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Dynamics of Human Renal Tumor Colony Growth in Vitro*

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Summary. Two-layer soft agar cultures from 26 patients with renal cell carcinoma, 21 renal primary lesions and 5 metastatic lesions, were evaluated for tumor colony formation using both dynamic growth curves and static single time point colony counting. Dynamic growth curves markedly increased the number of evaluable tumor cultures. There was no relationship between colony formation and TNM stage of the tumor or renal vein invasion. However, there was a significantly ($p < 0.02$) higher rate of colony formation from younger (< 50 y.) patients than from older patients. Only 2 of 5 tumors tested showed response to one or more cytostatic agents in vitro, both tumors showing response to Adriamycin and one to Cis-platin. Dynamic evaluation of tumor colony formation in soft agar may increase the clinical applicability of the human tumor cloning system both by increasing the number of evaluable cultures and by providing more information about the processes involved in tumor colony formation in vitro.

Key words: Renal carcinoma, Soft agar culture, Growth-curve.

Introduction

The application of the two layer soft agar Human Tumor Cloning System (HTCS) to solid tumors by Hamburger and associates [1, 12] led to extensive investigation of the system's suitability as an in vitro test of tumor chemosensitivity. Subsequently, other clinical applications of the HTCS were proposed, including assessment of persistent or recurrent viable tumor after therapy [2, 9, 10] and as a prognosticator analogous to tumor grading and staging [6, 7]. Because of technical problems in the routine performance of HTCS, evaluation of tumor colony formation with time, i.e., colony "growth curves" were introduced [8]. Single point measurements of colonies for the growth evaluation have been used for renal cell carcinoma by others [11, 13]. The present study compares dynamic evaluation of tumor colony formation with single-point measurement of colony growth in renal cell carcinoma. Particular attention is focused on the implications of this comparison for the clinical application of the HTCS.

Material and Methods

Routine surgical specimens of renal cell tumors were transported sterile and unfixed to the Department of Pathology. Using a sterile technique, a pathologist examined the specimen and chose material for soft agar culture (approximately 1–20 g), as well as for routine histopathologic study. Tissue was chosen to include all macroscopically identifiable patterns of tumor but excluding necrotic and hemorrhagic areas. Tissue selected for soft agar culture was further processed according to the detailed description published by Hamburger [1]. All samples were processed for in vitro culture within 4 h of collection. In brief, the tissue was minced in a sterile centrifuge tube with scissors and incubated for 2 h with a mixture of collagenase Type II (1,176 U/ml, Worthington, Freehold, N.J., USA) and DNase Type I (200 U/ml, Sigma, St. Louis, Mo., USA) at 37 °C. After incubation, McCoy's wash (Gibco, Paisly, Scotland, U.K.) was added and the cell suspension was passed through a metal sieve (100 holes/cm^2), a 25 gauge needle and a gas sterilised nylon filter with 40–70 μ holes (Ortho Diagnostics, Beerse, Belgium). The cells were washed and resuspended in 2–10 ml McCoy's Wash for a stock solution. From this stock solution 0,1 ml was taken for determination of cell concentration (hemocytometer) and an additional 0,1 ml for determination of viability by trypan blue exclusion. From the stock solution, 18–24 35 mm double layer soft agar culture plates at 500,000 cells/dish were immediately plated without further processing, for assessment of temporal course of colony development. For a drug sensitivity test 4,5 x 10E6 tumor cells in 3 ml were incubated at 37 °C with McCoy's Wash for one hour as a control and a similar cell number was incubated for 1 h at 37 °C with each drug solution (Adriamycin, Cis-platin, 5-Fluorouracil, Methotrexate and occasionally some experimental drugs) in a concentration approximately 10% of the in vivo achievable peak plasma level. After the 1 h incubation the cell suspensions were centrifuged, washed with McCoy's

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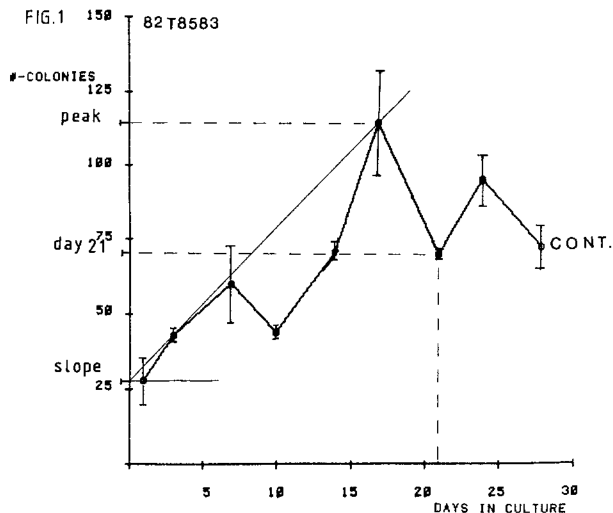

