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Effects on Labeling Index as a Predictor of Response to Chemotherapy in the 13762 Adenocarcinoma

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Summary. *A series of 24 experiments was carried out, in which treatment effect was determined in vivo on rats bearing measurable implants of 13762 adenocarcinoma treated with a variety of chemotherapeutic agents, alone or in combination. In 11 experiments, a reduction in mean tumor area was observed after treatment (response), while 13 showed no such effect (no response).*

For each experiment, cells from tumor-bearing animals were placed in suspension culture (10 experiments) or in two-layer soft-agar culture (14 experiments) after 24 h of exposure to drug-containing plasma or control plasma. Cells were harvested from cultures at 24-h intervals thereafter for determination of the thymidine labeling index in treated versus control populations. The sum of labeling indices in control plasma divided by that in treated plasma was determined to combine the available data over time (overall ratio). Critical values for this ratio were defined, which demonstrated significant depression of the labeling index.

Significant labeling index depression occurred in nine of 11 experiments where response in vivo occurred, and in one of 13 in which no response was seen ($P = 0.0004$ by Fisher's exact test). When experiments carried out in soft agar culture or in suspension culture were examined separately, the association of labeling index depression in vitro and response in vivo remained statistically significant. In this model system, labeling index depression by drug-containing plasma is highly predictive of response to therapy.

Introduction

Previous results from our laboratory demonstrated an association between labeling index depression after treatment in vivo and response to chemotherapy [4], as well as a relationship of in vitro effects by drug-containing plasma on labeling index and the likelihood of an in vivo response [3], in patients with a variety of tumors. The studies reported here were initiated to determine whether, in an experimental solid tumor system, the usefulness of an in vitro assay based on labeling index depression could be prospectively demonstrated as a predictor of antitumor effects. Secondary objectives included: (1) development of a satisfactory method of tumor cell dispersion and slide deposition for labeling index determination; and (2) optimization of conditions for short-term tissue culture of 13762 tumor cells. Because we had noted that the previously developed criterion for significance of a change in labeling index [4] was not equally satisfactory at all levels of labeling index, an additional objective was to better define what degree of variation in this parameter could be considered significant at different labeling index values.

The 13762 rat 'mammary' adenocarcinoma was chosen because of its defined transplantability, growth properties, and varied sensitivity to different chemotherapeutic agents, and because it was felt to be a relevant animal model system for human solid tumors, such as breast cancer [1, 7].

Materials and Methods

In vivo. Tumor transplantation was carried out prior to each experiment in the following manner: A crude suspension containing $1-4 \times 10^6$ viable 13762 tumor cells (by Trypan blue dye exclusion) in 0.2–0.3 ml sterile saline was inoculated SC into the lower mid-back region of Fisher 344 female rats aged 42–47 days. Inoculations were done with a 1-ml syringe and 20-gauge needle, and 24 rats were inoculated per experiment. The original 13762 tumor line was obtained from the Mason Research Institute (Worcester, Massachusetts, USA): in subsequent experiments the cell suspension used was a portion of that prepared from tumor-bearing rats for tissue culture in the preceding experiments.

At approximately day 14 after inoculation (when tumor implants became palpable), baseline measurements of tumor length and width were obtained with calipers, and 20 rats were divided into groups of 10 each: One group received the drug(s) being studied in a single dose (see Table 1 for drug doses used),

Table 1. Drug doses used in vivo

Drug(s)	Dose (mg/kg)	
Adriamycin	5	(IP)[a]
Cytoxan	120	(IP)
L-Phenylalanine Mustard	5	(PO)
Methotrexate	10	(IP)
Vincristine	0.8	(IP)
5-Fluorouracil	125	(IP)
Adriamycin/Cytoxan (combination)	4 100	(IP)
Vincristine/Cytoxan (combination)	0.8 120	(IP)

Drug doses administered to rats bearing 13762 adenocarcinoma in each experiment. Only one dose was given, at day 14–21 after implant (when palpable tumors could be measured at the implant site)

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and the other served as an untreated control. Treated and control groups were earmarked for subsequent identification before the drugs were given, but no effort was made to identify to which group an animal belonged during the period of tumor measurements after treatment. The length and width of individual tumors in each group were determined at 2- to 3-day intervals over the next 12 days (6 measurements), after which all animals were sacrificed. On the day of treatment, two to four additional rats were sacrificed and their tumors excised to furnish the cells for in vitro assay. Three to 4 days prior to the day of treatment in each experiment, blood to furnish plasma for the in vitro assay was obtained from healthy, non-tumor-bearing Fisher 344 rats by cardiac puncture. Drug-containing blood was collected 15 min after IP injection of the drug(s) to be studied, or 45 min after administration PO (melphalan only) at the doses listed in Table 1, and non-drug-containing ('control') bloods were obtained at the same time.

