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Clonal Heterogeneity in Gastric Cancer Cells

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A heterogeneitic human gastric cancer cell line was isolated and established as a UMK-1 line. Eight clones were selected for studies on morphology, growth pattern, cellular DNA and RNA contents and drug sensitivity in comparison with those in parent cell line of UMK-1.

There were differences in morphology, growth pattern, saturation density, cloning efficiency and cellular DNA and RNA contents among the 8 clones. The drug sensitivity to Mitomycine C (MMC), Adriamycine (ADM) and Cis-diamindichloroplatinum (CDDP) varied in each of the clones although that of UMK-1 line had been in-between them. Such is a reflection of a presence of clonal heterogeneity. Selection of drugs time by time offers the greatest effectiveness on tumor regression due to responses to accidental loss of sensitivity to drugs used.

INTRODUCTION

It has been believed that cancer cell is monoclonal cell originated from a single cell.[1)2)3)4)] However, in accordance with propagation of a single cell, it constructs the tumor mass which is composed of numerous cancer cells.[5)]

These cells propagated have varying variety of individual properties. It has become recognized that malignant cells are so heterogenous that the drug sensitivity of these cells is not uniform. On the other hand, to enhance the chemotherapeutic effect on cancer cells, it is necessary to know the sensitivity of each of cancer cells to various anticancer drugs because the clones in the tumor have individually high heterogeneity to response to drugs.

And also recurrence of the tumor probably begins from regrowth of natural resistant cells to drugs previously used. It would be speculative to say that these regrowing cells

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have less sensitivity or natural resistance to the drugs used.[6)7)] The study on heterogeneity of cancer cells has been advanced in rats and mice. In contrast, in humans there are a few reports in regard to colon[8)9)10)] cancer and brain tumor.[11)12)] Little information is available for this aspect.

The aim of this study is to clarify the characteristics of human cancer cells derived from gastric cancer with respect to morphology, growing pattern, cellular DNA and RNA, and anticancer drug sensitivity.

MATERIALES AND METHODS

Cell lines

About 500ml of ascitic fluid from the patient who underwent non-curative surgery for gastric caner were taken (Fig. 1), (Table 1) centrifuged for 5 min at 600 cpm. The pellet was resuspended in growth medium and 20 ml of the resulting cell suspension was plated in each 75 ㎠ tissue culture flask (*Corning Co*) at 37°C in a humidified cabinet containing 5% CO_2 in air, supplemented with 20% Fetal Calf Serum (*GIBCO Co*) in growth medium RPMI 1640 (*DIFCO Co*), adding 1×10^5 U./L penicillin (MEIJI SEIYAKU Co) and

Table 1. Obtaining and culturing of gastric cancer cell UMK-1

Case : 56 year-old man
Operation : total gastrectomy for stomach cancer, Borrman type 3 with peritoneal implant, in Jul. 8, 1982.
Pathological diagnosis : moderately differentiated adenocarcinoma
Cancer cells were obtained from ascitic fluid in Jan, 1983.

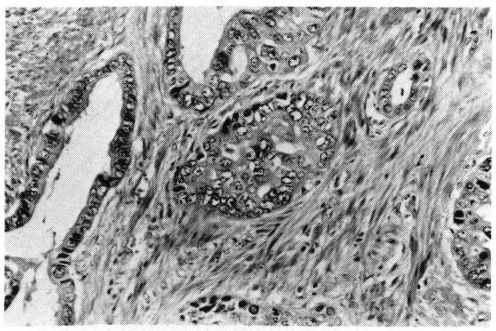


Fig. 1. Microscopic feuture of primary tumor : moderately differentiated adenocarcinoma.

