) and a commercially available,

biotinylated, affinity-purified goat anti-pleiotrophin polyclonal antibody to detect the captured antigen. The standard curve showed that the minimum concentration of pleiotrophin detected was 5–10 pg/mL (Fig. 1, A). The intraassay coefficient of variation was 1.67%, and the interassay coefficient of variation was 2.10% based on five repetitions with a given sample.

The specificity of the ELISA was tested against the pleiotrophin homologous protein midkine (22), as well as basic fibroblast growth factor, another heparin-binding growth factor, and no cross-reaction was observed in either case. Furthermore, co-incubation of pleiotrophin with a 500-fold excess of human midkine did not alter the signal generated by pleiotrophin alone (Fig. 1, B). Because secreted mouse and human pleiotrophin proteins have the same amino acid sequence (23), murine and human pleiotrophin proteins cross-react in the assay.

To test whether the ELISA assay could detect pleiotrophin in serum samples, we did experiments in the absence or the presence of human serum. The standard curve for the detection of pleiotrophin in the presence of serum was similar to the curve in the absence of serum (Fig. 1, C). However, the background level in the presence of serum was higher due to low levels of pleiotrophin present in normal adult human serum. In our present study, we found pleiotrophin serum concentrations of $27 \pm 32\ \text{pg}/\text{mL}$ in blood donors (mean \pm standard deviation; $n = 28$). The serum levels of pleiotrophin that we ob-

Fig. 1. Characteristics of the pleiotrophin (PTN) sandwich enzyme-linked immunosorbent assay (ELISA). **A**) Sensitivity. Standard curve for pleiotrophin concentrations from 0.005 to 20 ng/mL. Results are from a representative experiment done in duplicate. **Inset**) Retention of pleiotrophin by 4B7, the anti-pleiotrophin monoclonal antibody (MAb) used as a capturing antibody. Pleiotrophin (50 ng) was incubated in ELISA wells that had been preadsorbed without (–) or with (+) 4B7 MAb and bovine serum albumin. Bound pleiotrophin was eluted with sodium dodecyl sulfate–polyacrylamide sample buffer and subjected to electrophoresis and western blotting with a goat anti-pleiotrophin antiserum (R&D Systems, Inc., Minneapolis, MN). **B**) Specificity. Pleiotrophin (1 ng/mL), midkine (10 ng/mL), basic fibroblast growth factor (10 ng/mL), or pleiotrophin (1 ng/mL) plus midkine (500 ng/mL) was subjected to the ELISA. Results are from a representative experiment done in duplicate. **C**) Influence of serum. Pleiotrophin concentrations ranging from 0.005 to 20 ng/mL were analyzed in the absence (circles) or presence (triangles) of 50% of normal human serum. Results are from a representative experiment done in duplicate. Standard error bars are shown if they are larger than the symbol size.



served confirm an earlier qualitative study in which the pleiotrophin protein was purified and sequenced from human post-heparin plasma samples (24). In contrast to human serum, the serum of athymic nude mice contained pleiotrophin levels that were below the detection limit of the ELISA. Thus, these results indicate that serum components other than pleiotrophin do not interfere with the detection of this growth factor by the ELISA used. Furthermore, the assay appears to be sensitive enough to detect constitutive levels of pleiotrophin in normal human serum.

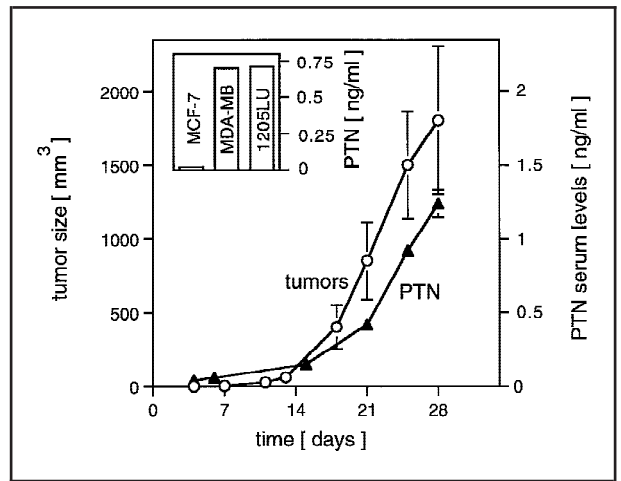
Detection of Pleiotrophin in the Supernatants of Human Tumor Cell Lines

Human tumor cell lines of various origins were screened for the presence of pleiotrophin in their culture medium. We detected 6.3 ± 0.1 and 6.9 ± 0.2 ng of pleiotrophin/mL of conditioned medium from subconfluent 1205LU melanoma and MDA-MB 231 breast cancer cells, respectively. Both cell lines had been shown previously by our laboratory to express pleiotrophin messenger RNA (mRNA) and protein (12,19,25). In other human tumor cells that were deemed pleiotrophin mRNA-negative based on northern blot analysis (MCF-7 breast cancer cells and ME-180 squamous cell cancer cells), no pleiotrophin protein was detected in the culture supernatants.

Pleiotrophin Serum Levels in Mice With Human Xenograft Tumors

We next asked whether xenografts grown from pleiotrophin-positive human tumor cells (MDA-MB 231 breast cancer cells and 1205LU melanoma cells) would generate increased serum levels of pleiotrophin in experimental animals. MCF-7 human breast cancer cells transfected with a fibroblast growth factor expression vector, FGF-4 (to give MCF-7/FGF-4 cells), served as a negative control because these cells do not produce pleiotrophin (12). They grow into well-vascularized tumors due to their expression and secretion of FGF-4 (22). Serum samples from mice bearing tumors of at least 1500 mm³ were analyzed (Fig. 2, inset). Pleiotrophin levels in the serum of mice bearing pleiotrophin-negative MCF-7/FGF-4 tumors were below the detection limit of the assay. In contrast, the serum from

Fig. 2. Xenograft tumor growth and serum levels of pleiotrophin (PTN) in athymic nude mice. Subcutaneous tumor growth of 1205LU human melanoma xenografts (open circles) and serum levels of pleiotrophin (solid triangles) in the mice are shown. Serum was collected from the orbital sinus of subgroups of the mice at different days. Mean \pm standard deviation values are shown (n = 18 tumors; three to six independent serum samplings at each time point). **Inset** Serum levels of pleiotrophin in mice with 1500 mm³ subcutaneous human tumor cell xenografts of various origin. Tumors were grown from pleiotrophin-positive human breast cancer cells (MDA-MB 231) and melanoma cells (1205LU), as well as from pleiotrophin-negative FGF-4-transfected MCF-7 breast cancer cells (22).



mice with the pleiotrophin-positive tumor xenografts had a pleiotrophin concentration of 0.7 ng/mL, which is greater than 100-fold above the limit of detection of the assay.