In vitro. Cell suspensions of tumor for subsequent assay were prepared from at least two whole tumors, which had been grossly excised. A stainless steel, fine-mesh grid was placed in a petri dish containing artificial medium (enriched RPMI with 2% antibiotic-antimycotic added). The tumors were minced with scissors and forceps in the grid, and the fragments were then compressed with a pestle. The crude tumor cell suspension in RPMI was transferred to a sterile, disposable 50-ml test tube; remaining tumor debris was rinsed twice and the resultant cell suspension added. Concentrations were determined with a Coulter counter and the cell suspensions were adjusted to a concentration of 12×10^6 cells/ml, then diluted 1 : 1 with plasma, either containing drug(s) ('treated' plasma) or no drugs ('control' plasma); at the resulting concentration of 6×10^6 cells/ml, 3 ml each of treated and control cell suspensions were incubated for 24 h in Erlenmeyer flasks at 37° C in a CO_2 incubator. (Plasma was obtained by centrifugation from whole blood stored at −70° C until the day of treatment, then thawed, recentrifuged, and filter-sterilized prior to use.)

After 24 h, the contents of each Erlenmeyer flask were removed and layered on 3 ml Ficoll-Hypaque solution (2.4 parts 9% Ficoll to 1 part 33.9% Hypaque, resultant density, 1.08 g/ml) in Ki_{max} centrifuge tubes, then centrifuged at 2,000 rpm for 12 min. A buffy layer of mononuclear cells with high Trypan blue viability was formed in each tube at the interface between Ficoll-Hypaque and the overlaid medium. These layers were removed, and the resultant concentrates from treated and control suspensions were then placed in a two-fold volume of phosphate-buffered saline, centrifuged at 1,500 rpm for 10 min, and resuspended at a concentration of 0.5×10^6 cells/ml. The washed cell suspensions were then placed either in suspension culture (experiments 1−10) or in culture on a soft agar base plate (experiments 11−24).

Suspension Culture. Two milliliters each of treated and control cell suspensions in enriched RPMI was placed in Falcon T-flasks for subsequent harvest at each of two time points (72 and 96 h). Tritiated thymidine (specific activity, 6 C/mmol) was added at 5 μCi/ml 1 h prior to harvest, after cell counts and Trypan blue viability had been determined. Exposure to thymidine was terminated, slides prepared, and cytocentrifugation carried out as described previously [3, 4]. Four slides were made for treated and control at each time point.

Soft Agar Culture. One milliliter each of treated and control cell suspensions in double-enriched RPMI was pipetted over duplicate agar base plates for subsequent harvest at daily intervals (72, 96, 120, 144, 168, and 192 h). Agar base plates were made as described by von Hoff et al. [8]. Briefly, just prior to use, 3% agar was taken from the refrigerator and reheated to the liquid state at 50° C in a water bath: Two milliliters of liquid agar was added to 10 ml plating medium with a pipet, mixed quickly, and the mixture immediately dispensed into 35-mm petri dishes as 1-ml aliquots, where it became semi-solid. Twenty-four hours prior to harvest for each time point, 0.2 ml was removed from the fluid overlayer on each of the two base-plates for determination of cell counts by Coulter counter and percent viability by Trypan blue dye exclusion; tritiated thymidine was then added as described above, but the plates were replaced in the CO_2 incubator and allowed to remain in contact with the tritiated thymidine for the remainder of the 24 h. The plates were removed from the incubator and the fluid overlayer harvested with a Pasteur pipet. The base-plates were then washed with 1 ml phosphate-buffered saline, and the wash added to the previously harvested material. The resultant cell suspension was then used to make cytocentrifuge slide preparations as previously described [3, 4] with four slides for treated and four for control suspensions at each time point.