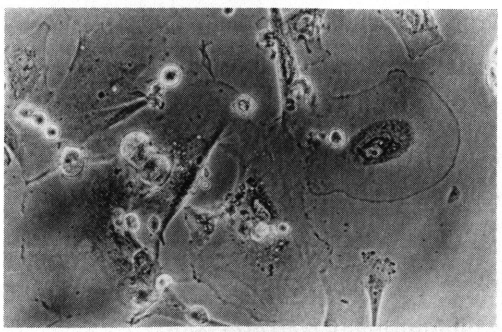


Fig. 2. Primary culture of UMK-1

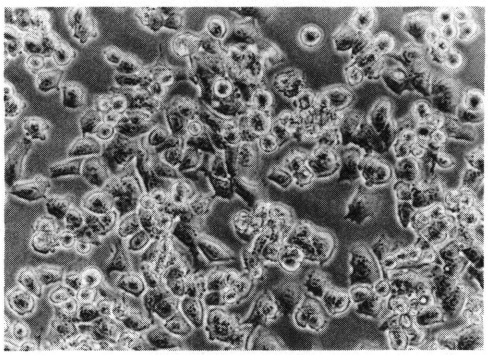


Fig. 3. Microscopic feuture of parent cell line UMK-1.

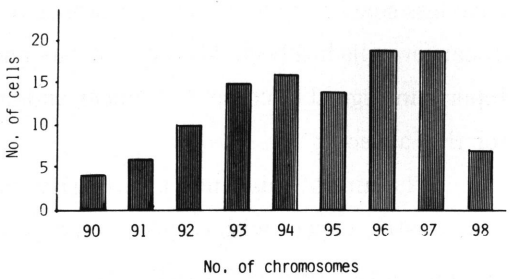


Fig. 4. Chromosomal analysis of UMK -1.

100 mg/L Kanamicin sulf. (MEIJI SEIYAKU Co).

Half of growth medium used herein was refreshed every 2 days followed by changing once a week at 3 weeks when floating and dead cells were no longer seen (Fig. 2).

At 3 months when confluent culture was achieved, routine maintenance of the culture was carried out by trypsinization (0.25% trypsin DIFCO co). After the first subculture the speed of growth gradually increased at 11 month. After the fiftieth subculture was achieved, October, 1983, the established cell line (P-50) was called UMK-1 (Fig. 3). The cells were frozen in every 5 generations following prepared for this study.

Cell staining

UMK-1, corresponding to proliferative stage were grown in 60 ml culture dish (*Falcon Co.*), supplemented with 0.02 μg/ml colsemid (*GIBCO co*) and cultured for 5 hours so as to be identical to M stage.[13] After washing with PBS (-), 0.075M KCl was added to make the cells enlarge, the cells were fixed in Carnoy's solution (absolute methanol : glacial acetic acid, 3 : 1). Air-dried preparation were stained with Giemsa solution. Chromosome analysis was made by histogram, counting 110 cells (Fig.4).

Transplantation to nude mouse

5×10^5cells / 0.5 ml of UMK-1 cells was transplanted on the back of nude mice, aged 8 weeks, male. A comparison was made with respect to tumorgenecity, latency period to grow up to a tumor of 5mm in diameter and morphologic feature. (Fig. 5)

UMK-1 cloning procedure

After thawing of P-10 UMK-1, all cells were incubated for 1 week. Confirming their growths, suspended cells were diluted to 3 cells/ml and seeded into a flat bottom 96 well multiplate (*Corning Co).* At 7 day incubation at 37°C in a humidified cabinet containing 5% CO_2 in air, wells with single colony alone were selected. Reached confluency, the cells were continued to subculture and the 200 clones were established (Table 2). For

Table 2. Cloning of UMK-1 cells

thawing frozen cells cultured in 10 generations
↓ culture for 7 days
cell suspension (3 cells/ml)
↓
0.15ml of samples into 96 wells
↓ selection of plates including single colony after 7 days
about 200 clones

further examination, line 101, 112, 118, 201, 206, 271, 279 and 287 were subjected with respect to different morphology, cellular DNA and RNA content and drug sensitivity to MMC and ADM. Samples were frozen and prepared for this study. Clonal heterogeneity of human gastric cancer cells

1) *Morphology*: 1×10^5 cells/2ml of UMK 1 and each clone were plated in a 6 well multiplate (*NUNC Co*). The cell growth was plotted on semilogarithmic paper and a plateau of growth was tested with the aid of a light microscope.

2) *Cell proliferation*: 1×10^5 cells/2ml of UMK-1 and each clone were plated in 6 well multiplate and 10 days growth curves were illustrated to determine doubling times. Saturation desity was also calculated from the cell numbers in confluent dishes that the plateau regions of the growth curves provided the maximum cell numbers.

