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Cancer biology for individualized therapy: Correlation of growth fraction index in native-state histoculture with tumor grade and stage

(histological autoradiography/biological grading/clinical validation)

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ABSTRACT **There is a need for individualization of all aspects of cancer therapy. Because of significant heterogeneity within a tumor class, there is a need to develop an *in vitro* test to accurately gauge tumor aggressiveness. Such a measurement would greatly aid treatment decision making. Current methodologies such as flow cytometry, which lacks unambiguous interpretation of cell-proliferative data, and determination of the thymidine-labeling index, which measures nucleotide uptake in a nonphysiological state, have not reproducibly attained this goal. We have developed an *in vitro* native-state three-dimensional gel-supported histoculture system that allows the growth of all human solid tumor types for relatively long time periods. The native-state system was used to identify the percent of cells capable of incorporating [^{3}H]thymidine over a 4-day period, which we term the growth fraction index (GFI). We have compared the ability of cancer tissue to proliferate in native-state culture to the stage and histological grade of four major types of human carcinomas: breast, ovarian, colon, and lung. Eighty percent of tumor explants could be evaluated, even when sent from across the country. We have determined that the GFI correlates with tumor stage and grade for breast and ovarian carcinoma. In colon carcinoma, there is a trend toward higher GFIs in tumors of more advanced stage and grade. In non-small cell lung carcinomas, GFI, stage, and grade do not correlate. These results suggest the applicability of gel-supported three-dimensional native-state histoculture for prognostic purposes in patients with breast and ovarian cancers and demonstrate the clinical relevance of the native-state histoculture system.**

An accurate means of determining prognosis for cancer patients at the time of primary tumor resection would greatly improve and individualize medical management. In addition to clinicopathologic staging, which measures the extent of tumor invasion, determination of tumor aggressiveness is necessary to increase predictability of survival and to determine modality of treatment. Histological and nuclear grading is used with some success but interobserver variation occurs frequently (1). In addition, clinical aggressiveness often varies among tumors of the same histological classification, stage, and grade.

Flow cytometry has been used for the measurement of cellular DNA content to determine the percentage of diploid vs. aneuploid tumor cells and to measure the percentage of cells in S phase. A good correlation between DNA content abnormalities and grade for breast tumors has been observed (2). Tumor aneuploidy has been predictive of shorter disease-free survival in most studies (3–5). However, in a series of 490 patients with node-negative breast cancer, DNA aneuploidy did not have independent prognostic significance (6). Similar studies have shown ploidy to be a significant prognostic indicator in patients with colon and ovarian cancers (5, 7–9). The use of DNA image cytometry can help exclude benign cells from being counted in the assay that may improve the accuracy of measurements (10). However, in the many tumors with multiple cell types, overlapping DNA content values may preclude accurate S-phase measurements. Highly differentiated breast tumors have lower S-phase values than undifferentiated ones (2). Increased tumor S-phase percentage determined by flow cytometry correlates with decreased survival in patients with node-positive breast cancer but is not an independent predictor of outcome (6). In node-negative breast tumors, S phase is an independent predictor of decreased disease-free survival in diploid but not aneuploid tumors (4).

NEU protooncogene amplification initially showed promise as an indicator of poor prognosis in patients with node-positive breast cancer and ovarian carcinoma (11, 12). However, other studies have failed to uphold this correlation (13).

The monoclonal antibody Ki-67, specific for a nuclear antigen present in non-G_0 cells, has been used to indicate cell proliferation (14, 15). However, a study of the human breast cancer cell line MCF-7 indicated that expression of the Ki-67 antigen may be undetectably low at the onset of DNA replication and that nonproliferating cells may retain the antigen for a period of time. Thus the Ki-67 antigen may not be a good indicator of the growth fraction (16). In one study, nuclear staining was noted in only 56% of breast carcinomas but, when present, did correlate with poorer histological grade and higher S-phase content (17). Ki-67 immunoreactivity did not correlate with known prognostic parameters in 108 patients with colorectal carcinoma (18), however.

