[CANCER RESEARCH 47, 403–406, January 15, 1987]

Biological Effect of Epidermal Growth Factor on the *in Vitro* Growth of Human Tumors[1]

S. Eva Singletary,[2] Fraser L. Baker, Gary Spitzer, Susan L. Tucker, Barbara Tomasovic, William A. Brock, Jaffer A. Ajani, and Anne M. Kelly

Departments of Surgery [S. E. S.], Hematology [G. S., B. T., A. M. K.], Biomathematics [S. L. T.], Experimental Radiotherapy [W. A. B.], and Medical Oncology [J. A. A.], The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030, and LifeTrac, Ltd., Irvine, California [F. L. B.]

ABSTRACT

The effect of epidermal growth factor (EGF) on the *in vitro* growth of 186 malignant human tumor specimens (45 melanomas, 32 sarcomas, and 56 lung, 16 gynecological, 14 breast, 12 genitourinary, and 11 gastrointestinal carcinomas) was evaluated in the cellular adhesive matrix human tumor culture system supplemented with transferrin, insulin, hydrocortisone, and estradiol. EGF increased tumor growth by at least 50% in 81% of the 186 tumors and by over 100% in 54%. The enhanced growth induced by EGF was related to an accelerated cellular division independent of tumor type and not to an increase in the actual number of clonogenic units. The drug concentrations of cell cycle-independent Adriamycin and cisplatin needed to achieve a 90% tumor cell kill were not altered by the responsiveness of the tumor to EGF.

INTRODUCTION

It has been shown that murine and human myeloid leukemia cells are frequently still dependent on known hematopoietic growth factors for cell division and also that they will differentiate under the influence of these factors (1). Because hematopoietic malignant cells retain responsiveness to growth factors, we examined the histological range of and the frequency of growth response of nonhematopoietic human tumors to EGF.[3]

The biological properties and role of EGF in normal mammalian development have been extensively studied (2, 3). Preliminary *in vitro* studies on a small number of human tumors in the clonogenic agar assay indicated that EGF significantly increased tumor growth of both epithelial and mesenchymal human malignancies (4, 5). Previously, we investigated the effects and interactions of EGF, insulin, hydrocortisone, and estradiol on the growth of human tumors in our clonogenic agar assay cultured at a reduced serum concentration. Under the conditions of our agar assay, EGF when supplemented with all three factors was the most effective combination in stimulating colony growth (6). Recently, we reported that greater than 80% of human tumor specimens grew successfully *in vitro* in the CAM monolayer culture with EGF hormone-supplemented medium (7). Tumor origin of increased cell growth was confirmed by morphology with Papanicolaou stains and cytogenetics. Using this primary human tumor culture method, we demonstrated that, like myeloid malignancies, human tumors are frequently dependent on EGF for *in vitro* growth and that this growth is an enhancement of cellular division. We also showed that the addition of EGF to cultures does not affect the chemosensitivity of cell cycle-independent drugs, such as Adriamycin and cisplatin.

Received 5/27/86; revised 8/18/86; accepted 10/8/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Contract JMV:bg 11783 from LifeTrac, Ltd., Irvine, CA.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: EGF, epidermal growth factor; CAM, cell-adhesive matrix; IC_{90}, drug concentration achieving a 90% tumor cell kill.

MATERIALS AND METHODS

Materials. Enzyme disaggregation medium was Ham's F-12 (K. C. Biologicals, Lenexa, KS) with 10% fetal bovine serum (K.C. Biologicals), 0.75% collagenase III (Cooper Biomedical, Malvern, PA), and 0.005% DNase (Sigma Chemical Company, St. Louis, MO). Culture medium was Ham's F-12 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (2.7 mg/ml) (Sigma), 10% swine serum (J. R. Scientific, Woodland, CA), penicillin-streptomycin (100 units/ml) (GIBCO, Grand Island, NY), and hormone supplementation: EGF (5 ng/ml); transferrin (10 μg/ml); insulin (5 μg/ml); hydrocortisone (0.5 μg/ml) (Collaborative Research, Lexington, MA); and estradiol (0.27 μg/ml) (Sigma). Attachment medium was the culture medium with 0.6% Methylcellulose 4000 (Fisher Scientific, Houston, TX). Phosphate-buffered saline was Dulbecco's (GIBCO) and Hanks' balanced salt solution (Fisher). Culture dishes were 24-well plates (Costar, Cambridge, MA) subsequently coated with CAM (LifeTrac, Ltd., Irvine, CA).