To test whether the levels of pleiotrophin detected in the serum of tumor-bearing mice reflect the tumor size, we compared serum levels and tumor size of 1205LU melanoma xenografts over a period of several weeks. Most surprisingly, at first detection of a palpable tumor nodule, we already detected increased pleiotrophin levels in the serum of the animals (Fig. 2). At this early time, the tumors had reached less than 1 mm³ and, thus, were less than 0.01% of the total body mass. Subsequently measured serum levels of pleiotrophin increased in parallel with further tumor growth ($P < .0001$). Surgical removal of subcutaneous tumors in some

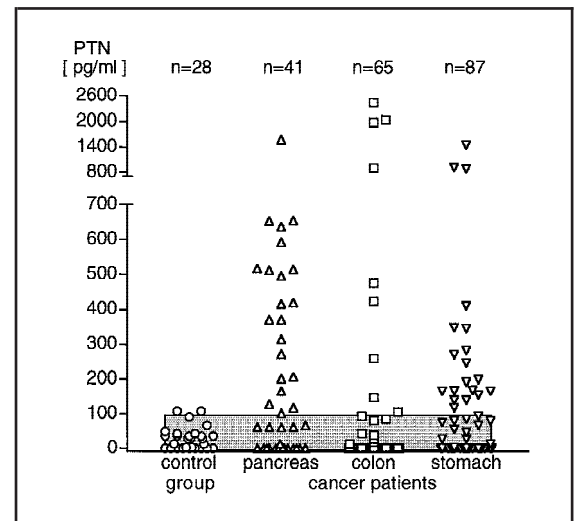
of the animals (n = 6) was reflected in reduced serum levels of pleiotrophin (data not shown).

Pleiotrophin Serum Levels in Cancer Patients and Healthy Subjects

A nonselected group of healthy blood donors served as control subjects. In this group, only eight of 28 subjects showed pleiotrophin serum levels below the sensitivity of our assay (Fig. 3). The data were normally distributed with the highest level at 107 pg/mL and an average of 27 pg/mL (95% confidence interval = 16–41 pg/mL).

Pleiotrophin serum levels in patients (n = 193) with various cancers of the gastrointestinal tract are shown in Fig. 3. Relative to samples from control subjects, we found statistically significantly el-

Fig. 3. Serum levels of pleiotrophin (PTN) in healthy volunteers and patients with various types of gastrointestinal cancers. Shaded area indicates the normal range (mean \pm 2 standard deviations of control values).



evated serum levels in patients with pancreatic cancer ($n = 41$; $P < .0001$) and colon cancer ($n = 65$; $P = .0079$) but not in patients with stomach cancer ($n = 87$; $P = .42$). The respective P values were derived from a Mann-Whitney U test due to a lack of normal distribution of the patients' values. If one assumes that only values above the normal range (i.e., mean ± 2 standard deviations) of healthy subjects are informative, half of the values from the patients with pancreatic cancer (20 of 41; $P = .0002$; Fisher's exact test), 15.4% of the patients with colon cancer (10 of 65; $P = .23$), and 24.1% of the patients with stomach cancer (21 of 87; $P = .039$) showed serum levels of pleiotrophin above the control range.

To understand whether elevated serum levels of pleiotrophin indicate gene expression in the patients' tumors, we immunohistochemically stained the sections of pancreas, colon, and stomach tumors

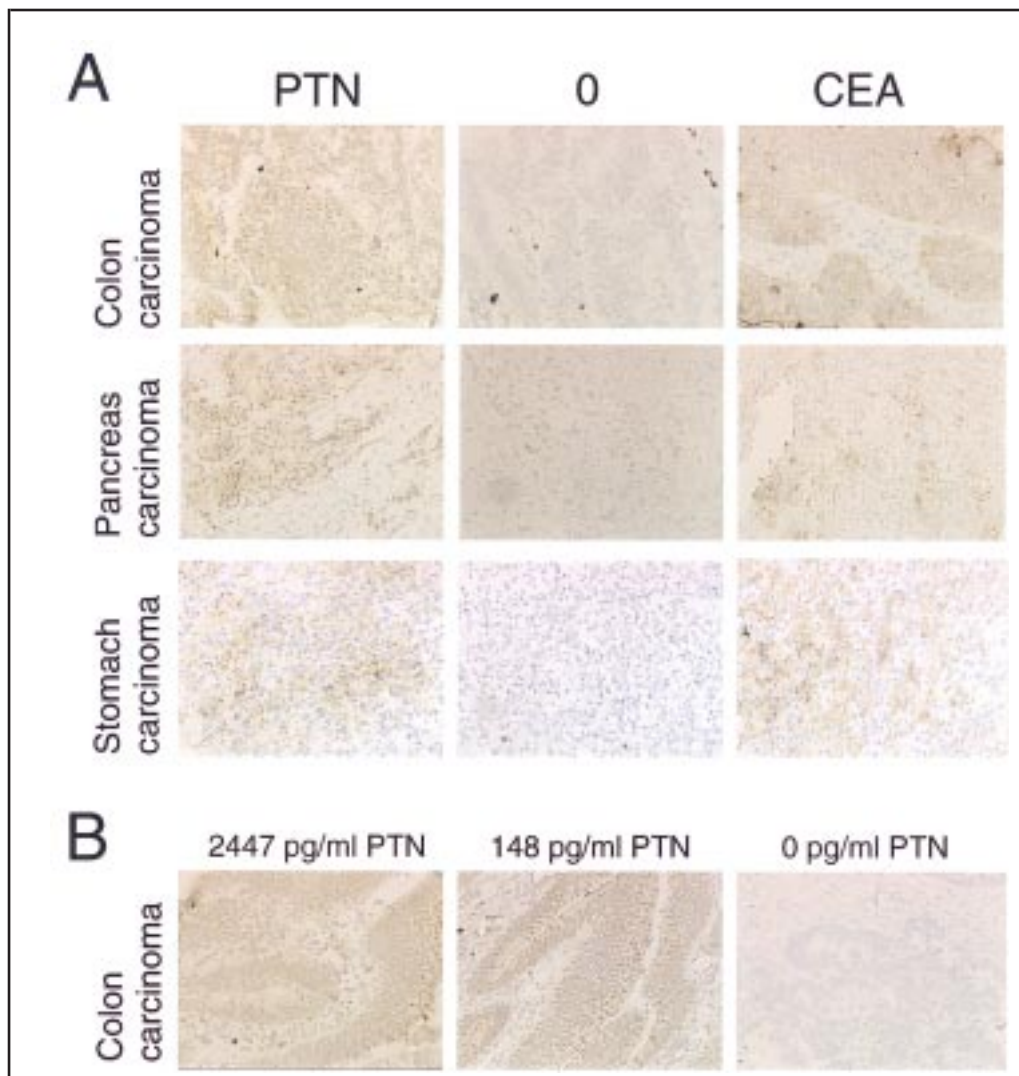
for pleiotrophin and compared the findings with pleiotrophin serum levels obtained from the corresponding patients before surgery. Examples of the stained sections are shown in Fig. 4 (note that the staining is in the tumor cells). The analysis of this dataset shows a strong correlation between the presence (25 of 47 or 53%) or absence (none of 17 samples = one pancreas sample, four colon samples, and 12 stomach samples) of pleiotrophin in the tumors and elevated or normal serum levels at the time of surgery ($P < .0001$; Fisher's exact test). Pleiotrophin was detected immunohistochemically in seven of nine pancreas sections (78%), in nine of 16 colon sections (56%), and in nine of 22 stomach samples (41%) from individuals who had elevated levels of pleiotrophin in their serum. In particular, patients with pleiotrophin-negative tumors do not have elevated pleiotrophin serum levels, and elevated