Monoclonal antibodies can also be used to identify proliferating cells that have incorporated bromodeoxyuridine *in vivo* or *in vitro* (19). Bromodeoxyuridine staining closely correlated with thymidine labeling in human tumor xenografts and has the advantage of a shorter assay period (20). Initial studies have indicated increasing bromodeoxyuridine incorporation in gastric tumors of advanced stage and grade (21). However, technical difficulties, expense of antibodies, and lack of a large clinical correlation with this technique persist.

The thymidine labeling index (TLI), where tumor fragments are briefly incubated with [^{3}H]thymidine in a saline

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Abbreviations: TLI, thymidine labeling index; GFI, growth fraction index.

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solution, has been shown in numerous studies to have a strong correlation with prognosis in women with breast and ovarian cancer (22–26). In fact, TLI was reproducibly shown to have greater predictability of disease-free survival than estrogen receptor content in primary breast cancer (23, 25) and in one study was more important prognostically than tumor size or lymph node metastasis (25). However, TLI is measured under relatively nonphysiological conditions for only 1–2 hr (22–24, 27), giving rise to the need for a more native-state and practical model for measuring growth potential in national and international series of human cancers.

Thus we have developed a native-state technique of histoculturing tumors in three dimensions on flexible collagen-containing gels with the maintenance of native tumor histology and function and with a high success rate of sustained growth in culture (28–30). In the native-state system, long-term growth can be achieved, which can in some instances be 100 days or more. Consequently, the percentage of tumor cells undergoing cell division over a 4-day labeling period with [^{3}H]thymidine can be easily measured and is termed the growth fraction index (GFI). Since only cells replicating during a brief 2-hr labeling period are usually used to measure the TLI, cells with a slower replication cycle may be underrepresented by the TLI technique. Since these slower replicating cells may be equally malignant, the use of a longer labeling period coupled with more physiological conditions may improve the prognostic capabilities of cell labeling. In addition the physiological conditions used in the GFI methodology allow tissue to recover even after relatively long time periods in transit to the laboratory.

Our results indicate that the GFI of breast and ovarian cancer correlates with clinical stage and grade. This suggests that the GFI may have prognostic significance for these tumor types. In colon carcinomas there is a trend toward