Primary Human Tumor Cell Culture Method. Sterile, freshly obtained solid human tumor specimens with histology confirmed by a pathologist were collected from patients undergoing diagnostic or therapeutic surgical procedures. The tumor specimens were mechanically dissociated with a scalpel to the size of 1 mm and then treated with 0.75% collagenase III and 0.005% DNase in Ham's F-12 with 10% fetal bovine serum for 16 h at 37°C under continuous agitation to form single cell suspensions. Cells were then washed in Hanks' balanced salt solution. The number of viable cells was determined by a hemocytometric count of trypan blue-negative nucleated cells greater than 10 μm excluding lymphocytes, granulocytes, and mesothelial cells using a phase-gradient imaging microscope at ×400 magnification.

The single-cell suspension was diluted with the attachment medium in 4 inoculum titrations (3,125; 6,250; 12,500; and 25,000 cells) per well in 24-well plates precoated with CAM. All remaining wells were seeded with 25,000 cells per well. In the experiment comparing colony number and size, the cell dose inoculum was reduced to 2,000; 4,000; 8,000; and 16,000 cells to prevent tumor cell overgrowth secondary to EGF response and to allow clear delineation of colony size. All assays were performed in duplicate.

Cultures were incubated at 37°C in a humidified 5% CO_2- air atmosphere. After 24 h of incubation, the medium was aspirated. The adherent cells remaining in the wells were washed with Dulbecco's solution. The controls were fed with hormone-supplemented Ham's F-12 culture medium including EGF at 5 ng/ml (the optimal concentration in a dose-response curve of 1 to 50 ng/ml of EGF as determined in 10 human tumor specimens). The experimental wells were fed with the same hormone-supplemented culture medium but without any added EGF. In addition, one control well was fixed with 95% ethanol for 10 min to record the starting well population, and one control well had 5 μCi/ml of tritiated thymidine to provide a record of the background cell population. A tritiated thymidine control rather than an ordinary Day 2 culture control was used to measure the tendency of nondividing cells toward cellular enlargement during *in vitro* culture.

Cultures were incubated in a humidified 5% CO_2-air atmosphere for 5 days. On the sixth day, all cultures were refed by a 100% medium exchange. On the 14th day (total incubation period, 13 days), the cultures were washed with Dulbecco's phosphate-buffered saline, fixed in 70% ethanol for 20 min, and stained with 0.05% crystal violet. Tumor cell growth was determined quantitatively by the absorbance of crystal violet-stained cultures integrated over the surface of the monolayer cultures with a Nikon/Joyce-Loeble Magiscan2 image analysis

system with subtraction of the absorbance of the tritiated thymidine background cell population. The number of cells per colony and cell size were measured at ×400 magnification using a bright-field imaging microscope. The data were analyzed using the nonparametric sign test (8).

In Vitro Drug Sensitivity. To evaluate the drug sensitivity of cell cycle-independent drugs, Adriamycin and cisplatin were included in the medium with and without EGF in 4 different concentrations (as defined by the drug's sensitivity against normal bone marrow cells) (9) after the removal of nonadherent cells on Day 2. All wells were initially inoculated with 25,000 cells per well. Cultures were then incubated for 5 days in a humidified 5% CO_2-air atmosphere. After a 5-day drug exposure, the chemotherapy drugs were removed, and all cultures were refed by a 100% medium exchange. On Day 14, the cultures were washed, fixed, and stained as described above in the primary human tumor cell culture method. Using the Magiscan2 image analysis, tumor cell survival was measured by the absorbance of the control and drug-exposed cultures minus the absorbance of the tritiated thymidine background cell population.

RESULTS

Human Tumor *in Vitro* EGF Growth Response. The addition of exogenous EGF (5 ng/ml) significantly stimulated the *in vitro* growth as measured by absorbance image analysis of 186 human tumor specimens in the CAM-tumor culture assay at a concentration of 25,000 tumor cells per well: 56 lung carcinomas ($P < 0.001$); 45 melanomas ($P < 0.001$); 32 sarcomas ($P < 0.001$); 16 gynecological malignancies ($P < 0.001$); 14 breast carcinomas ($P < 0.001$); 12 genitourinary carcinomas ($P < 0.006$); and 11 gastrointestinal carcinomas ($P = 0.001$).