serum levels of pleiotrophin predict pleiotrophin-positive tumors. However, in almost half (47%) of the patients with pleiotrophin-positive tumors (22 of 47), the amount of pleiotrophin protein produced by the tumor was not sufficient to result in elevated pleiotrophin serum levels. Finally, some of the serial serum samples were analyzed for pleiotrophin in the first 10 days after surgery. Whereas we observed an increase or no change of serum pleiotrophin levels in two patients with only palliative surgery, we observed a significant decrease ($>90\%$ drop; $P < .001$; t test) of serum pleiotrophin levels in two patients with successful removal of the tumor ($n = 3$ postsurgical samples from each patient).

DISCUSSION

We have described a sensitive sandwich ELISA for the growth factor pleio-

Fig. 4. Detection of pleiotrophin (PTN) by immunohistochemistry of various gastrointestinal cancers. **A)** Sections from tumors of colon, pancreas, and stomach cancer patients with elevated pleiotrophin serum levels are shown. Negative control sections (0) without primary anti-pleiotrophin antibody are shown in the center. Sections stained for carcinoembryonic antigen (CEA) are shown to the right. **B)** Sections from tumors of colon cancer patients with various pleiotrophin serum levels are shown. Note the staining for pleiotrophin in the tumor cells and not the stroma.



pleiotrophin that can detect pleiotrophin concentrations in the range of 20 pg/mL to 10 ng/ and that was specific for pleiotrophin because it did not recognize the pleiotrophin homolog midkine or basic fibroblast growth factor. Normal human serum did not interfere with the detection of pleiotrophin by the ELISA, and the assay was sensitive enough to detect constitutive concentrations of pleiotrophin in the serum of most of the healthy subjects. In contrast to the findings with human serum samples, pleiotrophin levels in the serum from athymic nude mice were below the detection limits of the assay, although in the assay human and murine pleiotrophin cross-react.

The most surprising finding in this study in experimental animals was the fact that even a very low tumor load of less than 0.01% of the body mass led to a statistically significant increase in the serum levels of pleiotrophin. Furthermore, it appears that the tumor cells themselves and not the tumor stroma are the most likely sources of the pleiotrophin appearing in serum samples. This conclusion is supported by the lack of an increase in pleiotrophin serum levels when pleiotrophin-negative tumor cells are grown into xenografts in animals (Fig. 2, inset, with MCF-7/FGF-4 tumors).

In the clinical studies, only the groups of patients with pancreatic and colon cancer showed significantly elevated levels of pleiotrophin in their serum. Serum levels of pleiotrophin in patients with stomach cancer were essentially not elevated. Our immunohistochemistry data may explain this finding. Only 17% (five of 30) of the tumor sections from the patients with pancreas and colon cancers showed no staining for pleiotrophin, whereas twice that portion, 35% (12 of 34), of the tumor sections from patients with stomach cancer were negative for pleiotrophin. Thus, a correspondingly lower frequency of elevated levels of pleiotrophin in the serum of patients with stomach cancer is to be expected. In addition, the highest portion of patients with pancreatic cancer showed pleiotrophin serum levels above the range of concentrations seen in healthy subjects (Fig. 3). This may reflect the typically advanced state of this disease and the short median survival time after diagnosis of patients with this particular cancer.

In conjunction with our studies in experimental animals, it is tempting to speculate that monitoring the levels of pleiotrophin in serum of patients could be a useful measure of residual tumor burden and/or recurrence of tumors after therapy. This notion is supported by the drop in pleiotrophin levels in serum after successful tumor removal and the lack thereof with a residual tumor mass. Furthermore, in clinical trials with drugs targeting pleiotrophin for therapeutic purposes (e.g., synthetic ribozymes), monitoring pleiotrophin serum levels would be a very attractive way of assessing pharmacologic efficacy of the particular therapeutic molecule or its mode of delivery. Ongoing animal studies in our laboratory suggest the feasibility of this approach.