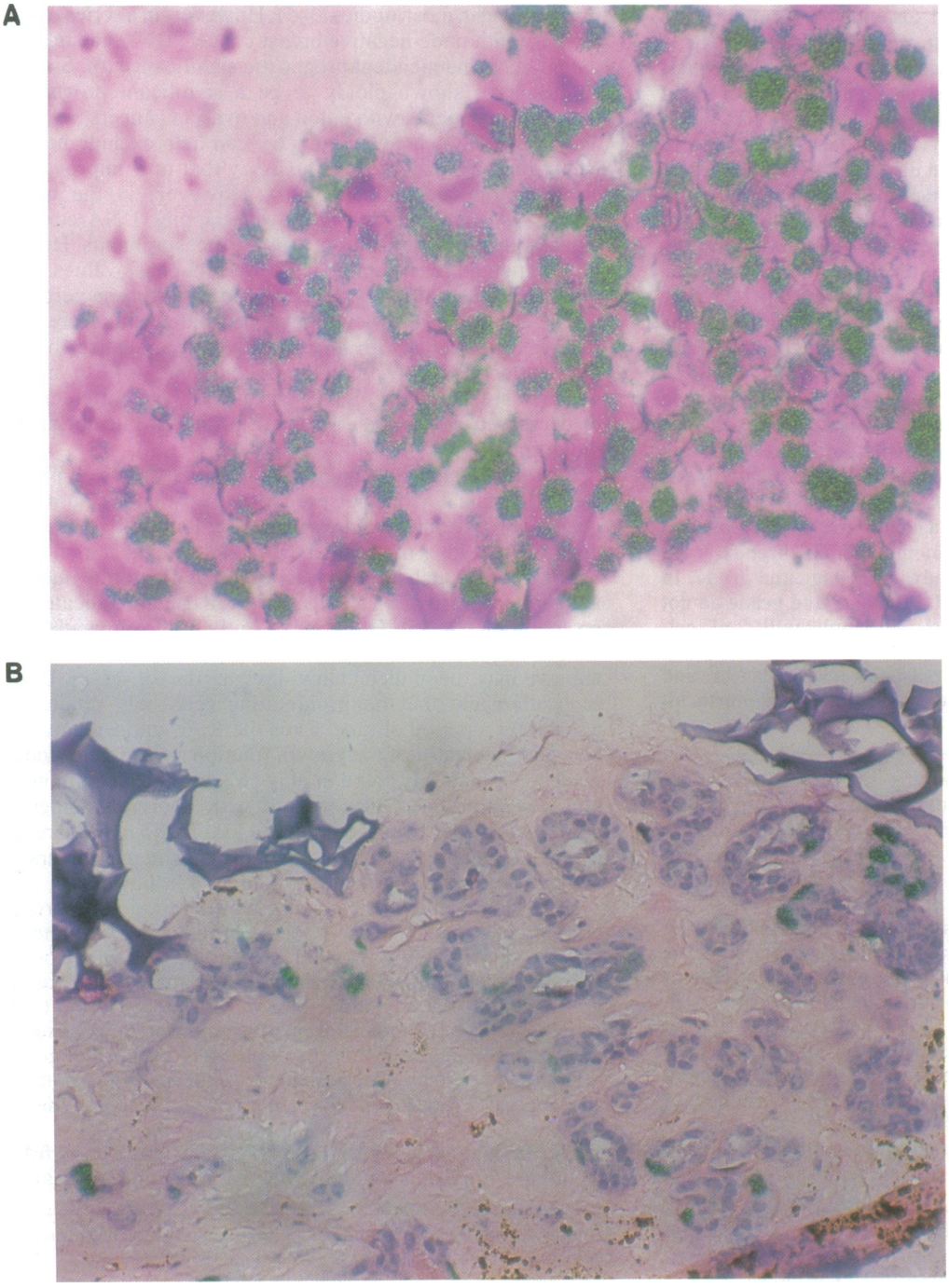

FIG. 1. Representative histological autoradiograms of tumor explants from two patients with node-negative breast cancer. Tumors were in culture 14 days and labeled with [^{3}H]-thymidine and [^{3}H]deoxyuridine for the last 4 days. Specimens were analyzed with epi-illumination polarization microscopy. Bright green grains over cells indicate uptake of [^{3}H]thymidine and, therefore, cell proliferation. Note the very high extent of cell proliferation in *A* (×5200) and the low level of proliferation in *B* (×2600). This emphasizes the range of biological aggressiveness in patients with similar-staged breast cancer.

higher GFI when the tumors are of high stage and grade. In lung carcinoma, GFI, stage, and grade do not correlate.

MATERIALS AND METHODS

Tumor Tissue Acquisition. Tumor samples were obtained from various hospitals in the San Diego region as part of a study to determine which *in vitro* biological parameters correlate with clinical data. In addition specimens came from Alabama and Los Angeles by overnight delivery systems.

Tumor Histoculture. Tumors were removed surgically, placed into culture medium [Eagle's minimal essential medium containing Earle's salts, L-glutamine (0.3 mg/ml), 10% (vol/vol) fetal calf serum, nonessential amino acids (1:100 dilution of stock solution from Irvine Scientific), and gentamicin (0.2 mg/ml)] by the pathologist, and transported to the laboratory.

The tumors were minced into pieces 1–2 mm in diameter and placed on hydrated pigskin collagen gel matrices (Health Design Industries, Rochester, NY) within 48 hr of removal from the patient. After allowing tumor cell growth in culture for 3–11 days, [^{3}H]thymidine (4 μCi/ml; 1 Ci = 37 GBq) was added to label replicating cells for 4 additional days.

The cultures were then washed with isotonic phosphate-buffered saline, placed in histology capsules, and fixed in 10% (vol/vol) formalin. The histocultures were then dehydrated, embedded in paraffin, sectioned, and placed onto slides. The slides were deparaffinized and then coated with Kodak NTB2 emulsion in a dark room and exposed for 5 days at 4°C before developing. After rinsing, the slides were stained with hematoxylin and eosin.

Determining GFI. The slides were then analyzed with a polarizing microscope at ×400 power in a blinded fashion (30). Replicating cells were easily determined by the presence of bright-green-reflecting silver grains over the cell nuclei. Benign stromal cells were excluded by their morphologic appearance. The percentage of cells undergoing DNA synthesis was determined in at least three visual fields with the heaviest labeling for each tumor piece. The GFI was calculated by dividing the number of labeled tumor cells by the number of unlabeled tumor cells.

