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Clonal Heterogeneity in Drug Sensitivity of Primary and Metastatic Murine Tumor Cells Using a Clonogenic Assay[1]

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SUMMARY

The heterogeneity with respect to the sensitivity to a cytotoxic agent of clones derived from a mouse fibrosarcoma tumor (MCA-F[3]) and its individual metastatic lung colonies has been examined. After 1 hr exposure of mitomycin C (MMC), the plating efficiency (PE) of parental tumor cell clones and metastatic cell clones were measured respectively, suggesting that the degree of clonal heterogeneity of primary tumor cells which showed different ancholage-independency was lower than that of metastatic tumors. The drug sensitivity of parental tumor cell clones also showed their heterogeneity and in the individual metastatic lung tumor cell clones as well, but with a rather high resistance.

One (MCA-F-M2) out of 4 colonies in the lung, especially showed significantly high % PE in a clonogenic assay using 1 hr MMC exposure at 0.1 $\mu g/ml$ concentration (mean % PE=19.7; $P<0.005$) and its clones showed almost the same homogeneous sensitivity within a growing colony in the lung. While, there existed not a small population of drug sensitive clones within these metastatic lesions. These findings indicate the clonal chemotherapeutic heterogeneity in metastatic lesions, and may provide serious implications for the administration of antitumor drugs on the early differentiation of tumors.

Key words: Drug sensitivity, Clonal heterogeneity, Clonogenic assay

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3. The abbreviations used are: MCA-F, 3-methylcholanthrene-induced mouse fibrosarcoma; MMC, mitomycin C; PE, plating efficiency; MCA-F-M1 through MCA-F-M4, lung metastatic clones of MCA-F.

INTRODUCTION

The *in vitro* soft agar culture system, screening antitumor drugs by cell culture techniques, is more economical and more time-saving than *in vivo* system such as a xenograft in nude mice and provides an accurate estimation of the proportion of the tumor cell population directly that is killed by drug treatment as well. Since Hamburger and Salmon(1) reported that a soft agar culture system was suitable for direct bioassay of tumor stem cells from a variety of human cancers, there has been a great interest in colony-formation assay technique to evaluate the potential of antitumor agents and many researchers have studied antitumor activities of various agents either human tumor cells in primary cultures(2-4) or established human tumor cell lines(5, 6). Recently, this *in vitro* procedure, tumor cell colony-forming assay has been developed as the human tumor clonogenic assay and come into good use clinically for predicting the effectiveness of antitumor agents against an individual patient's cancer(7, 8).

At first *in vitro* murine tumor experiment, the colony-forming assays for transplantable tumor cells have been shown to indicate a therapeutic response *in vivo*(9). Essentially, the cellular tumorigenicity of tumors, which mean their degree of malignancy *in vivo*, has been known as the capacity of tumor cell colony-forming in soft agar. The ability of tumor cells to form colonies in soft agar has been closely correlated with tumorigenic potential(10, 11) and recognized that the anchorage-independent growth of tumor cells in soft agar was available for study of the biological characterization of various murine and human tumor cells on *in vitro* culture(12-17).

There are convicing evidences to show that a primary malignant tumor is markedly heterogeneous in respect of metastatic capacity(18, 19) and a metastatic tumor as well(20).

In addition, the heterogeneity concerned with the sensitivity to cytotoxic drugs in primary(21, 22) and metastatic tumor(23-25) is demonstrated to be heterogenous. Therefore, the existence of these cellular heterogeneity in tumors has many important therapeutic implications and help to explain the failure of some therapeutic regimens and the success of others.

In this study, we have evaluated the mitomycin C (MMC)-sensitivity of several clones of MCA-F and metastases when they were at the most early culture stage in an attempt to minimize the diversity which may have arisen during the growth of the clones. MMC was found to have a broad spectrum of activity against various tumors and significant clinical antitumor effects with a high response rate(26). The predominant lethal efficacy of MMC which inhibits DNA synthesis in tumor cells has been producing many useful therapeutic protocols in single administration and / or

combination with others(27).

Consequently, we succeeded in cloning of metastatic lung tumors which were spotaneously produced from primary tumor produced in the footpad by injection of mouse fibrosarcoma cell, MCA-F, and in determining the tumorigenicity relate to clonal antitumor drug sensitivity using a clonogenic assay by modified Hamburger's soft agar assay in primary and metastatic tumor clones.