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NOTES

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Specific Genetic Predictors of Chemotherapeutic Response and Survival in Patients With Anaplastic Oligodendrogliomas

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Background/Methods: Gliomas are common malignant neoplasms of the central nervous system. Among the major subtypes of gliomas, oligodendrogliomas are distinguished by their remarkable sensitivity to chemotherapy, with approximately two thirds of anaplastic (malignant) oligodendrogliomas responding dramatically to combination treatment with procarbazine, lomustine, and vincristine (termed PCV). Unfortunately, no clinical or pathologic feature of these tumors allows accurate prediction of their response to chemotherapy. Anaplastic oligodendrogliomas also are distinguished by a unique constellation of molecular genetic alterations, including coincident loss of chromosomal arms 1p and 19q in 50%–70% of tumors. We have hypothesized that these or other specific genetic changes might predict the response to chemotherapy and prognosis in patients with anaplastic oligodendrogliomas. Therefore, we have analyzed molecular genetic alterations involving chromosomes 1p, 10q, and 19q and the TP53 (on chromosome 17p) and CDKN2A (on chromosome 9p) genes, in addition to clinicopathologic features in 39 patients with anaplastic oligodendrogliomas for whom chemotherapeutic response and survival could be assessed. **Results/Conclusions:** Allelic loss (or loss of heterozygosity) of chromosome 1p is a statistically significant predictor of chemosensitivity, and combined loss involving chromosomes 1p and 19q is statistically significantly associated with both chemosensitivity and longer recurrence-free survival after chemotherapy. Moreover, in both

univariate and multivariate analyses, losses involving both chromosomes 1p and 19q were strongly associated with longer overall survival, whereas CDKN2A gene deletions and ring enhancement (i.e., contrast enhancement forming a rim around the tumor) on neuroimaging were associated with a significantly worse prognosis. The inverse relationship between CDKN2A gene deletions and losses of chromosomes 1p and 19q further implies that these differential clinical behaviors reflect two independent genetic subtypes of anaplastic oligodendroglioma. These results suggest that molecular genetic analysis may aid therapeutic decisions and predict outcome in patients with anaplastic oligodendrogliomas. [*J Natl Cancer Inst* 1998;90:1473–9]

Gliomas are common central nervous system neoplasms. The major subtypes of glioma are astrocytomas, oligodendrogliomas, and ependymomas; each subtype has characteristic histologic features. The importance of correct and specific glioma diagnosis became apparent several years ago with the observation that oligodendrogliomas, unlike ependymomas and the common astrocytic gliomas, were particularly sensitive to chemotherapy. In particular, many anaplastic oligodendrogliomas respond dramatically to combined treatment with procarbazine, lomustine, and vincristine (termed PCV) (1,2). For unknown reasons, however, approximately one third of gliomas with oligodendroglial

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morphology are resistant to chemotherapy (3) and, to date, clinical and histopathologic differences have not predicted which oligodendrogliomas will respond and which will not respond to treatment (4,5). The inability to predict the chemosensitivity of oligodendrogliomas complicates advising early chemotherapy and delaying customary radiation therapy, even if early chemotherapy is frequently effective and devoid of toxic effects to the brain. In addition, the inability to predict chemoresistant tumors causes some patients receiving PCV to have unrealistic expectations of therapy and to experience side effects with little or no benefit. Thus, the ability to predict the response of brain tumors to therapies would have important benefits for oncologists and their patients.

Oligodendrogliomas, in addition to their sometimes unusual chemosensitivity, have specific molecular genetic alterations that distinguish them from astrocytic neoplasms and other subtypes of glioma (6,7). Allelic losses of chromosomes 1p and 19q are a molecular signature of oligodendrogliomas and occur in 50%–70% of both low-grade and anaplastic tumors (8–11). Furthermore, anaplastic oligodendrogliomas show losses of chromosome 9p (including the CDKN2A gene region) and chromosome 10q (11), as well as occasional TP53 gene mutations (12,13).

Molecular diagnostic approaches have already made inroads in oncologic diagnosis and prognosis (14,15). We hypothesized that molecular differences might explain why some oligodendrogliomas respond to PCV but others do not. To test this hypothesis, we performed molecular genetic and clinicopathologic analyses of 39 anaplastic oligodendrogliomas in which the chemotherapeutic response and survival status of the patients were known.

MATERIALS AND METHODS

Clinical Parameters

The 39 patients (18 males and 21 females) had a mean age of 46 years at diagnosis (range, 25–75 years) and a median Karnofsky performance score of 70 at the start of chemotherapy (range, 60–90). All patients received chemotherapy; 37 received PCV, one received carmustine, and one received cisplatin and etoposide.

At the time of chemotherapy, patients had tumors that were either newly diagnosed ($n = 24$) or recurrent after radiation therapy ($n = 15$). All tumors

were visible by neuroimaging; 34 showed contrast enhancement, with six displaying ring enhancement. All tumors with ring enhancement were newly diagnosed.

Response to chemotherapy was assessed by sequential neuroimaging and was defined as a decrease in tumor size of 50% or greater (16); care was taken to control for steroid effects (17,18) and other false-positive responses (16). The median time to maximum radiographic response was 4 months (range, 2–8 months). Of the 30 patients (77%) who responded to chemotherapy, 28 responded to PCV, one responded to carmustine, and one responded to cisplatin and etoposide. Among the 30 responding patients, there were 14 complete responses (16). Among newly diagnosed patients, nine received radiation therapy after responding to chemotherapy, and seven nonresponders were irradiated. The median follow-up was 9.47 years (range, 0.5–14.5 years).

These investigations have been approved by the Massachusetts General Hospital Subcommittee on Human Studies and the Review Board for Health Sciences Research Involving Human Subjects at the University of Western Ontario.

Diagnosis and DNA Extraction

All 39 tumors were classified by at least two neuropathologists (R. R. Hammond and D. A. Ramsay) according to World Health Organization (WHO) cri-

teria (19), with a final central review by one of the authors (D. N. Louis). At diagnosis, 37 tumors were classified as anaplastic oligodendrogliomas, WHO grade III (Fig. 1, A and C). Tumors with necrosis, including pseudopalisading tumor cells around areas of necrosis, were included; these two histologic features were recorded separately. Two of the tumors were symptomatic enlarging and enhancing lesions, pursuing aggressive clinical courses, and were classified at diagnosis as WHO grade II oligodendrogliomas. Tumor DNA was extracted from microdissected, formalin-fixed, paraffin-embedded sections (20,21). Constitutional DNA was extracted from blood leukocytes or from formalin-fixed, paraffin-embedded sections of adjacent, uninvolved brain or from other tissues (20,21).