Determining Histological Staging. Breast, ovarian, and lung cancers were staged in accordance with the American Joint Committee on Cancer (31). The Astler–Coller modification of the Dukes system was used to stage the colorectal carcinomas (31). Histological grade was used only if included in the original pathology report. Pathologic staging and histological grading were then matched to the GFI in a blinded manner. Statistical analysis was performed using the Student's *t* distribution test.

RESULTS

Four tumor types were used for GFI analysis. Fig. 1 depicts two typical histological autoradiograms visualized by the polarization microscopy used to determine GFI. The bright-green nuclear staining is due to silver grains being exposed due to [^{3}H]thymidine incorporation. The polarized light is then reflected as bright green (30). Fig. 1*A* depicts a tumor with high labeling and Fig. 1*B* is from a tumor with low labeling. Despite the marked difference in labeling, both tumor explants came from patients with node-negative breast cancer with the high GFI measured in a high-grade tumor and with the low GFI measured in a low-grade tumor.

Breast Cancer. Eighty percent of the explants from 152 individual cases of breast cancer proved cultivatable. Of these, 120 specimens had been assigned a histopathological stage. These were divided into nonmetastatic (101 cases), which could include local node involvement, and metastatic to distant sites (19 cases). The mean GFI for all breast tumors was 31 ± 17% (mean ± SEM) and the median was 29% (range 5–88%). Patients with nonmetastatic disease had significantly lower GFIs (28 ± 15%) compared to patients with metastatic disease (37 ± 15%) ($P < 0.025$). Fig. 2*A* demonstrates that with increasing GFI there is an increasing percentage of metastatic tumors. The histological grade was available for 96 patients. Thirty-nine patients had tumors with well or moderate differentiation with a mean GFI of 23 ± 11%. This was significantly lower than the mean GFI of 34 ± 16% determined in 57 patients with poorly differentiated cancer ($P < 0.0005$). As the GFI increased, there was a greater percentage of undifferentiated tumors (Fig. 2*B*).

Ovarian Cancer. Seventy-one ovarian tumors were cultured successfully with a mean GFI of 34 ± 19% (median = 34%; range = 1–79%), representing 88% of the specimens explanted. Stage I–II disease, present in 4 patients, was associated with a mean GFI of 13 ± 15% compared to the mean GFI of 40 ± 17% in 40 patients with abdominal or distant metastasis. The differences in GFI between limited and advanced (stage III–IV) disease was highly significant ($P < 0.005$). Fig. 3*A* demonstrates that with increasing GFI there was an increasing percentage of metastatic tumors. Seven patients had tumors of well or moderate differentiation compared to 24 patients with poorly differentiated carcinoma. The mean GFIs were 33 ± 18% and 43 ± 17%, respectively, for these two groups, but despite the trend, this was not statistically significant ($P = 0.15$). Fig. 3*B* shows that as GFI increased the percentage of poorly differentiated tumors also increased.

Colorectal Cancer. Of 114 colorectal carcinomas, 75 were cultured successfully (66%) with a mean GFI of 43 ± 18%