Of the 56 lung carcinomas (30 adenocarcinomas and 14 squamous cell, 7 small cell, and 5 poorly differentiated carcinomas), EGF increased cell growth by at least 50% in 44 (79%) and by 100% in 21 (38%) (Fig. 1*A*), independent of histology. In the 45 melanoma specimens (all of metastatic origin), EGF enhanced growth (Fig. 1*B*) by 50% in 38 (84%) and by 100% in 28 (62%). Similarly (Fig. 1*C*), a 50% growth increment by EGF was seen in 26 of 32 sarcomas (24 soft tissue, 8 bone) (81%) and a 100% growth increase in 17 (53%). As shown in Fig. 1*D*, the growth effect of EGF was particularly striking for breast carcinomas [13 tumors (93%) with 100% growth increase] but less impressive for genitourinary carcinomas [only 4 specimens (33%) experienced growth doubling]. All 11 gastrointestinal carcinomas (7 colon, 3 stomach, and 1 pancreas) responded to EGF by a greater than 50% growth increase, and 8 of these tumors (73%) had a greater than 100% growth increment. Of the 16 gynecological tumors (13 ovarian, 3 endometrial), 13 (81%) increased growth by 50% and 9 (56%) by 100%. Most important, 59 of these 186 tumors (32%) would have had inadequate *in vitro* growth, as defined by an absorbance of less than 500 above that of the background cell population, without EGF supplementation.

Effect of EGF on Colony Size. We next examined if the growth effect of EGF was the result of an increase in the number of clonogenic units or an enhancement of colony size compared with colony size in cultures without EGF. In 23 human tumors (10 lung carcinomas, 8 melanomas, and 5 sarcomas) plated with and without EGF supplementation at 4 reduced tumor cell-dose titrations of 2,000 to 16,000 cells per well, EGF did not significantly affect the total number of colonies but did markedly increase the actual colony size. A critical examination with a bright-field microscope at ×400 magnification revealed that this phenomenon was an increase in the number of cells per colony and not in individual cell size. As demonstrated in Fig. 2*A*, the *in vitro* growth of 23 tumors at a dose of 2,000 cells per well without EGF resulted in a cumulative total of 174 colonies with a median colony size of approximately 32 cells per colony. In contrast (Fig. 2*B*), the addition of EGF (5 ng/ml) in the same 23 tumors at the same concentration of 2,000 cells per well resulted in a cumulative total of 170 colonies with a median colony size of approximately 512 cells per colony. As shown in Table 1, this shift to an increase in colony size with a similar total number of colonies was also seen in a human lung adenocarcinoma specimen at higher cell dose titrations of 4,000; 8,000; and 16,000 cells per well. This EGF effect was identical within the 3 histological subtypes—lung carcinoma, melanoma, and sarcoma.

Effect of EGF on Drug Survival Curves. If EGF only accelerated cellular division, then the drug sensitivity of cell cycle-independent drugs, such as Adriamycin and cisplatin, should not be significantly affected. Five parallel cultures with and without EGF were performed. Tumor cell growth was quanti-