Molecular Genetic Assays

Allelic loss was assessed by loss-of-heterozygosity assays (10,20–22) in constitutional DNA/tumor DNA pairs by use of microsatellite markers located on chromosome 1p (D1S508 and D1S2734), chromosome 10q (D10S88, D10S109, and D10S169), and chromosome 19q (D19S219, D19S412, and D19S596). These microsatellite markers span the regions of chromosomes 1p, 10q, and 19q that are commonly lost in anaplastic oligodendrogliomas; the loss of all markers from each chromosomal arm typically represents loss of the entire arm in anaplas-

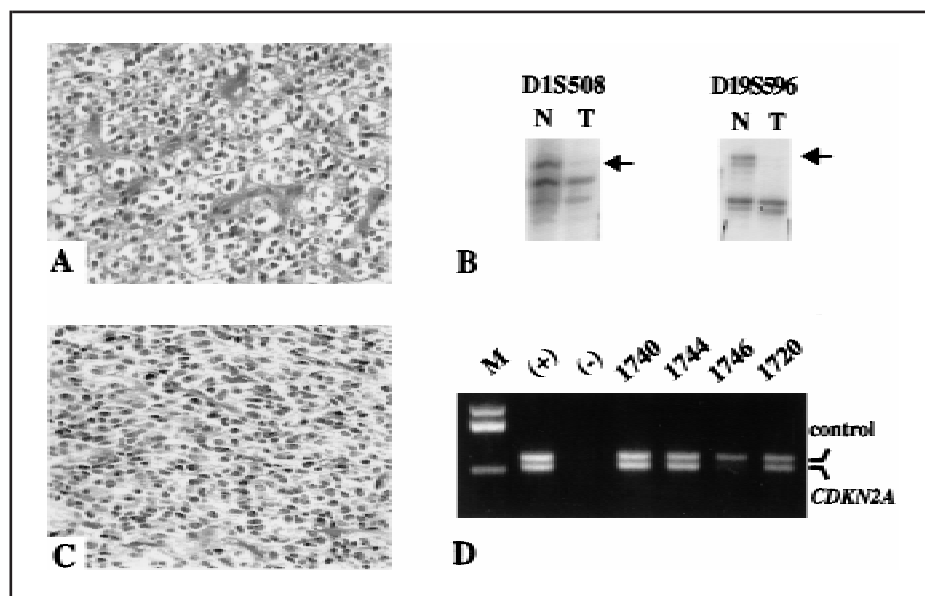


Fig. 1. Representative histopathologic and molecular genetic characteristics of oligodendrogliomas from the patient population. **A**) Tumor from patient 1720 had histologic features characteristic of oligodendroglioma (hematoxylin–eosin stain, original magnification $\times 400$). Other regions of the tumor from patient 1720 show anaplastic features similar to those in panel C. **B**) Allelic loss was assessed in a polymerase chain reaction (PCR)-based loss-of-heterozygosity assay. Tumor 1720 displayed allelic loss of chromosome 1p (loss of microsatellite D1S508) and chromosome 19q (loss of microsatellite D19S596) (lane N, constitutional DNA; lane T, tumor DNA; arrows, allelic loss in the tumor) but retained CDKN2A gene (see panel D, lane 1720). Patient 1720 responded completely to combined procarbazine, lomustine, and vincristine chemotherapy (termed PCV) and is alive without recurrence of disease 6.4 years after diagnosis. **C**) The tumor from patient 1746 has characteristic histologic features of anaplastic oligodendroglioma (hematoxylin–eosin, original magnification $\times 400$). **D**) Tumor 1746 had a CDKN2A gene deletion, as shown by the absence of a CDKN2A gene-specific band in lane 1746. The upper band is the control PCR product; the lower band is the CDKN2A gene PCR product. Lanes: M, DNA molecular size marker; (+), normal DNA control; (-), “no DNA” control. Note that tumors 1740 and 1744 retained the CDKN2A gene (lanes 1740 and 1744, respectively). Tumor 1746 retained alleles on chromosomes 1p and 19q (data not shown), and patient 1746 did not respond to PCV chemotherapy and died 1.7 years after diagnosis.

tic oligodendrogliomas (10,11,22,23). TP53 gene mutations were detected by single-strand conformation polymorphism analysis of exons 5–8 and by direct sequencing of aberrantly migrating bands from the polymerase chain reaction (PCR)-amplified target DNA (24,25). Homozygous deletions of the CDKN2A gene were evaluated with a comparative multiplex PCR assay (26).

Immunohistochemistry

For immunohistochemistry, the avidin–biotin complex technique was used to assess the MIB-1 proliferation index and the expression of p53 protein (encoded by the TP53 gene) and p16 protein (encoded by the CDKN2A gene) (24–28). MIB-1 immunoreactivity was evaluated with an image analysis system composed of a Sony 3 charge-coupled device color video camera and an Olympus BX60 microscope interfaced with a PowerComputing Pro 200 computer. The percentage of MIB-1 immunoreactive tumor cells was calculated after 1000 cells from the region of highest labeling were counted by use of Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Levels of immunoreactive p53 protein and p16 protein were estimated visually, and the results were scored as 0 (no staining), 1 (<5% of cells positive), 2 (5%–50% of cells positive), or 3 (>50% of cells positive).

Statistical Analyses

The primary goals of the analyses were to determine whether molecular genetic, pathologic, and clinical features 1) predicted chemosensitivity, as measured by radiologic response to chemotherapy

and recurrence-free survival from the start of chemotherapy, and 2) were associated with the clinical outcome of overall survival from the time of initial diagnosis. The association between dichotomous variables was tested with Fisher's exact test. The analysis of survival and recurrence-free survival was made with Cox regression models. For survival analyses, patients were censored at their last follow-up. Patients who were alive and who had no evidence of disease progression were treated as censored for the analysis of recurrence-free survival. Survivor function curves were calculated with the Kaplan–Meier method. Multivariate Cox models were fit by use of a stepwise (forward and backward) regression to select the combination of variables that independently predicted the outcome. For all Cox models, the relative risk (RR) and 95% confidence interval (CI) for significant predictors are reported. This estimate reflects the increased hazard for failure for patients with the prognostic factor versus those without. All reported *P* values are two-sided.