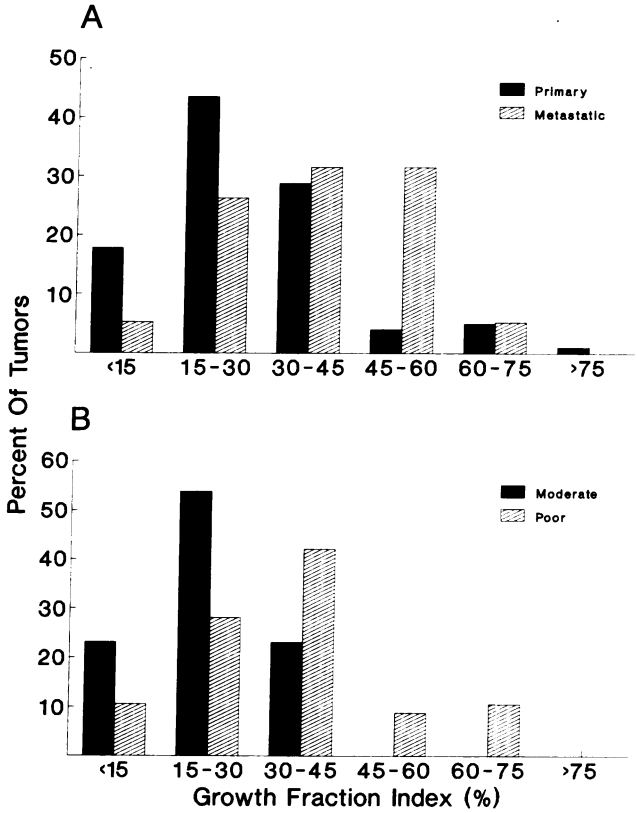

FIG. 2. (*A*) Distribution of percent GFI in tumor explants from 120 breast cancer patients with respect to stage (local tumors include lymph node involvement). Mean GFI = 28% for local tumors and 37% for metastatic disease ($P < 0.025$). (*B*) Distribution of percent GFI in tumor explants from 96 breast cancer patients with respect to histological grade. Mean GFI = 23% for moderately differentiated tumors compared to 34% for poorly differentiated tumors ($P < 0.0005$).

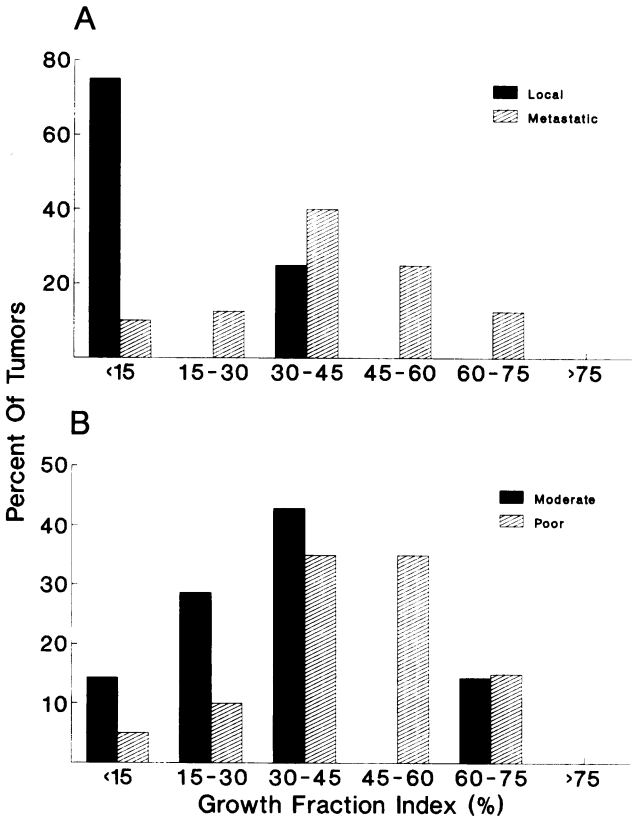

FIG. 3. Distribution of percent GFI in tumor explants from 44 ovarian cancer patients with respect to stage. Mean GFI = 13% for stage I–II disease and 40% for stage III–IV disease ($P < 0.005$). (*B*) Distribution of percent GFI in tumor explants from 31 ovarian cancer patients with respect to histological grade. Mean GFI = 33% for moderately differentiated tumors compared to 43% for poorly differentiated tumors ($P = 0.15$).

(median = 46%; range = 5–80%). Unfortunately, 35 cultures were not evaluated because of microbial contamination. Mean GFIs were 37 ± 14% for 15 stage A/B tumors, 40 ± 19% for 15 stage C, and 44 ± 20% for 19 stage D tumors. Fig. 4*A* depicts a trend correlating advancing stage with increasing GFI although this was not statistically significant. Histologic grade was obtainable for 49 patients, only 2 of whom had tumors that were well differentiated (GFI = 26 ± 8.0%). Moderate differentiation in 34 tumors was associated with a mean GFI of 44 ± 19% compared to 40 ± 17% in the 13 patients with poorly differentiated tumors. Fig. 4*B* demonstrates a trend correlating poorer histologic grade with increasing GFI.