MATERIAS AND METHODS

Animals and tumors

The murine fibrosarcoma (MCA-F) was induced by 3-methylcholanthrene in female C3H/HeJ mice(28), and was used in the seventh *in vivo* passage generation. Tumors were maintained by serial subcutaneous (s. c.) passage is 4-6 week old specific-pathogen free female C3H/HeN (MTV^-) mice (Charles River, Kingston, NY), as previously described(28).

Cell preparation

5×10^5 of MCA-F lines were injected into the right hind footpad (i. f. p.) of mice. When the resulting s. c. tumors reached an average diameter of 1.0 cm, the tumor-bearing leg, including the popliteal lymph node was resected at midfemur. Three weeks after resectios, spontaneous metastatic lung colonies of 1-2 mm diameter were aseptically removed. Of the same mouse, 4 colonies were isolated from each individual lung nodules and single cell suspensions were prepared, respectively as described(29).

Cell cloning

The MCA-F line and its lung metastatic cell lines designated as MCA-F-M1 through MCA-F-M4 were cloned twice *in vitro* by the limiting dilution technique as reported previously(30). Briefly, a viable sigle tumor cell was implanted into 24-well culture plates (Costar 3524, Costar, Cambridge, MA) and maintained in Eagle's minimal essential medium (MEM) containing nonessential amino acids, vitamins, L-glutamine, sodium pyruvate, and 10% fetal bovine serum (FBS).

Cultures were incubated in 5% CO_2 at 37 ℃. Two weeks later, those cultures showing cell growth were harvested by brief incubation in 0.05% trypsin-0.02% EDTA and a second single cell cloning was done for each clone. Then clones were obtained from each 4 lung colonies and used for the clonogenic assay.

Clonogenic assay

A modification of the *in vitro* double layer soft agar assay described by Hamburger and Salmon's method(1) was used. 1×10^3 cells/m*l* in a tube for

MCA-F clones and metastatic clones were respectively incubated with MMC at 0, 0.1 and 1.0 μg/m*l* concentrations for 1 hr, then the cells were washed 3 times with Hank's balanced salt solution. The effect of MMC on the anchorage-independent growth of resultant cells was studied for 10^3 cells/m*l* inocula in 35 mm petri dishes (Corning 25000, Corning, NY) in which was the seeder layer that was adjusted to a 1 m*l* of 0.3% bacto-agar (Difco Laboratories, Detroit, MI) in MEM supplelmented 10% FBS. The underlayer as a feeder on which the cells were plated consisted of 1 m*l* of 0.6% agar in 2 fold concentration of MEM and FBS. The anchorage-independent growth capability of cells was scored microscopically at 2 to 3 weeks after plating, and the colony formation was expressed as the % PE: (number of cluster−number of original cell aggregates)×100/number of viable nucleate cells plated. Cells forming clusters appeared as early as the sixth day of culture and grew to form colonies with more than 30 cells until fourteenth day after plating. Prior to plating, the cell viability was checked by trypan blue exclusion and also cell aggregation was examined, resulting in the fact that all suspensions exhibited more than 95% cell viability and no aggregation. All assays were set up in triplicate.

Drug

MMC was purchased from Sigma Chemical Co. St. Louis, MO. Two mg of drug powder were reconstituted in 10 m*l* of 0.9% NaCl solution, and appropriate drug concentrations were made with MEN.

Statistics

Statistical differences in % PE of MCA-F cells and the other clones were determined by the Student's t test.

RESULTS

Clonogenic assay in parent clones

Twenty clones from MCA-F parent cell line were obtained by limiting dilution technique. As shown in Table I, % PE of clones were determined at 2 and 3 weeks after plating cells, resulting that meas % PE were 20.4±3.8 and 29.6±3.0, respectively. Statistically the differences betwees mean % PE of parent cells and each % PE of clones were examined and 7 out of 20 clones were significant ($p<0.05$-0.005) in both weeks. However, in comparison with mean % PE of clones, there were no signficance. On the other hand, in order to compare the activity of antitumor drug against these clones, we used MMC in different concentration of 0.1 μg/m*l* and 1.0 μg/m*l*.