3) *Cellular DNA and RNA*: 1×10^6 cells/5ml of UMK-1 and each clone were plated in 60 mm culture dish and colony isolation was achieved by trypsinization and centrifuged for 5 min. at 1000cpm (HITACHI 20PR-52D). After discarding the supernant, cells were fixed in ethanol-acetic acid 1 : 1. And also the lymphocyte were separated from the peripheral blood by a linear gradient of Ficoll-Conray and fixed with the similar manner. Normal human lymphocytes were used as the inner control when measuring the content of cellular DNA and RNA by using flow-cytometry (Becton Dickinson FACS IV)[14] after incubation at 4°C for 7 days (Table 3). Staining was carried out by the two step acridine orange technique. The acridine orange binds to double helical DNA by intercalation and fluorescens green in blue light, and to single stranded RNA is denatured to its single stranded by EDTA. The fluorescence emission at the two different wave-length bands is separated optically and quantified for each of the 15000 cells per sample.[15] The cells fixed with ethanol-acet acid were centrifuged, washed and adjusted to 5×10^5 cells/ 0.2ml adding 0.4ml acid detergent (0.01% Triton-X, 0.08N HCl, 0.15M NaCl, pH 0.79). By keeping the cells on ice, the cells were plated in Falcon 2054, adding 1.2ml acridine orange solution (10 μg/ml AO, 10^{-5} M EDTA, 0.15M NaCl,

Table 3. Two step acridine orange technique

UMK-1 or clones
↓ single cell suspension
ethanol-aceton
(48 hours～)
cell suspension (5×10^5 cells/0.2ml)
↓
0.01% Triton×100, 0.08N HCl (0.4ml at 0°C)
0.15N NaCl, 10^{-4} EDTA-Na
↓ (after 30 sec.)
10^{-3} M EDTA Na, 0.15N NaCl (1.2ml)
0.1M phosphate-citrate buffer (pH 6.0)
acridine-orange 10μg/ml
↓ (after 5 min.)
Flowcytometry (FACS IV)

excitation ……Argon ion laser 488nm
emission……DNA(530nm) green fluorescence
RNA(640nm) red fluorescence

0.1M Sitrate buffer pH 6.0) after 30 sec, left at room temperature for 5 min and filtrated through Nylon mesh to remove tissue fragments and cell groups. As a rule, the cellular DNA and RNA measurement was set up within 3 min. Cell cycle was analysed DNA histogram, namely G_2M was twice in DNA content of G_1, the region over it was polyploid (P. P) and S was between G_1 and G_2M, and the region of below G_1 was dead cell (D). The cell numbers were counted. The rate of G_1+S+G_2 M to $S+G_2M$ was expressed as a proliferative index (P I) and the peak values of DNA and RNA were shown in the histogram. (Fig. 9, 10, 11)

Drug sensitivity test

1) *Regrowth assay*

1×10^5 cells/2ml of UMK-1 and each clone were plated in 6 wells multiplate and after 2 days culture, anticancer drugs were given for 1 hour. After washing twice with PBS (-), the cells were incubated for 5 day in fresh medium, separated with trypsin and counted. All procedures were made in the triplet. The control values were normalized to 100% survival for calculation of the percentage of survivors. The drugs and their concentrations used in this study were as followed; 0.1μg/ml, 0.5μg/ml and 0.25μg/ml Mitomycin C (MMC, KYOWAHAKKO Co), 0.02μg/ml, 0.1μg/ml and 0.5μg/ml Dexorubicin Hydrochloride (ADM, KYOWAHAKKO Co), 0.02μg/ml, 0.1μg/ml, 0.5μg/

ml Cis-diamindichloroplantinum (CDDP, NIHONKAYAKU Co). All drugs were dissolved in saline and diluted 50 : 1, adding growth medium of 1 : 50 volume and growth medium was refreshed every two days.