Cell Dispersion, Slide Preparation, Staining, and Labeling Index Determination. Just prior to dispensing aliquots into the cytocentrifuge cups, the treated and control cell suspensions were centrifuged at 1,000 rpm for 8 min to obtain cell pellets. The supernatant fluid was decanted and 1 ml 0.25% trypsin (containing 0.25% EDTA) was added. The mixture was then vortexed and placed in a Branson ultrasonic cleaner, exposed to ultrasound for 3 min, allowed to stand for 3 min, and then exposed to ultrasound for an additional 3 min. This dispersed cell suspension was then immediately dispensed in 0.2 ml aliquots into the cytocentrifuge cups. After overnight drying, the cytocentrifuge-prepared slides were treated with trichloroacetic acid and methanol, dipped in emulsion, exposed for 24 h, fixed, developed, and stained as previously described [3, 4]. In general, the cell population represented on the slides was > 90% tumor cells. A total of 400 tumor cells were counted per slide, and all slides were read blind.

Statistical Methods. An overall ratio between index values in control and those in treated plasma was determined by the following calculation:

$$\text{overall ratio} = \frac{\text{sum of labeling index values in control plasma}}{\text{sum of labeling index values in treated plasma}}.$$

To avoid mathematical difficulties and to be consistent with earlier methods (cited in the *Appendix* to this paper), one was added to both the numerator and the denominator.

A *critical* ratio was defined as one such that values at this level or above imply with 95% confidence that the two labeling index values are representative of kinetically different populations. Levels for this ratio were determined by reference to the mean values of the labeling indices in plasma control for each experiment (see *Appendix*). For experiments done in suspension culture, the time points used to obtain the ratio were at 72 and 96 h, while for those in soft agar culture, available data for labeling index were taken at 72, 96, 120, 144, 168, and 192 h.

Antitumor effect in vivo was defined in either of two ways: (1) as a reduction in mean tumor area among animals in the treated group, compared with the baseline values, at any time from day 4 after treatment to the termination of the experiment; and (2) as a reduction in the ratio of mean tumor area of treated versus control tumors at day 7 after treatment, again compared with baseline. In either case, mean tumor area was calculated as follows:

$$\text{mean tumor area} = \frac{\text{sum of length} \times \text{width measurements}}{\text{number of animals}}.$$

The former criterion implies an overall reduction in size of tumor. It should be noted that day 4 after baseline measurements was chosen because in serial measurements on tumors in 240 control animals a reduction was never seen in an individual tumor after that date. The latter criterion implies at least a slowing in rate of growth compared with control. To determine the statistical significance of this Student's *t*-test was used, with $P < 0.05$ considered significant.

We examined the relationship between biological effect in vivo and an effect of treated plasma on the labeling index in vitro. This relationship was examined for all experiments, and separately by culture utilized. For the comparison of various proportions and testing for statistical significance, Fisher's exact test was used.

Results

Overall, an effect on in vitro labeling index was seen in nine of 11 courses, with biological effect defined as reduction in mean tumor area, versus one of 13 courses without this effect (Table 2). This difference in sample proportions is highly significant by Fisher's exact test ($P = 0.0005$). When treated plasma effects on labeling index in vitro were compared with in vivo effects of chemotherapy separately for the two cultures, the results were essentially the same. For the soft agar culture, an effect on in vitro labeling index was seen in five of six courses with biological effect defined as reduction in mean tumor area, versus one of eight courses without this biological effect. This difference in sample proportions is significant by Fisher's exact test ($P = 0.03$). Similarly, for the cell suspension culture, an effect on in vitro labeling index was seen in four of five courses with biological effect defined as reduction in mean tumor area, versus zero of five courses without this biological effect. The difference in sample proportions is significant by Fisher's exact test ($P = 0.05$).

When the more sensitive criterion of antitumor effect related to reduction in the *rate* of treated versus control tumor area at day 7 was applied, 16 of the 24 experiments demonstrated an effect of treatment, rather than 11. None of these five experiments with continued overall tumor growth in the treated group but at a slower rate showed associated depression of tumor cell labeling index values in drug-containing plasma: therefore, the correlation of in vitro and in vivo results, using Fisher's exact test, yields a difference in sample proportions which is not statistically significant ($P = 0.08$, all experiments).