As a result, on 0.1 μg/m*l* assay 4 drug resistant and 7 sensitive clones at 2 weeks incubation and 7 drug resistant and 3 sensitive clones at 3 weeks assay

Table 1 *Effect of MMC on Colony Formation of Clones from MCA-F Parent*

Cells	% Plating Efficiency					
MMC (μg/ml)	2 nd wk 0	0.1	1.0	3 rd wk 0	0.1	1.0
Parent	21.0±1.1	3.0±0.6	0.7±0.5	30.6±0.8	4.0±0.8	0.9±0.7
Clone No. 1	20.4±2.7	3.1±0.8	0[c]	30.6±1.7	3.9±0.7	0.8±1.2
2	19.1±3.1	2.3±0.5[a]	1.0±0.9	28.6±1.3[b]	2.4±0.7[c]	1.2±0.9
3	21.6±2.7	1.7±0.9[b]	0.9±0.8	26.6±0.6[d]	1.7±1.2[d]	1.3±0.4
4	16.9±1.8[d]	2.7±1.0	0[c]	26.6±0.9[d]	3.2±0.6	0[d]
5	17.7±1.8[b]	1.7±0.5[b]	0.7±0.5	26.0±3.2[d]	3.1±1.2	1.4±1.0
6	21.7±2.8	2.0±0.9[b]	0[c]	31.3±1.7	3.2±0.4	0[d]
7	20.9±4.6	2.9±0.8	0[c]	31.9±2.0	4.2±1.2	0.7±0.9
8	20.4±2.7	4.1±0.4[a]	1.4±0.5[a]	29.9±4.0	5.8±0.8[b]	1.4±0.4
9	24.5±1.0[b]	3.2±1.0	1.0±0.9	32.0±1.4	3.5±1.0	1.3±1.2
10	23.9±4.5	2.0±0.9[b]	0.7±0.5	31.8±1.5	4.2±0.8	1.1±0.9
11	15.0±0.9[d]	1.7±0.5[b]	0[c]	30.2±1.7	2.7±0.4[c]	0.7±0.5
12	19.2±1.8	3.7±1.2	0[c]	30.2±5.1	5.6±2.4[b]	0[d]
13	24.9±3.5[b]	6.4±0.4[d]	1.8±0.6[d]	27.1±0.8[d]	8.9±1.6[d]	2.6±0.5[d]
14	16.9±1.1[d]	2.0±0.8[b]	0[c]	29.4±0.7	4.3±0.6	0[d]
15	22.6±1.4	2.7±0.9	1.0±0.7	31.0±3.0	4.9±0.3[b]	1.3±0.9
16	23.2±1.3	5.3±0.5[b]	1.1±0.8	31.1±2.7	7.8±2.6[c]	1.8±0.6[b]
17	19.4±2.6	3.4±1.3	0[c]	29.9±0.7	4.5±0.8	1.0±0.8
18	17.2±3.3[c]	5.7±0.6[d]	1.4±0.4[a]	28.0±2.5[b]	8.2±1.7[d]	2.1±0.9[b]
19	21.6±3.4	3.1±1.5	1.3±0.5	27.9±1.3[c]	4.4±2.1	1.4±1.0
20	21.7±1.5	3.1±0.9	1.2±0.9	32.1±3.3	5.5±1.1[b]	1.6±1.2
Mean[e]	20.4±3.8	3.1±1.6	0.7±0.8	29.6±3.0	4.6±2.3	1.1±1.0

10^3 cells were inoculated into a 0.3% agar in triplicate cultures and % PE were calculated by the formula described in Materials and Methods (mean ± SE). Statistically significace were determined by Student's t test between clones and parent.
a) $p<0.05$ b) $p<0.025$ c) $p<0.01$ d) $p<0.005$ e) Average of clones.

were shown and a heterogeneous trend was also seen on 1.0 μg/m*l* level. Considering the relation between drug concentration and incubation time, the drug sensitivity seemed to influence the duration of incubation. Especially, all drug resistant clones such as No. 8, 13, 16, and 18 were shown to maintain their qualities and they were seen to be almost dose-dependent. In contrast, some drug sensitive clones such as No. 2, 3 appeared to have a weak instability against MMC in terms of drug concentration and incubating time.