RESULTS

Allelic Losses of Chromosomes 1p and 19q as Positive Predictors of Chemotherapeutic Response

Allelic loss of chromosome 1p occurred in 24 (67%) of 36 informative DNA pairs (Table 1; Fig. 1, B). Allelic loss of chromosome 19q was found in 28 (82%) of 34 informative DNA pairs

(Table 1; Fig. 1, B). No DNA pairs had partial deletions of chromosome 1p or 19q, which is consistent with earlier studies showing that oligodendrogliomas typically display allelic loss of the entire short arm of chromosome 1 (11,23) and of the entire long arm of chromosome 19 (10,22). Furthermore, allelic losses of chromosomes 1p and 19q were closely associated with one another (*P* = .008), which is consistent with previous reports (9,11); 22 of 34 patients informative for markers on chromosomes 1p and 19q had combined allelic losses of chromosomes 1p and 19q.

Loss of chromosome 1p was associated strikingly with radiologic response to chemotherapy (*P* < .001; Table 2). Those tumors that responded to chemotherapy were likely to have lost chromosome 1p (24 of 27, or 89%) and those tumors with chromosome 1p loss were all chemosensitive (24 of 24, or 100%). Conversely, of the nine chemoresistant tumors that could be evaluated for allelic loss of chromosome 1p, all nine (100%) retained both copies of chromosome 1p (data not shown). The association of chromosome 19q loss and chemotherapeutic response was not statistically significant. However, patients whose tumors had lost both chromosomes 1p and 19q had a significantly greater chance of responding to chemotherapy than patients whose tumors lacked such combined losses (*P* < .001; Table 2). Of those patients whose tumors had lost both chromosomes 1p and 19q, 100% (22 of 22) responded to chemotherapy. Similarly, those patients who responded to chemotherapy were likely to have combined losses of alleles on chromosomes 1p and 19q (22 of 26, or 85%; Fig. 1, B).

The association between losses of chromosomes 1p and 19q and response to chemotherapy also was reflected in significantly longer recurrence-free period of survival after chemotherapy. Those patients whose tumors retained both copies of chromosome 1p had an RR of recurrence or death after chemotherapy that was 4.3 times greater than that of patients whose tumors had lost chromosome 1p (*P* = .0014). Furthermore, patients whose tumors retained chromosome 19q alleles had an RR of recurrence or death after chemotherapy that was 5.6 times greater than that of patients with tumors lacking

Table 1. Clinical, pathologic, molecular genetic, and outcome characteristics of the patient population

Characteristic variable	Result
Clinical	
Age at diagnosis, y	46 (mean; range, 25–75)
Karnofsky performance status	70 (median; range, 60–90)
Enhancement, No./total No.	34/39 (87%)
Ring enhancement, No./total No.	6/39 (15%)
Pathologic	
Necrosis, No./total No.	19/39 (49%)
Necrosis with pseudopalisading, No./total No.	9/39 (23%)
MIB-1 labeling index	18% (mean; range, 0%–43%)
p16 immunoreactivity	
Absent, No./total No.	2/38 (5%)
<5%, No./total No.	11/38 (29%)
5%–50%, No./total No.	9/38 (24%)
>50%, No./total No.	16/38 (42%)
p53 immunoreactivity	
Absent, No./total No.	0/39
<5%, No./total No.	3/39 (8%)
5%–50%, No./total No.	19/39 (49%)
>50%, No./total No.	17/39 (43%)
Molecular genetic	
Chromosome 1p loss, No./total No.	24/36 (67%)
Chromosome 19q loss, No./total No.	28/34 (82%)
Chromosome 1p and 19q loss, No./total No.	22/34 (65%)
Chromosome 10q loss, No./total No.	8/34 (24%)
CDKN2A gene deletion, No./total No.	8/38 (21%)
TP53 gene mutation, No./total No.	6/39 (15%)
Outcomes	
Response, No./total No.	30/39 (77%)
5-y survival	66%
Median survival	6.76 y
Median recurrence-free survival after chemotherapy	2.0 y

Table 2. Univariate genetic predictors of response and risk of death in patients with anaplastic oligodendrogliomas*

Variable	Chemotherapeutic response		Risk of death		
	Response rate, No./total No. (%)	<i>P</i>	RR	<i>P</i>	95% CI
Chromosome 1p					
Allelic loss	24/24 (100)	<.001	0.059	<.0001	0.018–0.199
Intact	3/12 (25)				
Chromosome 19q					
Allelic loss	23/28 (82)	.126	0.250	.0116	0.085–0.734
Intact	3/6 (50)				
Chromosomes 1p and 19q					
Allelic loss of both	22/22 (100)	<.001	0.121	<.0001	0.044–0.331
No allelic loss of both	4/13 (31)				
Chromosome 10q					
Allelic loss	5/8 (63)	.126	1.817	.2549	0.650–5.081
Intact	23/26 (88)				
CDKN2A gene					
Deleted	5/8 (63)	.363	4.901	.0009	1.924–12.487
Intact	24/30 (80)				
TP53 gene					
Mutant	3/6 (50)	.123	2.586	.0724	0.917–7.293
Wild-type	28/34 (82)				

*Risk of death was calculated as RR (relative risk) and was determined for all Cox models. Corresponding *P* values are shown with significant values in boldface type. All *P* values are two-sided. CI = confidence interval.

alleles on chromosome 19q (*P* = .0017). Finally, those patients whose tumors retained both chromosomes 1p and 19q had an RR of recurrence or death after chemotherapy that was 5.7 times greater than that of patients with combined allelic losses on chromosomes 1p and 19q (*P* = .0003).

Neither age at chemotherapy nor Karnofsky performance status was significantly associated with response to chemotherapy. Of the remaining clinical, pathologic, and molecular genetic factors analyzed (Table 1), only ring enhancement on neuroimaging was associated with chemotherapeutic response and recurrence-free survival after chemotherapy. Although this was an uncommon feature, none of the six patients whose tumors had ring enhancement responded to chemotherapy.