2) *Colony forming assay*

3×10^4 cells/2ml UMK-1 and each clone were plated in 12 well multiplate (*Linbro Co*). At 2 days anticancer drugs were given for 1 hour, thereafter, washed and isolated with 0.25% trypsin. All cells were suspended in 3 ml growth medium containing 0.3% Bactoagar (*DIFCO Co*). One ml of this suspension was seed as upper layer in a 6 well multiplate layered 1 ml growth medium containing 0.5% Bactoagar as lower layer in advance.[16] The numbers of colony composed of 50 or more cells were counted and expressed as a survival rate to the control.

RESULT

Establishement of UMK-1 cell line and cloning

Cancer cells from ascitic fluid of gastric cancer slowly grew and it was possible to obtain a cell line at 4 months (Fig. 2). After the first subculture cancer cells showed a stable growth at doubling time of 28 hours. Consequently UMK-1 was establised at 9 months from the intial cell culture through P-50 courses (Fig. 3). The number of chromosome was 90 to 98, especially concentrating to 93 to 97, with a peak of 96 and 97 and it situated in the region of tetraploid and/or hypertetraploid (Fig. 4). Tumorgenecity to the nude mouce was 40% (2/5) and latency period to grow into a tumor of 5mm diameter ranged from 42 to 98 days at an inoculation of 5×10^5 cells.

1) *Morphology of UMK-1*

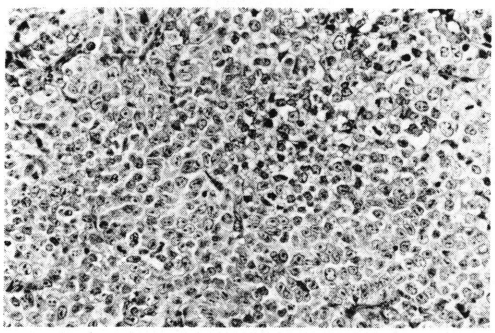


Fig. 5. Microscopic feuture of tumor appeared after transplanted UMK-1 cells to nude mouse.

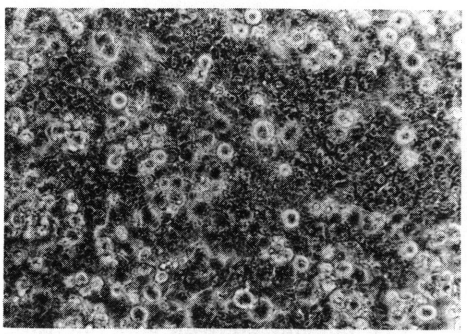


Fig. 6. Microscopic feuture of line 112.

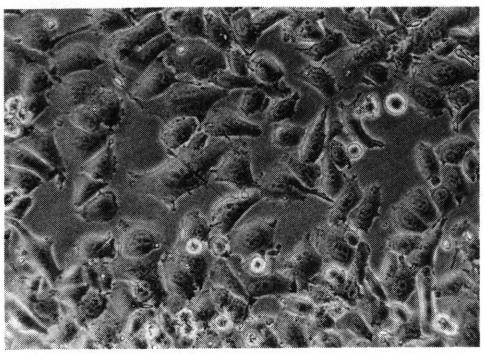


Fig. 7. Microscopic feuture of line 118.

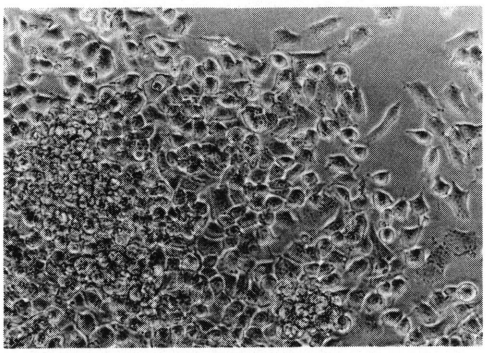


Fig. 8. Microscopic feuture of line 287.

Table 4. Growth caracteristics of UMK-1 and clones

	Doubling time (hours)	Saturation density ($\times 10^5$/cm^2)	Cloning efficiency (%)
UNK-1	28.0	6.4	26.7
101	38.6	5.7	20.7
112	41.0	4.6	10.9
118	33.8	3.5	23.9
201	31.3	4.8	35.8
206	32.5	4.3	33.3
271	43.7	6.7	63.6
279	28.7	3.9	61.5
287	35.6	6.8	49.0

The parental P-15 cell population composed of various cells in size, arranging like a paving stone. When it had become confluent, there was a tendency for the cells to be piled up (Fig. 3). Unclearness of the borders of line 112 has become visualized (Fig. 6). Line 118 was composed of the largest cells among the 200 kinds of clones separated in this series, demonstrating less pile up of the cells (Fig. 7). Fig 8 showed the minimum sized cell of line 287. In addition, the intercellular borders were kept clear.