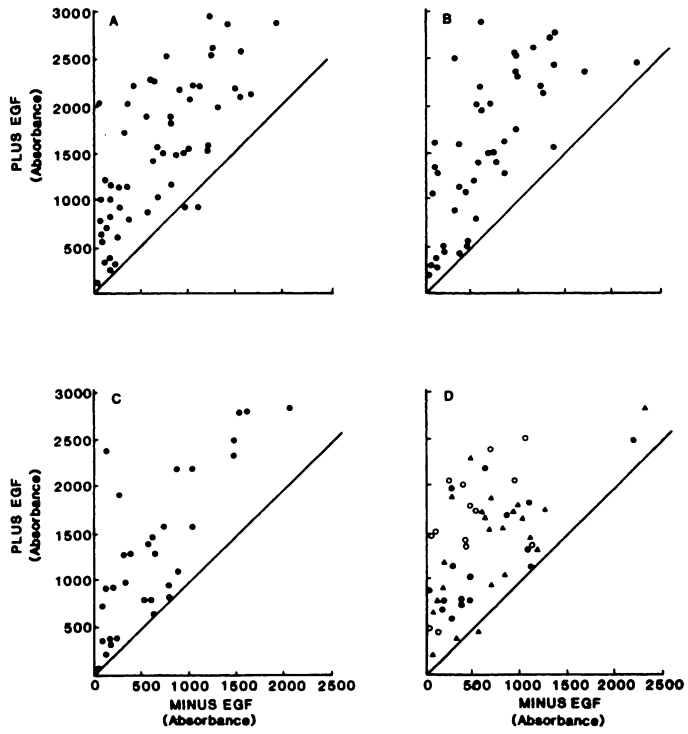

Fig. 1. *In vitro* growth (absorbance) of 56 lung carcinomas (*A*); 45 melanomas (*B*); 32 sarcomas (*C*); and 16 gynecological (●), 14 breast (○), 12 genitourinary (▲), and 11 gastrointestinal (△) carcinomas (*D*) cultured with EGF (*ordinate*) and without EGF (*abscissa*).

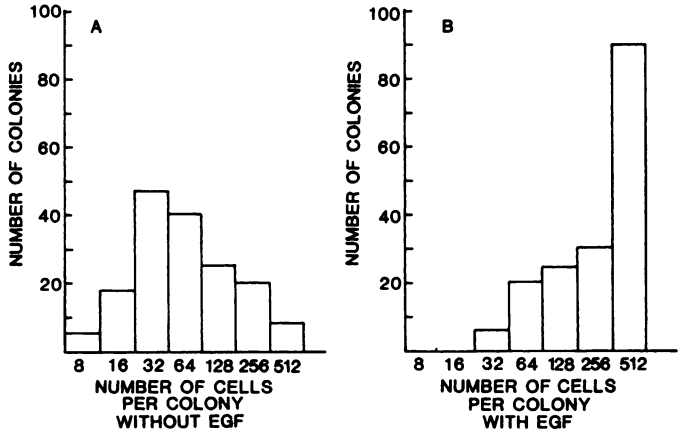

Fig. 2. Total number of colonies (*ordinate*) and approximate number of cells per colony (*abscissa*) in cultures without EGF (*A*) and with EGF (*B*).

Table 1 *Effect of EGF on colony size and colony number of a human lung adenocarcinoma plated at reduced cell dose inoculum titrations in the CAM-human tumor culture assay*

Approximate no. of cells/colony	No. of colonies							
	2,000 cells/well		4,000 cells/well		8,000 cells/well		16,000 cells/well	
	−EGF	+EGF	−EGF	+EGF	−EGF	+EGF	−EGF	+EGF
8	3	0	2	0	2	0	2	0
16	2	0	3	0	4	0	5	0
32	7	0	13	0	20	0	25	0
64	0	2	6	12	15	2	18	5
128	0	4	0	0	0	20	0	17
256	0	4	0	9	0	11	0	17
512	0	0	0	2	0	6	0	10
Total	12	10	24	23	41	39	50	49

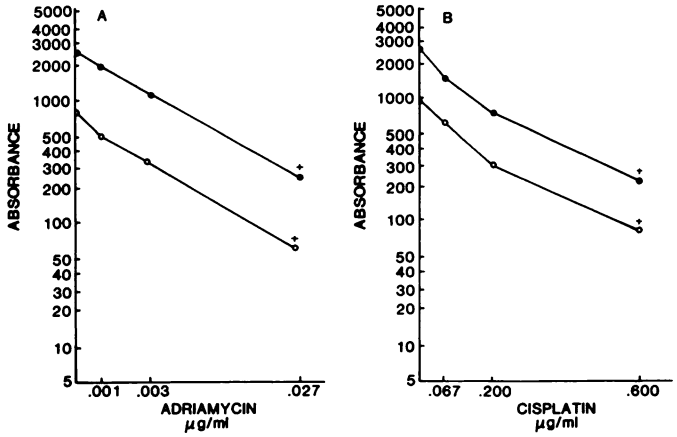

Fig. 3. Drug survival curves and IC_{90} concentrations (+) of Adriamycin in a lung squamous cell carcinoma (*A*) and cisplatin in a malignant fibrous histocytoma (*B*) cultured with EGF (●) and without EGF (○).