There were false-negative results in two experiments (see Table 2) and a false-positive result in one, if the criterion of reduction in mean tumor area among treated animals is used as the measure of in vivo response. Both the false-negatives involved the two- drug combination of adriamycin and cyclophosphamide, with a value for critical ratio in one which was equal to, but did not exceed, the critical value. The false-positive experiment involved the single drug L-PAM: there was no evidence of in vivo antitumor effect in this experiment, even by the more sensitive criterion of a reduction in tumor growth rate.

Table 2. Summary of results obtained in individual experiments

Drug(s) tested	Culture conditions	Mean LI, control plasma	Overall ratio: LI in control plasma / LI in treated plasma	Overall ratio > critical value:	Reduction in tumor area, treated group
[a]ADR + CTX	Suspension	12.5	2.25	Yes	Yes
5-FU	Suspension	9.0	6.67	Yes	Yes
5-FU	Suspension	14.0	10.00	Yes	Yes
MTX	Suspension	17.0	0.44	No	No
L-PAM	Suspension	9.0	1.00	No	No
VCR	Suspension	17.0	1.44	No	No
CTX	Suspension	9.5	7.00	Yes	Yes
ADR	Suspension	9.0	1.18	No	No
MTX	Suspension	2.5	0.50	No	No
ADR + CTX	Suspension	16.0	1.62	No	Yes
ADR + CTX	Soft agar	11.0	1.88	No	Yes
CTX	Soft agar	9.7	1.73	No	No
5-FU	Soft agar	31.8	1.67	Yes	Yes
CTX + VCR	Soft agar	18.2	1.57	No	No
MTX	Soft agar	41.5	1.28	No	No
VCR	Soft agar	37.5	0.86	No	No
CTX	Soft agar	35.3	1.90	Yes	Yes
ADR	Soft agar	34.7	1.03	No	No
L-PAM	Soft agar	31.7	1.82	Yes	No
ADR + CTX	Soft agar	31.0	1.83	Yes	Yes
MTX	Soft agar	36.3	1.12	No	No
CTX + VCR	Soft agar	27.6	2.38	Yes	Yes
5-FU	Soft agar	21.5	2.60	Yes	Yes
VCR	Soft agar	32.8	1.06	No	No

The overall ratio, LI in control plasma/LI in treated plasma, reflects the effect of drug-containing (treated) plasma on DNA synthesis in the tumor cell population: higher ratios reflect greater effects. 'Critical value' for an overall ratio is one such that it or a higher ratio would be expected to occur on the basis of chance variation less than 5% of the time. Therefore, an overall ratio greater than the critical value may be taken as statistically significant evidence of effect on tumor cell DNA synthesis in vitro. Reduction in mean tumor area among the treated animals was the measure of minimum in vivo effect to qualify as a response

[a] ADR, adriamycin; CTX, cyclophosphamide; 5-FU, 5-fluorouracil; MTX, methotrexate; L-PAM L-phenylalanine mustard; VCR, vincristine

Discussion

The results indicate that plasma obtained from a treated animal shortly after administration of chemotherapy will produce effects on tumor cell DNA synthesis (as measured by the labeling index) which predicts for subsequent success or failure of treatment. The overall frequency of false-negative

Table 3. Duplicate sample haves: 95th percentile of labeling index ratios for selected levels of labeling index

Level of LI[a]	N	95th Percentile
0–10	27	2.27
10–20	62	1.88
20–30	36	1.54
30–40	45	1.48
40–50	35	1.43
50–60	11	1.29

The 95th percentile of the observed distribution of LI ratios is that value above which ≤ 5% of the ratios fell when the population sampled was known to be the same (duplicate sample halves). It can be seen that this critical ratio becomes lower at higher values of LI, so that a greater than 2-fold difference is required for statistical significance in the range of LI from 0 to 10, while at LI values above 30 a difference less than 1.5-fold is significant

[a] LI, labeling index; N, number of specimens in this range of LI which were subjected to duplicate sample half LI determinations

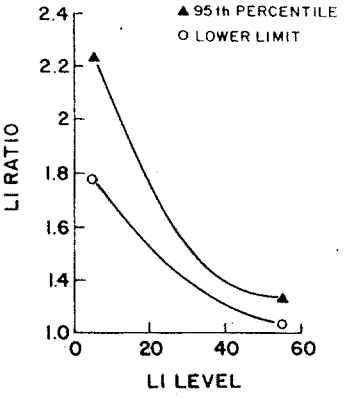

Fig. 1. Quadratic curves of the 95th percentile of labeling index (LI) ratios from halves of the same slide and the lower 97.5% confidence limits for those percentiles (▲) 95th percentile; (O) lower limit

results (no effect in vitro but in vivo effect observed, as reduction in mean tumor area) is 2/11. The observed frequency of false-positive results (effect in vitro but none in vivo) is 1/13. Among all courses, the in vitro results demonstrated a positive correlation with in vivo effect, defined as a reduction in mean tumor area, in 87.5% of the observations.