Fig. 1. Growth curve of a renal cell carcinoma in HTCS. *Ordinate:* number of colonies; *abscissa:* days in culture. Slope of the growth curve, peak count and day 21 count are indicated. Standard error bars of duplicate counts

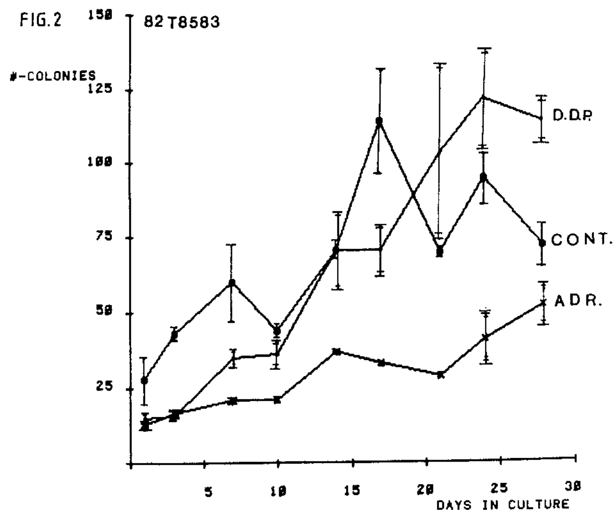

Fig. 2. Growth curves from the same renal cell carcinoma as shown in Fig. 1, including control culture and cultures pre-incubated with Cis-platin (DDP) or Adriamycin (ADR). Note "catch-up" growth in both drug-treated samples. Standard error bars of duplicate counts

wash and seeded in 35 mm double layer soft agar culture dishes at 500,000 cells/dish exactly as described by Salmon [12], except that conditioned medium was not used. The cultures were then incubated at 37 °C and at 6% CO_2 in a humidified atmosphere. All colony counting was performed using the Omnicon Fas II automated colony counter (Bausch and Lomb, Inc, Rochester, N.Y., USA) as previously described [3].

Assessment of in vitro colony formation was performed using three different methods. The dynamic growth of colonies cultured in vitro was detected and followed by counting two dishes twice a week over a period of three to six weeks [8]. Using this method, the growth potential in culture is described by growth curves, indicating number of colonies counted as a function of time (Fig. 1, 2). Colony formation was checked by optical control with a Leitz inverted microscope. Colonies were counted and characterized based on optical density, shape and diameter ranging between 60 and 400 μm in size. For a culture to be accepted as growth positive, three criteria had to be met: (1) more than 30 colonies over 60 μ in diameter at the peak of growth, that is, the highest number of colonies counted; (2) a maximum colony number at peak growth at least twice the Day 1 count or twice the minimum colony count after an initial decline in the number of clumps seeded into culture as determined by the growth curve; (3) the trend of the curve before peak colony count should show a consistent increase in the number of colonies counted on at least two successive counts. In addition to evaluation of growth curves, the single point colony count on day 21 was evaluated, using this technique, the Day 1 count has to be less then 30 counts in a dish to exclude dishes seeded with clumps. In addition to the types of growth evaluation described above, drug tests were also evaluated by measuring the slope of the growth curve, that is, the peak number of colonies counted, minus the day one count or the lowest count during the culture period, divided by the total number of days necessary to achieve the peak number of colonies in culture (Fig. 1). In defining the effect of the cytostatic drugs on the cultures the same criteria for the three methods of evaluation were used. Sensitive cultures showed less than 50% of the control value for each method and resistant cultures showed more than 50% of the control value. Clinical and histopathologic data were used for staging of the tumors according to the TNM-classification [14].

Table 1. Correlation of growth curves with single time point (day 21) colony counting in assessing presence or absence of tumor colony formation in HTCS

	All growth-curves			
	+	–	N.E.	
All day 21 count				
+	21	3	0	24
–	5	25	0	30
N.E.	16	35	0	51
	42	63	0	105

+ Growth in culture, – No growth in culture, N.E. Not Evaluable; For Day 21 cound = > 30 "colonies" counted on day 1

Results

Twenty-six patients were evaluated in the present study. From 21 patients the primary tumor in the kidney was cultured and from 5 patients lymph node metastatic lesions were cultured. From this material, 105 cultures of 18 to 24 dishes each were plated and evaluable (not lost due to infection). These included direct growth curves, drug tests and drug controls. The first evaluation of the cultures was a comparison between the growth curves and single point day 21 counting as a way of evaluating presence or absence of colony formation (Table 1). Of a total of 105 cultures, 51 were not evaluable by day 21 counting because of seeding of > 30 clumps into cultures as assessed by day 1 counting. Of the 54 cultures in which growth curve and single point counting could be compared, the two methods agreed in assessing presence or absence of colony formation in 46

Table 2. Relation of colony growth to the TNM classification and the presence (V+) or absence (V–) of renal vein invasion in primary tumors only. Number of cultures showing growth by analysis of temporal growth pattern/total number of cultures