2) *Growth caracteristicus of UMK-I and clones* (Table 4)

The doubling time of each clone varied from 28.7 hours of line 279 to 43.7 hours of line 271 clone. In the parent cell line of UMK-1 it was shorter as being 28.0 hours. The nadir of saturation density was 3.5×10^6/cm^2 in line 118 which possessed the largest cytoplasma, showing less pile formation of cells. In contrast, it was a minimum of 6.8×10^5/cm^2 in line 287 clone which contained the smallest cytoplasma.

Cloning efficiency in agar showed the maximum of 63.6% in line 271 clone and the

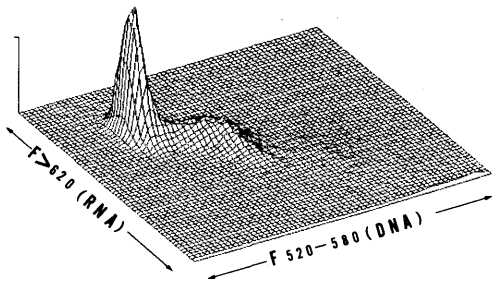


Fig. 9. Computer plotted three dimensional histogram of UMK-1.

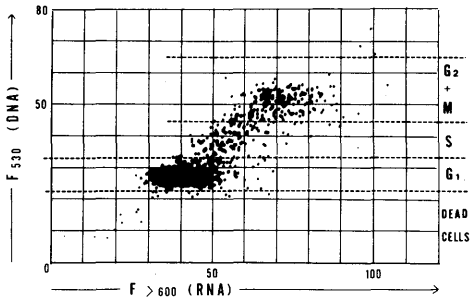


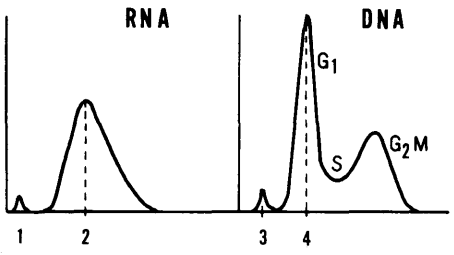
Fig. 10. DNA and RNA dot plot diagram of UMK-1.

minimum of 10.9% in line 112, such in UMK-1 being 26.7%.

3) *DNA RNA analysis*

Fig. 9 showed the three dimention histogram of DNA and RNA of 3×10^4 UMK-1. The highest peak existed in G_1, showing a two fold increase in G_1, the second peak in G_2 and the ridge between G_1 and G_2 lay in S phase.

Accurate evaluation of DNA measurement in each stage of cell cycle was made by a Dot-plot exhibition as shown in Fig. 11, 12. Table 5 illustrated sizes of the cells,



1 RNA peak of lymphocytes
2 RNA peak of cancer cells
3 DNA peak of lymphocytes
4 DNA peak of Cancer cells
※ PI=(S+G_2M) (G_1+S+G_2M)

Fig. 11. DNA and RNA analysis

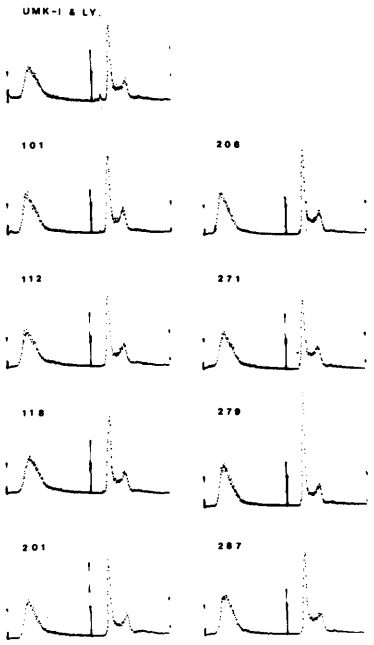


Fig. 12. DNA and RNA histogram of UMK-1 and eight clones.