Non-Small-Cell Lung Cancer. GFIs were determined for 79 of 98 patients with non-small-cell lung cancer (81%). The mean GFI for all non-small-cell lung cancer was 39 ± 18% (median = 37%; range = 8–91%). There was almost no difference in mean GFIs of tumors obtained from patients with regard to tumor stage (Fig. 5*A*). The mean GFIs were as follows; 37 ± 16% for the 20 patients with stage I disease, 36 ± 16% for the 15 patients with stage II/III disease, and 34 ± 15% for the 6 patients with stage IV disease. Furthermore, histological grade had little influence on mean GFI being 39 ± 17% for the 3 well-differentiated tumors, 37 ± 15% for the 11 moderately differentiated tumors, and 36 ± 18% for the 24 poorly differentiated tumors (Fig. 5*B*).

DISCUSSION

There is a need to develop accurate individualized prognostic indicators at the time of primary tumor resection to optimize treatment. As an example, recent studies indicate a disease-free survival advantage if women with estrogen receptor-negative tumors or estrogen receptor-positive tumors greater than 3 cm in size are treated with postoperative chemotherapy (84% disease-free survival at 3 years vs. 69% for the untreated control group) (32). However, since only 30% of women with node-negative breast cancer have disease recurrence after surgery (33), the use of postoperative adjuvant chemotherapy subjects many women to toxic chemotherapy needlessly. A method to better identify the patients at higher risk of having tumor recurrence could improve the selection of patients who require adjuvant chemotherapy (34).

The TLI has been demonstrated to have independent prognostic significance in breast and ovarian carcinoma (22–25). Our native-state technique of measuring the GFI of tumor explants in histoculture has a number of important advantages over TLI. By growing the tumors in histoculture, the amount of time the cells are exposed to tritiated thymidine is increased to 4 days, in contrast to the 2 hr used for TLI where tumors are not cultured. Since many tumor cells may not cycle into the G_0 phase of cell growth during the brief 2-hr labeling time allocated by the TLI method, many cells capable of cell proliferation may not be assayed. The 4-day labeling period used to determine the GFI will consequently allow the detection of even slowly dividing cells. This difference may be dramatic as evidenced by the mean GFI of 28% for primary and 37% for metastatic breast carcinoma compared to the mean TLI of 6.8% and 8.8%, respectively, in similar patients. Thus, slowly dividing tumor cells are probably underrepresented by the TLI yet these cells may be equally malignant and possibly the most unlikely to respond to chemotherapy. The technique of measuring the GFIs in

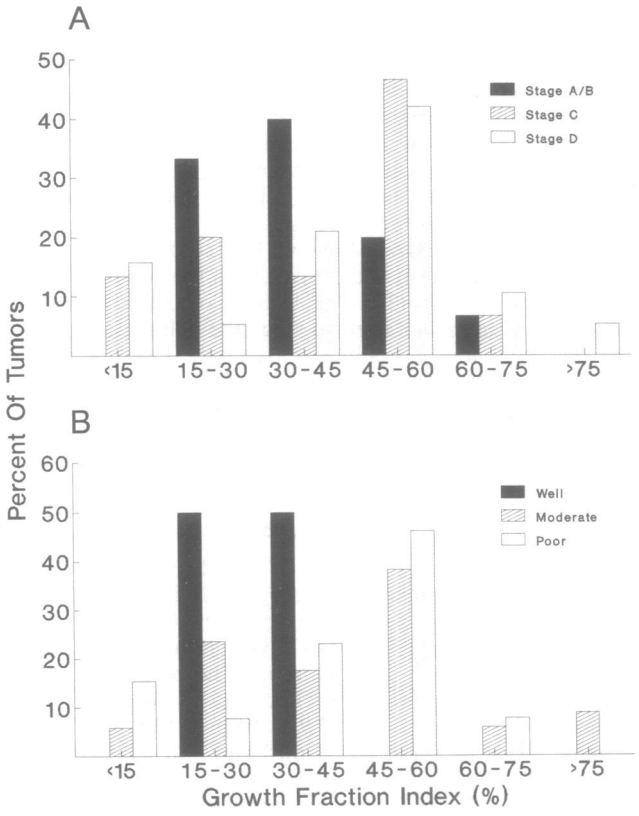

FIG. 4. (*A*) Distribution of percent GFI in tumor explants from 49 colorectal cancer patients with respect to modified Astler–Coller staging. Mean GFI were 37%, 40%, and 44% for stage A/B, C, and D disease, respectively. (*B*) Distribution of percent GFI in tumor explants from 49 colorectal cancer patients with respect to histological grade. Mean GFI values were 26%, 44%, and 40% for well, moderately, and poorly differentiated tumors, respectively.