Association of Allelic Loss of Chromosomes 1p and 19q With Longer Survival and Association of CDKN2A Gene Deletions and Ring Enhancement With Shorter Survival

The loss of chromosome 1p was a statistically significant predictor of survival from the time of initial diagnosis (Table 2; Fig. 2, A); the RR of death for patients whose tumors retained chromosome 1p microsatellite markers was 17-fold greater than that for patients whose tu-

mors lost chromosome 1p alleles (*P* < .0001; Table 2). Loss of chromosome 19q markers was also a significant predictor of survival; the RR of death for patients whose tumors retained chromosome 19q was four times greater than that for patients whose tumors had 19q loss (*P* = .0116; Table 2). When combined as a single variable, allelic loss of chromosomes 1p and 19q showed a remarkably strong association with survival; the RR of death for patients whose tumor retained alleles from both chromosomes 1p and 19q was 8.3 times greater than that for patients whose tumors showed this combined change (*P* < .0001; Table 2). The 5-year survival rate for patients whose tumors had lost chromosomes 1p and 19q was 95% (Fig. 2, A).

Homozygous CDKN2A gene deletions were detected in eight (21%) of 38 cases (Table 1; Fig. 1, D). CDKN2A deletions occurred preferentially in tumors that retained chromosome 1p or 19q (*P* = .048), suggesting the presence of at least two distinct genetic subsets of anaplastic oligodendroglioma. Accordingly, in contrast to allelic losses of chromosomes 1p and 19q, deletions of the CDKN2A gene predicted reduced survival (Table 2; Fig. 2, B). The RR of death for patients whose tumors carried a CDKN2A gene deletion was 4.9 times greater than that for patients whose tumors lacked such deletions (*P* =

.0009). In turn, patients with tumors containing a CDKN2A gene deletion had a 5-year survival rate of only 38% (Fig. 2, B). In contrast to the molecular genetic data, immunohistochemical assessment of the p16 protein, encoded by the CDKN2A gene, was not predictive of outcome (Table 1).

To determine whether other molecular genetic events were characteristic of this group of oligodendrogliomas, we performed loss-of-heterozygosity assays by using chromosome 10q markers and examined tumor DNAs for TP53 gene mutations. Chromosome 10q loss occurred in eight (24%) of 34 informative cases. Although chromosome 10q loss was associated with the presence of perinecrotic pseudopalisading (*P* = .0035), chromosome 10 status was not associated with survival. TP53 gene mutations were found in six (15%) of 39 cases, with mutation types characteristic of other malignant gliomas (29). However, neither TP53 gene mutation status nor p53 immunohistochemical results were associated with survival (Tables 1 and 2).

Characteristic of malignant glioma, patients 45 years of age or younger fared better than those older than 45 years (*P* = .0264). However, Karnofsky performance status was not associated with survival. In addition, contrast enhancement was not prognostically significant, but ring enhancement was a powerful negative predictor of survival (RR = 26.26; 95% CI = 6.94–99.28; *P* ≤ .0001), with no patients whose tumors had ring enhancement alive 5 years after diagnosis. Of the pathologic features evaluated, the presence of necrosis and necrosis with pseudopalisading was not associated with response or survival; the MIB-1 proliferation index was not associated with response, but higher MIB-1 indices were associated with decreased survival (*P* = .0253).

A stepwise Cox multivariate selection determined that the best model for predicting survival included combined allelic loss of chromosomes 1p and 19q (*P* = .0050) with CDKN2A gene deletion (*P* = .0002) and ring enhancement (*P* = .0008). Allelic loss of chromosomes 1p and 19q was associated with markedly better survival (RR = 0.136; 95% CI = 0.3–0.54), whereas CDKN2A gene deletions (RR = 10.331; 95% CI = 3.02–35.37) and ring enhancement (RR =

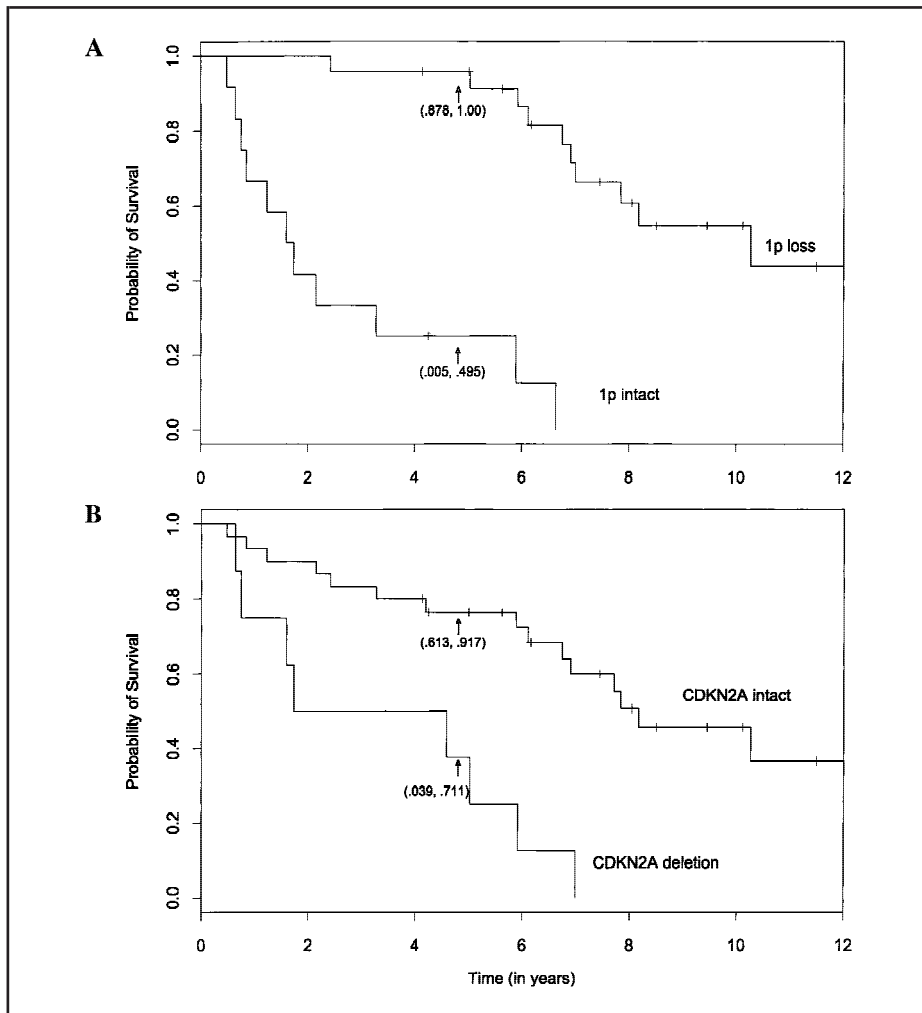


Fig. 2. Kaplan-Meier analysis showing the probability of survival among 39 patients with anaplastic oligodendroglioma. **A)** Those patients whose tumors have chromosome 1p loss have markedly better survival. **B)** Those patients whose tumors had CDKN2A gene deletions had worse survival. Vertical marks represent length of evaluation on individual patients alive at the time of last follow-up, and 95% confidence intervals for various times are shown in parentheses.