Table 5. DNA, RNA analysis of UMK-1 and Clones

	Size peak	DNA peak	RNA peak	PI
Lymphocyte		27	5	
UMK-1	69	58	64	41.9
101	70	58	62	42.9
112	60	58	59	44.8
118	78	58	70	44.5
201	68	60	65	42.7
206	69	56	55	43.3
271	65	54	64	43.8
279	66	53	61	35.3
287	57	58	63	44.7

peak DNA and RNA values and proliferative indices (PI) respectively. The sizes of the cells varied from 60 to 78. When those of the parent cell line of UMK-1 were normalized to 1.0 of the control, these ranged from 0.87 to 1.13.

Peak DNA values were a range of 53 to 60, 1.96 to 2.2 times as high as compared with 27 of the lymphocyte in normal humans. These values were almost the same with 90 to 98 of the chromosome number of UMK-1, 1.95 to 2.13 times higher than those in normal humans.

the lymphocyte values varied from 55 to 70, 11 to 14 fold as high as compared with 5 of peak RNA in normal humans. Proliferative index showed 42.7 to 44.7% with no wide variation, being as low as 35.3% in line 279 clone.

Drug sensitivity

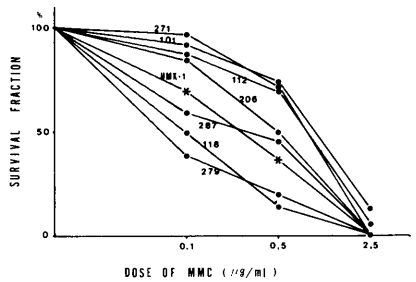
1) *Regrowth assay* (Fig. 13)

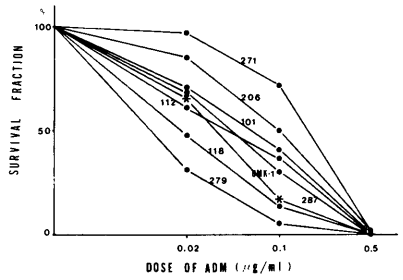
Cell line 118 and 279 revealed high drug sensitivity to MMC. In contrast, line 271, 101 and 112 were resistant although drug sensitivity of UMK-1 showed a medium. In contrast, cell 287 took a medium drug sensitivity to MMC but resistance to ADM. Line 118 and 279 were highly sensitive to CDDP whereas line 271 was resistant.

Average sensitivity to these drugs was seen in UMK-1 which was a collection of each clone.

2) *Colony forming assay* (Fig. 14)

Line 118, 279 and 287 were highly sensitive to MMC. In contrast, line 271, 112 and 101 were equally resistant. Line 279, 118 and 287 were highly sensitive to ADM, whereas line 101 271 and 2.0 were equally resistant. Line 118 and 279 were highly sensitive to CDDP and line 206 and 271 were equally resistant.





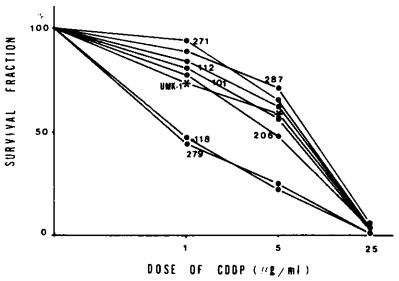
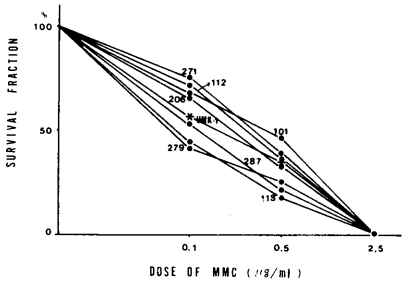
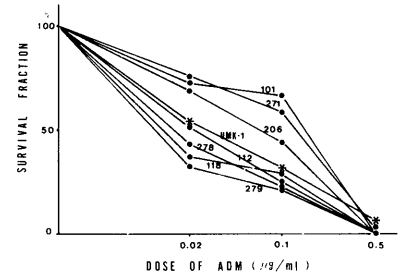


Fig. 13. Dose response curve of UMK-1 and clones by regrowth assay.