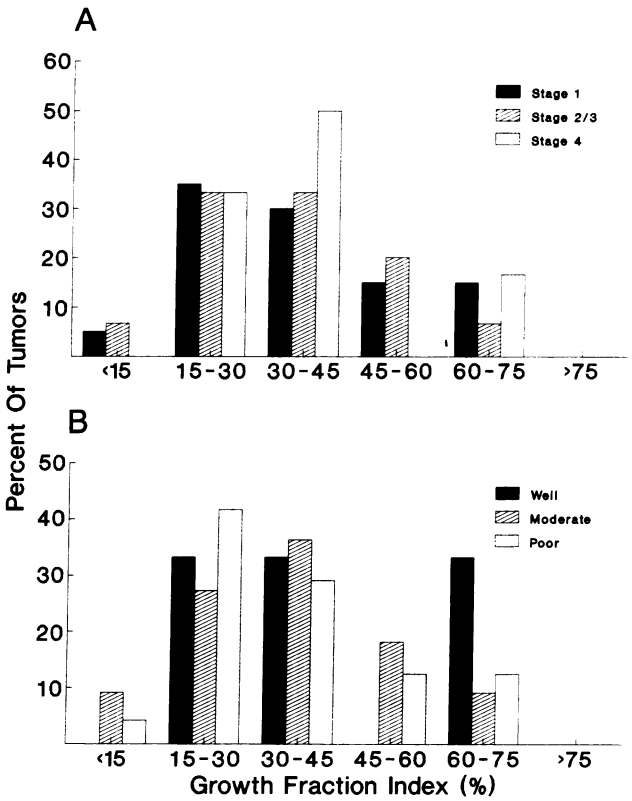

FIG. 5. (*A*) Distribution of percent GFI in tumor explants from 41 non-small-cell lung cancer patients with respect to stage. Mean GFI = 37%, 36%, and 34% for stage I, II–III, and IV disease, respectively. (*B*) Distribution of percent GFI in tumor explants from 38 non-small-cell lung cancer patients with respect to histological grade. Mean GFI = 39%, 37%, and 36% for well, moderately, and poorly differentiated tumors, respectively.

fields with the most active labeling may explain some of this increase. However, because of tumor heterogeneity, we believe the use of this technique is valid since it assures that the most proliferative and viable areas of the histoculture are used for determination of the GFI. Of similar importance, the native-state histoculture system allows specimens to recover even after 3–4 days in transport medium, allowing GFI to be measured on a mass scale for cancer patients. This stands in contrast to the relatively nonphysiological TLI methodology.

Our results show that for breast and ovarian cancers, advanced pathologic stage and histologically poor cellular differentiation correlates with a higher GFI. This suggests that the GFI has clinical relevance for these tumors. Similar prognostic significance has been demonstrated for the TLI in patients with breast cancer (22–25). In node-negative women treated surgically, the 5-year survival was 89% in patients with a TLI <3% compared to 66% in patients with a TLI >8% (22). Although the current recommendation is to treat all breast cancer patients with node-negative disease with chemotherapy (33), a low GFI might identify patients with a very low likelihood of tumor recurrence post-operatively and thus eliminate the need to use costly and toxic chemotherapy for these patients.

Although the mean GFIs did not show a statistical difference between stage and grade for colon cancer, the histograms indicate a trend toward increasing GFI with progressing stage and grade. In contrast, for lung tumors there was no correlation for stage, grade, and GFI. In addition, the mean GFIs for these tumor types were higher than for breast and ovarian tumors. These findings are consistent with those obtained by Meyer (27), showing higher TLIs in colon and lung tumors.

With the attainment of further patient follow-up data, the GFI should prove to be an important prognostic indicator for multiple tumor types and consequently improve individualized patient management.

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