17.867; 95% CI = 3.29–96.95) were associated with markedly worse survival. Including clinical features, such as age and performance status, or pathologic features, such as MIB-1 index, did not add to the predictive power of this model.

DISCUSSION

To identify more objective methods of establishing which anaplastic oligodendrogliomas respond to chemotherapy and which patients can expect longer survival, we analyzed a variety of characteristic genetic changes in patients with anaplastic oligodendroglioma who were selected on the basis of known radiographic response or lack of response to chemotherapy. Strikingly, allelic loss of chromosome 1p or combined allelic loss of chromosomes 1p and 19q predicted a far greater likelihood of chemotherapeutic response.

While the mechanism by which loss of genetic information on these chromosomes confers chemosensitivity remains unclear, allelic losses typically unmask inactivated tumor suppressor genes that reside on the remaining alleles (30). Understanding the biologic basis of anaplastic oligodendroglioma chemosensitivity and the direct or indirect effects associated with the loss of chromosomes 1p and 19q must await the isolation of the respective genes. Nonetheless, our results demonstrate, to our knowledge for the first time, that specific molecular genetic changes may be used as markers of relative chemosensitivity in malignant gliomas and could, therefore, guide therapeutic decisions in the management of these patients. Moreover, such genetic alterations appear to be more powerful determinants of a favorable chemotherapeutic

response in patients with anaplastic oligodendroglioma than younger age of the patient at diagnosis, better performance status, or absence of tumor necrosis.

In addition to predicting chemotherapeutic response, molecular genetic findings provide important prognostic information on overall survival for patients with anaplastic oligodendroglioma. Loss of chromosomes 1p and 19q, independently or in combination, augurs for a more favorable clinical course with markedly longer overall survival in patients whose tumors had these alterations (Fig. 2, A). Loss of chromosomes 1p and 19q occurred primarily in those anaplastic oligodendrogliomas without CDKN2A gene deletions, suggesting the existence of a CDKN2A gene deletion-dependent pathway to anaplastic oligodendroglioma that does not involve inactivation of tumor suppressor genes on chromosomes 1p and 19q (6). In contrast to the loss of chromosome 1p or 19q, CDKN2A gene deletions portend a worse prognosis, with median survival times of less than 2 years in patients whose tumors had CDKN2A gene deletions (Fig. 2, B). Curiously, however, CDKN2A gene deletions were not associated with higher MIB-1 proliferation indices, as has been reported for anaplastic astrocytomas and glioblastomas (26). Thus, the worse prognosis associated with CDKN2A gene deletions is not simply secondary to increased cellular proliferation. Regardless of the mechanism, anaplastic oligodendrogliomas that progress via the CDKN2A gene deletion pathway appear to represent a more resistant and aggressive subset of histologically indistinguishable tumors.

The only other parameter to prove predictive for the response to chemotherapy and survival was the presence of ring enhancement on neuroimaging. Although this was an uncommon finding, none of the six tumors with ring enhancement showed radiologic responses to chemotherapy, and patient survival was short. Examples of ring enhancement, however, did not overlap significantly with CDKN2A gene deletions; upon multivariate analysis, ring enhancement and CDKN2A gene deletions were independent, adverse prognostic factors. It is interesting that perinecrotic pseudopalisading (the histologic feature most characteristic of the highly malignant glioblastoma) was not associated with

survival, with CDKN2A gene deletion, or with ring enhancement. Thus, a tumor with CDKN2A gene deletions or ring enhancement does not simply reflect misdiagnosis of glioblastoma; instead, it represents a more malignant subtype of anaplastic oligodendroglioma. Furthermore, from a diagnostic neuropathologic point of view, perinecrotic pseudopalisading in an anaplastic oligodendroglioma is not synonymous with the ominous prognosis of glioblastoma. Oligodendrogliomas with perinecrotic pseudopalisading may still respond to PCV chemotherapy (3).

The prognostic significance of these molecular genetic alterations is highlighted by the final Cox multivariate model. If we include all of the assayed clinical, pathologic, and genetic parameters, only allelic loss of chromosomes 1p and 19q, CDKN2A gene deletions, and ring enhancement are simultaneous predictors. Even the age of the patient at diagnosis, although significant upon univariate analysis, is not independently predictive upon multivariate analysis. To our knowledge, this represents the first instance in neurooncology in which genetic factors supersede patient's age at diagnosis as a predictor of survival. Thus, it is predicted that a patient whose anaplastic oligodendroglioma has lost both chromosomes 1p and 19q would show substantial radiologic response to chemotherapy and would have better survival. Indeed, 95% of such patients are alive 5 years after diagnosis. As such, patients whose tumors have loss of chromosomes 1p and 19q might be candidates for post-surgical treatment strategies in which PCV-based chemotherapy is used while radiotherapy is delayed (31,32). A patient who has a histologically similar tumor with a CDKN2A gene deletion, however, would be expected to be less likely to respond to chemotherapy and could receive early radiation therapy. Such a patient would have an expected survival of only 2 years. Finally, a patient with a ring-enhancing anaplastic oligodendroglioma probably will not respond to PCV, has a prognosis of less than 1 year despite radiation therapy, and perhaps should be offered an investigational agent or a novel treatment.

While these results must necessarily be confirmed on a larger prospective series of patients with oligodendroglioma, the data nonetheless suggest that molecular

genetic alterations can provide powerful markers for the management of patients with anaplastic oligodendrogliomas. Furthermore, as the optimal timing and intensity of PCV chemotherapy are determined (3) and as other chemotherapeutic, radiotherapeutic, or surgical approaches are developed, the prognostic importance of molecular genetic analyses can be addressed. These findings raise the additional possibilities that molecular genetic analyses may have an impact on clinical decisions in the management of patients with other types of malignant glioma (6,33) and other human cancers for which a specific therapy is sometimes highly effective.

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