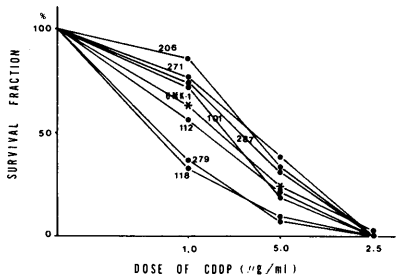


Fig. 14. Dose response curve of UMK-1 and clones by colony forming assay.

3) *Evaluation of* ED_{50} (Fig. 15)

ED_{50} was calculated with use of regrowth assay and colony formig assay methods. ED_{50} to MMC in line 101 assessed by regrowth assay method was a maximum of 0.96μg/ml and that in line 279 was a minimum of 0.065μg/ml, about 15 times lower. According to ED_{50} assessment by colony forming assay method, line 101 was a maximum of 0.38μg/ml, line 279 a minimum of 0.07μg/ml, making a 5.2 fold difference.

The drug sensitivities calculated by both assays showed a similar pattern in spite of lower values represented by colony forming assay method rather than by ragrowth one. The drug sensitivity to ADM by regrowth assay method a maximum of 0.16 μg/ml was in line 271 and a minimum of 0.011 μg/ml in line 279, which was 15 times lower.

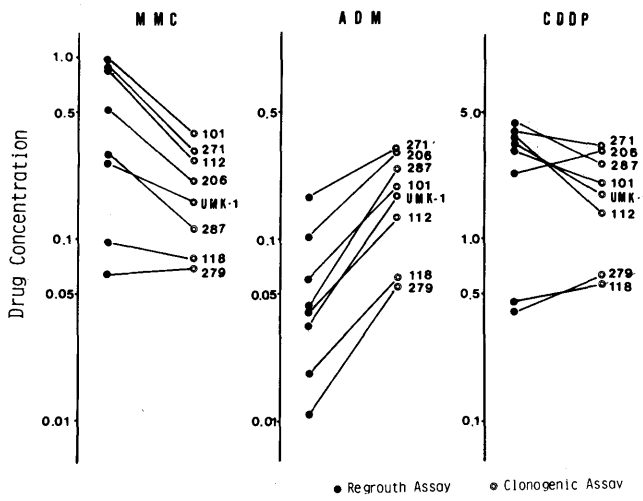


Fig. 15. ED_{50} of UMK-1 and clones.

The drug sensitivity to ADM by colony forming assay method was a maximum of 0.3 μg/ml in line 271, a minimum of 0.06 μg/ml in line 279, making a 5 times difference. Those of the other clones revealed the medium in parallel with both assays although there was a tendency for drug sensitivity assessed by colony forming assay to be higher than that by regrowth one.

The drng sensitivity to CDDP assessed by regrowth assay was a maximum of 4.2 μg/ml in line 287 and a minimum of 0.39 μg/ml in line 279, although about 11 times difference between them was found, there was no similar pattern between both assays

DISCUSSION

It is well known that histologic features vary at different parts of the tumor itself as well as recurrent and metastatic tumor masses. In the clinical course with administration of the anticancer drugs against progression of the tumor, the effectiveness of drugs used has not infrequently become disappeared although it was apparent at an initial administration. It is now obvious that cancer cells are not uniform and are composed of heterogenous cells with respect to morphology and drug sensitivity. One must take it into consideration that cellular heterogeneity of carcinoma influences on the effect of anticancer drugs. Fresh cancer cells derived from human carcinoma is needed for assessment of cellular heterogeneity. In this present study, P-10 cells from human gastric cancer were used. It is more likely that the established cells histologically reveal poor differentiation

in nude mouse against the original tumor with moderate differentiation. The reason for poor differentiation of the cells used herein is that the established cells are successfully propagated and inherently originated from poorly differentiated original cells. It is emphasized to mention that even the established cells are not so uniform as they represent conspicuous cellular heterogeneity.