tated at 5 exponential points—no drug and 4 increasing drug-dose concentrations. The IC_{90}s were then calculated and compared. In 6 human tumors (3 sarcomas, 1 melanoma, and 1 lung and ovarian carcinoma each), Adriamycin survival curves were constructed by plotting the log of the tumor cell survival in cultures with and without EGF against the drug dose. In all 6 tumor specimens, EGF supplementation did not significantly affect the Adriamycin drug concentration needed to achieve a 90% tumor cell growth reduction. The increase in cell growth by EGF only shifted the drug survival curve upward and parallel to the survival curve constructed from cultures without EGF. One example, the *in vitro* response of a lung squamous cell carcinoma to Adriamycin, is shown in Fig. 3*A*, in which the slope and IC_{90} values of cultures with and without EGF are unchanged. Similarly, in 4 human tumors (2 sarcomas and 1 ovarian and 1 lung carcinoma), cisplatin survival curves were determined. The IC_{90} cisplatin values of cultures with and without EGF were identical. As shown in Fig. 3*B*, the addition of EGF induced a pattern of an upward and parallel cisplatin survival curve with the same IC_{90} concentration.

***In Vitro* Culture Limitations of Tumor Growth.** In 9 of the 23 human tumors plated at the 4 cell concentrations of 2,000; 4,000; 8,000; and 16,000 cells per well with and without EGF supplementation, a decrease in cell growth was observed with increasing cell density. However, this reduction in growth was not related to inhibition by cellular overgrowth. The cultures without EGF exhibited the same growth plateau or decrease at the same cell-dose inoculum despite lower absolute growth compared with the cultures with EGF. As shown in Fig. 4, although cultures with EGF achieved a higher absolute number

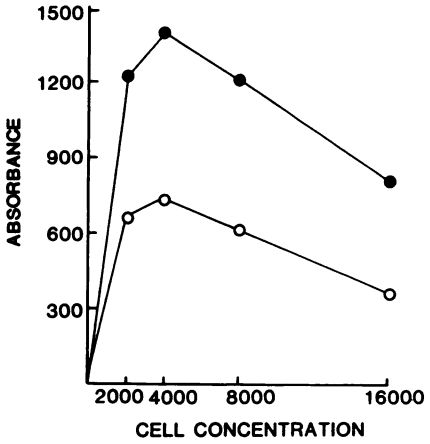

Fig. 4. Parallel *in vitro* growth inhibition (*ordinate*) of a lung adenocarcinoma at increasing cell density (*abscissa*) with EGF (●) and without EGF (○).

of cells as measured by absorbance, a critical culture condition or growth factor is still needed by some human tumors to show linear cell-inoculum growth response at higher cell concentrations.

DISCUSSION

Our data demonstrated that primary human tumors with *in vitro* culture supplementation with transferrin, insulin, hydrocortisone, and estradiol are frequently responsive to EGF and that this EGF response is related to accelerated cellular division and not to an increase in clonogenic units. In the 186 human tumors (81%) tested, the addition of exogenous EGF increased tumor growth by 50% or greater in 79% or more of each of the tumor cell cultures tested, except genitourinary carcinomas (only 5 of 12 cultures increased growth by 50% or more). Not only is this high frequency of human tumor responsiveness to EGF important, but the finding that some human tumors, such as breast carcinomas (64%), have little *in vitro* growth without EGF also suggests that particular hormone-responsive tumors may depend totally on growth factors like EGF for expansion. This potent mitogenic activity of EGF has been reported for many types of malignant human epithelial and mesenchymal-derived cells in liquid and agar cultures (4–6, 10). Furthermore, EGF has been shown to increase the frequency of anal squamous cell carcinoma more than 3-fold in 1,2-dimethylhydrazine-treated male mice (11). However, 75% of the genitourinary carcinomas in this study demonstrated satisfactory *in vitro* growth (absorbance above 500) without EGF supplementation, and a number of other tumors with a marked response to EGF also had significant *in vitro* growth without EGF. Whether growth without EGF represents an extreme sensitivity to the small amount of EGF present in serum or the independence of EGF as a growth factor requirement needs further investigation.