Stage	N0/M0	N+ or M+	V–	V+
T1–T2	5/9	1/2	6/11	0/0
T3–T4	2/3	4/7	4/6	2/4

Table 3. Relation of growth potential of the tumor to the age of the patient. Number of cultures showing growth by the temporal growth pattern/total number of cultures

Growth by TGP	Age class			
	30–39	40–49	50–59	60–69
+/Total	2/2	5/5	3/7	2/7

(90%) of the cultures. The three cultures which were evaluated as showing growth by day 21 counting but which were evaluated as growth negative by analysis of growth curves in fact showed no consistent increase in colony numbers over time although the day 21 count was above 30 colonies. The five cultures which showed growth, by growth curve evaluation but not by day 21 counting were more interesting. All five showed development of consistent peak colony counts, but in two cultures this occurred before day 21 with subsequent deterioration of the colonies and in 3 cultures the peak colony count occurred after day 21. If no growth of colonies was seen in the optical check of the culture plates this was obviously reflected in the dynamic growth curves by a decrease in the curves below the positive growth threshold of 30, even if a high day one count had occurred.

A comparison between growth of tumor colonies in culture evaluated by growth curves and other clinical and histopathological data was made. Of the 21 patients with a primary tumor, 12 showed growth in the HTCS system, while 4 of 5 metastatic lesions produced colonies in vitro (p = 0.67, Fischer exact test). In contrast, for the primary tumors no significant correlation could be detected between the growth potential of the tumor in the HTCS and the TNM stage (p = 1,000, Fischer exact test, Table 2) or the presence of renal vein invasion (p = 1,000, Fischer exact test, Table 2). No correlation with tumor size could be made due to the marked differences in cystic degeneration, necrosis and hemorrhage of the tumors. These features preclude reliable estimates of the amount of tumor tissue present. However, age of the patient seems to be important for the positive growth potential of primary tumors in the HTCS as there is a significance higher positive growth rate in the group of patients younger than 50 years compared with older patients (p = 0.013, Fischer exact test, Table 3).

Fifteen in vitro drug sensitivity tests from 5 patients with a primary tumor were evaluated. An example of one such test is shown in Fig. 2. In this example, Adriamycin is considered effective in vitro although the later peak colony count with Adriamycin (27 days) compared with the control culture (15 days) suggest a possible drug-induced lag phase followed by catch-up growth. This phenomenon is seen more clearly in the Cis-platin drug curve of Fig. 2. The peak growth of the culture incubated with Cis-platin is equal to that of the control culture but occurs a week later. In 14 of the 15 drug sensitivity tests the day 21 counts, peak colony number by growth curve and slope of the growth curve gave concordant results for tumor sensitivity or resistance. In one culture, the day 21 count showed sensitivity while both peak colony count and the slope of the growth curve indicated clear drug resistance. Of the 15 drug tests, sensitivity was identified in only 2 tumors, both renal primaries. Both of these tumors were sensitive to Adriamycin and one was sensitive to Cis-platin.

Discussion

The present study indicates that for human renal cell carcinoma, there is generally a correlation between dynamic evaluation of colony formation in soft agar and single point colony counting at day 21, for those tumors which are evaluable by both methods. A major contribution of the use of growth curves to evaluate in vitro colony formation is the increase in evaluable cultures, since growth curve evaluation is possible even in the presence of significant numbers of cell clumps seeded into culture [8]. Colony formation from tumors may be an additional prognostic indicator in renal cell carcinoma since the presence or absence of tumor colony formation was not related to TNM stage or renal vein invasion but was related to the age of the patient. These findings suggest that tumor growth in the HTCS reflects a biologically important potential [15]. In addition colony formation in agar did not occur significantly more frequently when metastases were cultured than when renal primary tumors were used as the source of material. In other studies in renal cell carcinoma [11, 13] about the same rate of positive growth and resistance to chemotherapeutic drugs were found as in our material. But the influence of the drugs was never studied over a period of time in culture which may explain some of the results of resistance to chemotherapeutic drugs as all studies were evaluated with a single point observation. Perhaps the most striking contribution therefore of dynamic assessment of colony formation in vitro will emerge from its use in in vitro chemosensitivity testing. The patterns of colony development over time in control and drug-treated cultures suggest that more complex drug effects such as drug-induced growth lag with subsequent catch-up may be identified in vitro (Fig. 2). In the present study, only 3 samples from two tumors of a total of 15 samples showed in vitro drug sensitivity ($>$ 50% growth inhibition by comparison with control).

It is of interest that both the sensitive tumors were sensitive to Adriamycin, one of the few drugs showing in vivo activity in treating sarcomas. The possible sarcomatous nature of renal cell carcinoma has been pointed out elsewhere [4] and confirmed by other investigators [5]. Whether or not these potential advantages of dynamic assessment of in vitro colony formation can be translated into more effective patient management is a subject of further investigation.

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