It is reported that human cancer cells reveal karyotypes ranging from 40 to 90 of the modal chromosome number in which 40 to 50 are called near-diploid and 70 to 90, near -tetraploid. In human cencer cells, the chromosome number of less than 30 is not frequently noticed.[17] WAI-KWAN noted that the 6 clones separated from human malignant melanoma revealed hypodiploid or near-tetraploid patterns.[16] Based on an evaluation of chromosome patterns of 50 Rono-Sarcomas in rats, MITTLEMAN clarified that diploid pattern was 80% in frequency and one side line or more were seen in 50%.[18] RALY also reported that the clones isolated from ascitic lymphoma in mice displayed the varying patterns of pseudodiploid, hyperdiploid and hypotetraploid. And also determination of the chromosome number of parent cell line revealed very significant difference ranging from 37 to 46 with a peak of 42, showing a range of hypodiploid to tetraploid cells.[9][19] In UMK -1 line established in this study, the chromosome number ranged from 90 to 98 with a narrow range of tetraploid to hypertetraploid without any side lines.

Since SANFORD had successfully achieved clonal propagation of L-cell in 1948[20], various devices have been developed to separate clones such as microdrip method as discribed by LWOFF,[21] heat-killing method by GOLDSTEIN,[22] colony-forming method by PUCK,[23] agar suspension culture method by MACPHERSON,[24] Replica method by GOLDSBY[25] and others.[26]

In this study, the limited dilution method was used. The eight clones among 200 clones were selected for drug sensitivity test. As already reported in in-vitro culture, the cell lines derived from epithelial origin were growing as an adherent type. A difference in the morphologic aspect between different clones was clearly made.[27][28]

Much has been said that although doubling time of human tumors generally varys from 19.7 hours to 632, that of cultured cells almost rangs from 20 hours to 72.[29] Of interest is the fact that doubling time of UMK-1 of exponential phase is far shorter rather than is expected. It is because cell interaction plays a role in promoting the cell cycle process. There are many influential factors on doubling time and cloning efficiency such as cell metabolism, optimal pH and temperature of the culture medium.

DNA analysis is widely applied in the field of cell cycle study since 1970.[30] Assessment of ploidy in also prevailing to clarify the cellular heterogeneity.[31] It is

recognized that the cellular DNA content is consistent with the chromosome number.[32] VINDELφV reported that DNA patterns in the 38 metastatic tumors arising from the 30 human small cell lung cancers revealed hypo-near tetraploid in 73%, hypotriploid in 12% and hypodiploid in 15%. And he detected the two peaks, lying in 20% on the DNA histogram.[33] NOMURA also cited that DNA patterns obtained from the 18 malignant gliomas of brain tumor varied with a range of hypodiploid to hypertetraploid.[34][35][36]

It is often believed that RNA peak values imply malignant potential of the tumors.[37][38] In this series, RNA peak value did not necessarily correlate with proliferative index, cell size, doubling time. It is clear that there are significant differences in drug sensitivity between cancer cells in the identical cancer tumor tissue. It is well known that the tumor exhibits varying variety of DNA patterns and the sensitivity to anticancer drugs is greatly variable. SIRACKY reported that drug sensitivity of the tumors to 5FU, MTX and VCR taken from colon cancers in 2 patients and ovary cancer in 1 varys among them.[40][41][8] PÄLYI also defined that chemosensitivity of the clones derived from the parent cell line of ascitic lymphoma in mice to ADM and VCR is not similar but that of parent cell line is in - between the two. This finding was consistent with a result in the present study. WAI-KWAN reported that ED_{50} assessment by using colonogenic assay method was benefical to know anticancer drug sensitivity of human malignant glioma.[12]

In this series, it was defined that drug sensitivity to MMC, ADM and CDDP was very different in each of the clones isolated from ascitic fluid of gastric cancer in humans. One possible explanation is that one is due to mutation of spontaneously acquired drug resistance and the other is due to loss of drug sensitivity incidentally induced by repeated administrations of drugs in the whole clones.[6] It is emphasized to say that induction of drug resistance is associated with selection of the cells that is tolerate to the drugs previously adminstered.[7] Such offers insight into significance of heterogeneity of cancer cells.

The present study also provides essential knowledge for heterogeneity of human gastric cancer cells. One must be aware of appropriate selection of anticancer drugs, considering drug sensitivity and recognizing its resistance to cancer cells.

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