Clonogenic assay in metastatic clones

Four spontaneous metastatic cultures in lung, namely MCA-F-M1 through

Table 2 *Effect of MMC on Colony Formation*

Cells	MMC (μg/ml)	% Plating Efficiency Clone No. 1	2	3	4	5
MCA-F -M1	0	25.1±2.0[c]	23.3±3.8	28.3±4.3[d]	28.8±3.3[d]	32.2±5.4[d]
	0.1	2.6±1.1	2.1±1.0[a]	0.9±0.8[c]	3.0±1.5	3.9±1.1[a]
	1.0	0.9±0.8	0.3±0.5	0[c]	0.6±0.8	0.7±0.5
MCA-F -M2	0	25.6±0.8[d]	26.6±1.8[d]	28.9±2.5[d]	32.8±1.4[d]	35.8±0.9[d]
	0.1	19.8±3.3[d]	23.8±1.9[d]	20.5±2.6[d]	19.5±1.3[d]	19.8±0.9[d]
	1.0	1.9±0.6[d]	2.7±1.9[d]	1.7±0.6[c]	2.1±0.9[d]	1.3±0.9
MCA-F -M3	0	24.6±5.3[b]	33.8±0.2[d]	35.9±1.6[d]	32.7±1.2[d]	30.5±1.9[d]
	0.1	2.3±0.9[a]	4.9±1.8[b]	3.5±1.1	4.6±1.4[b]	3.5±0.8
	1.0	0.3±0.4	0.7±1.0	0.7±0.5	1.7±1.2[c]	0.3±0.5
MCA-F -M4	0	33.2±7.1[d]	33.3±2.4[d]	28.7±2.8[d]	35.1±3.0[d]	32.9±2.4[d]
	0.1	4.2±0.6[b]	3.7±1.0	5.6±0.7[d]	5.3±0.8[d]	5.6±0.6[d]
	1.0	0.7±0.9	1.0± 0	0.7±0.5	0[d]	2.3±0.9[d]

Treatment of cells and their % PE determinations were identical with that in Table 1. Statistical significance was determined by Student's t test between clones and parent.
a) $p<0.05$ b) $p<0.025$ c) $p<0.01$ d) $p<0.005$

MCA-F-M4, were produced by a single i. f. p. injection of MCA-F parent cells and 10 clones derived from each of them were studied for clonogenic assay the same as in MCA-F parent clones in Table 1. According to the resuts of clonogenic assay related to 4 individual metastatic cell cultures, all showed significantly high mean % PE ($p<0.005$) compared with that of MCA-F parent (Table 2). The deta also demonstrated that the drug sensitivity of these clones were heterogeneous and dose-dependent like the parent cells and the mean % PE showed rather high values than that of the parent in both drug concentration of 0.1 and 1.0μg/m*l*, suggesting that almost all metastatic tumors is the lung were more resistant as compared to primary tumors. Above all, 1 culure (MCA-F-M2) out of 4 exhibited considerably high resistance against MMC and all of its clones as well. However, even in such metastatic tumors, on clonal examination there actually existed some cultures containing several drug sensitive clones such as MCA-F-M1 and MCA-F-M3 ($p<0.05$-0.01).