It is of interest that changes in labeling index were best correlated with a degree of antitumor effect which resulted in reduced tumor size, relative to baseline values before treatment, among the treated animals. In the clinical setting, treatment is generally discontinued when tumor growth occurs (there is, of course, no untreated control group for comparison, to allow a judgment as to slowing in the rate of growth). Thus, values for critical ratio appear to fit well with criteria for antitumor effect which are implicit in the clinical setting.

We believe that growth of 13762 tumor cells in soft agar culture is preferable to the use of suspension culture techniques, since it allows for a longer period of growth, and thus for more time points for labeling index determination. Additional advantages in the clinical setting might include the provision of a more homogeneous, primarily tumor cell population for counting, and the preferential growth of 'stem' cells critical to the tumor's reproductive integrity [5, 6].

Results obtained with this assay are encouraging. They appear, in an experimental model, comparable to those reported for stem cell colony counting [5, 6, 8] in the clinical setting. If the assay proves applicable to human tumors, it might be particularly advantageous in the setting where stem cell colonies grow out in soft agar, but in numbers which are insufficient to allow for determination of antitumor effect by colony-counting techniques. This may be the case in as many as 25% of clinical specimens [5]. In addition, the present technique is relatively simple and consumes less technician time.

Appendix

The differences between the LIs for halves of the same slide were determined as a measure of the error inherent in the experimental method. As before [2], the comparisons between LIs were expressed as a ratio after the addition of one to avoid the problem of a zero denominator. All ratios were defined in such a way that they were equal to or greater than 1. Since the variation in the LI ratios was greater for smaller LIs than for larger LIs, the distribution of these ratios was examined at several levels of LI. (Table 3). Specifically, the 95th percentile of the observed distribution of LI ratios and the lower confidence limit for a one-sided 97.5% confidence interval for that percentile are presented for each level of LI. In Fig. 1, these percentiles and lower confidence limits have been smoothed by means of quadratic regression to illustrate graphically the effect of the level of LI on the distribution of LI ratios. Table 3 or Fig. 1 can be used to determine for levels of LI less than 60 that value for which one could expect at least 95% of the ratios to be less than it.

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References

1. Bogden AE, Esber HJ, Taylor DJ, Gray JH (1974) Comparative study on the effects of surgery, chemotherapy, and immunotherapy, alone and in combination, or metastases of the 13762 mammary adenocarcinoma. Cancer Res 34: 1627–1631
2. Livingston RB, Ambus U, George SL, Freireich EJ, Hart JS (1974) In vitro determination of thymidine – ^{3}H labeling index in human solid tumors. Cancer Res 34: 1376–1380
3. Livingston RB, Titus GA, Heilbrun LK (1980) In vitro effects on DNA synthesis as a predictor of biological effect from chemotherapy. Cancer Res 40: 2209–2212
4. Murphy WK, Livingston RB, Ruiz VG, Gercovich FG, George SL, Hart JS, Freireich EJ (1975) Serial labeling index determination as a predictor of response in human solid tumors. Cancer Res 35: 1438–1444
5. Salmon SE (1979) Human tumor stem cells and adjuvant therapy of cancer. In: Jones SE, Salmon SE (eds) Adjuvant therapy of cancer, vol II. Grune & Stratton, New York, pp 27–36
6. Salmon SE, Hamburger AW, Soehnlen B, Durie BG, Alberts DS, Moon TE (1978) Quantitation of differential sensitivity of human tumor skin cells to anticancer drugs. N Engl J Med 298: 1321–1325
7. Segaloff A (1966) Hormones and breast cancer. Recent Progr Horm Res 22: 351–374
8. Von Hoff DD, Johnson GE, Glanbiger D (1981) Initial experience with the human tumor stem cell assay system; potential and problems. In: Salmon SE (ed) Human tumor cloning. Alan Liss, New York

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