EGF enhancement of the rate of cell cycle rather than increase in absolute number of competent stem cells by EGF suggests that it may act as an important progression signal for cellular proliferation. In the 23 human tumors plated at reduced cell concentrations with and without EGF supplementation, EGF was observed not to significantly affect colony numbers but to dramatically increase the number of cells per colony. This potentiation of cellular division was a generalized phenomenon unrestricted by the histological tumor type studied.

If the effect of EGF on tumor growth is only an acceleration of cellular division, then cell cycle-independent chemotherapeutic drugs should not be altered in their tumor cell kill activity.

As shown for the cell cycle-independent drugs Adriamycin and cisplatin, EGF did not change the drug concentration needed to achieve a 90% tumor cell kill. Whether EGF affects the *in vitro* chemosensitivity of cell cycle-dependent drugs like 5-fluorouracil is currently being examined.

We have no explanation why a number of some human tumor specimens have a growth plateau at the same cell-dose inoculum with and without EGF instead of being limited by the absolute total cell growth. Of the 25,000 cells originally seeded per well, only approximately 10% adhered in culture and generated growth. This growth plateau even at very low cell concentrations suggests that an inhibitory substance may not be the mechanism of growth inhibition but rather that the cells require some crucial culture condition or factor in addition to EGF supplementation.

These studies indicate that a large percentage of human tumors, epithelial and mesenchymal, have a dependency for EGF or EGF-like molecules for growth. Questions such as whether there are subpopulations within the tumor responsive to EGF, which stage of tumor differentiation is the exact stage responsive to EGF, and whether there are other important growth factors, like β-transforming growth factor, that interact and favorably affect tumor growth are being investigated.

REFERENCES

1. Burgess, A. W., and Metcalf, D. Characterization of a serum factor stimulating the differentiation of myelomonocytic leukemia cells. Int. J. Cancer, *26:* 647–654, 1980.
2. Cohen, S., and Carpenter, G. Human epidermal growth factor: isolation and chemical and biological properties. Proc. Natl. Acad. Sci. USA, *72:* 1317–1321, 1975.
3. Gospodarowicz, D. Epidermal and nerve growth factors in mammalian development. Annu. Rev. Physiol., *43:* 251–263, 1981.
4. Pathak, M. A., Matrisian, L. M., Magun, B. E., and Salmon, S. E. Effect of epidermal growth factor on clonogenic growth of primary human tumor cells. Int. J. Cancer, *30:* 745–750, 1982.
5. Hamburger, A. W., White, C. P., and Brown, R. W. Effect of epidermal growth factor on proliferation of human tumor cells in soft agar. J. Natl. Cancer Inst., *67:* 825–830, 1981.
6. Singletary, S. E., Tomasovic, B., Spitzer, G., Tucker, S. L., Hug, V., and Drewinko, B. Effects and interactions of epidermal growth factor, insulin, hydrocortisone, and estradiol on the cloning of human tumor cells. Int. J. Cell Cloning, *3:* 407–414, 1985.
7. Baker, F. L., Spitzer, G., Ajani, J. A., Brock, W. A., Lukeman, J., Pathak, S., Tomasovic, B., Thielvoldt, D., Williams, M., Vine, C., and Tofilon, P. Drug and radiation sensitivity measurements of successful primary monolayer culturing of human tumor cells using cell-adhesive matrix and supplemented medium. Cancer Res., *46:* 1263–1274, 1986.
8. Hollander, M., and Wolfe, D. A. The two-way layout. *In:* M. Hollander and D. A. Wolfe (eds.), Nonparametric Statistical Methods, pp. 138–149. New York: John Wiley & Sons, 1973.
9. Umbach, G. E., Singletary, S. E., Tomasovic, B., Spitzer, G., Hug, V., and Drewinko, B. Dose-survival curves of cisplatinum, melphalan, and velban in human granulocyte/macrophage progenitor cells. Int. J. Cell Cloning, *2:* 335–340, 1984.
10. Stoscheck, C. M., and King, L. E. Role of epidermal growth factor in carcinogenesis. Cancer Res., *46:* 1030–1037, 1986.
11. Kingsnorth, A. N., Abu-Khalaf, M., Ross, J. S., and Malt, R. A. Potentiation of 1,2-dimethylhydrazine-induced anal carcinoma by epidermal growth factor in mice. Surgery (St. Louis), *97:* 696–700, 1985.