DISCUSSION

Many tumors, whether of human origin or not, have been shown to be heteroge-

of Metastatic Clones from MCA-F Parent

at 2 nd Wk

6	7	8	9	10	Mean
30.5±6.2[d]	30.0±5.2[d]	29.8±2.4[d]	30.1±2.7[d]	28.9±5.0[d]	28.7±4.9[d]
4.3±1.9[b]	3.3±1.4	3.5±0.7	3.4±1.2	4.0±0.9[a]	3.1±1.6
1.6±1.2[c]	1.2±1.0	0.6±1.0	1.0±0.8	1.6±1.2[c]	0.9±1.0
36.3±3.0[d]	29.5±5.7[d]	28.8±2.6[d]	25.7±1.2[d]	33.3±2.9[d]	30.3±4.6[d]
18.1±2.0[d]	19.2±2.3[d]	18.9±1.2[d]	18.1±5.0[d]	18.9±3.8[d]	19.7±3.1[d]
0.8±0.3	1.7±0.5[c]	2.1±0.9[d]	1.6±0.9[d]	4.2±0.7[d]	2.0±1.3[d]
31.8±3.2[d]	32.7±0.8[d]	33.8±1.6[d]	30.3±2.3[d]	27.5±3.5[d]	31.4±4.1[d]
4.0±0.7[a]	5.1±1.4[c]	3.7±1.2	2.8±0.3	3.9±0.6[a]	3.8±1.4
1.4±0.5[a]	0.9±0.7	0[c]	0[c]	2.6±0.6[d]	0.9±1.0
30.2±3.2[d]	30.9±4.0[d]	36.4±1.9[d]	37.4±2.7[d]	32.2±1.8[d]	33.0±4.3[d]
5.8±0.9[d]	6.5±0.6[d]	5.8±0.6[d]	4.3±1.6[d]	4.8±1.8[b]	5.2±1.3[d]
2.0±0.1[d]	1.0±0.8	0.7±0.5	0[c]	0[c]	0.8±0.9

neous with respect to drug sensitivity(21, 23, 31, 33) and, *in vitro* and *in vivo* assay, numerous evidence to exhibit clonal variations in drug sensitivity existed(23, 25, 34, 35). As Heppner mentioned(36), recognition of tumor heterogeneity is essential to any theory of neoplastic development as well as to experimental design and clinical treatment. From a therapeutic point of view, it seems to be more important to clarify the nature of tumor cell and the mechanisms of its metastasis, in addition to the relation between these cells and antitumor agents.

Results from studies by Fidler and his co-workers(37, 38), using murine B 16 melanoma cells, UV-2237 fibrosarcoma cells, and their descendants, have indicated the existence of clonal diversity in their ability of tumor invasion and metastasis during culture *in vitro* as well as the stability in metastatic potential due to clonal interaction.

In other studies, *in vitro* cultivation of clones derived from mammary adenocarcinoma produced a phenotypic drift of metastatic property(39) and clonal interactions that maintained their metastatic potentinal among cultures was demonstrated using B16 melanoma cells(37). These would imply that the ability in metastasis of clone was not stable by itself but was stabilized in polyclonal cell populations owing to clonal cell interactions.

Of special interest clinically, the fact that the heterogeneous drug sensitivity existed in primary tumors and its metastatic tumors have provided a major impedi-

ment to the successful chemotherapy. Therefore, in order to prevent tumor metastases, it might be necessary to take the concept of heterogeneity of tumor cells in terms of the relation between metastatic ability and drug sensitivity into consideration. Recently, a clonogenic assay has been developed for predicting the chemotherapeutic effect of antitumor agents an individual tumor. Indeed, % PE of tumor cells obtained from this *in vitro* assay represented their *in vivo* drug sensitivity to fumors pre-eminently(4).

In the present study, we examined the clonal difference of tumorigenicity between the primary tumor, MCA-F, and its 4 metastatic tumors, MCA-F-M1 through MCA-F-M4, by measuring % PE in clonogenic assays. Furthermore, we evaluated the cytotoxic drug sensitivity using MMC in both clones, resulting in the findings that: 1). In primary tumor, the heterogeneity with respect to tumorigenicity represented as % PE as well as chemosensitivity was clearly observed. 2). In metastatic lesions, all colonies showed high tumorigenicity rather than in the primary tumor, however, their drug sensitivity were heterogeneous even in a clonal level as in primary tumor clones, besides one colony demonstrated significantly a high drug resistance. 3). In both clones, dose-dependent effects were obtained in the spite of their heterogeneity in drug sensitivity. In view of the difference of tumorigenicity between the primary tumor and the metastases, the present date were consistent with the results of Schlag and Schreml(40), while in terms of clonal drug sensitivity of the two, the data also supported the others(23, 24). Lung metastases have been thought to be the result from the single cells is the primary lesion and consequently clonal selection was considered in the process of metastasis from the primary tumor. Therefore, there was a good reason for metastatic tumors as well as their clones to explain their heterogeneity in drug sensitivity. Thus, the results of this assay should provide further insight into differences in sensitivity to antitumor drug of clonal tumor cells among primary tumors and metastatic tumors in regard to the therapeutic regimens.

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