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Originals

Studies on Relationship between nbl (S3a) mRNA Levels, Cell Density, and Chemosensitivity in Various Rat and Human Cell Lines

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SUMMARY

Cytocidal effects of anti - cancer drugs such as cisplatin, 5 - fluorouracil (5 - FU), and actinomycin D on various cell lines were investigated using Alamar Blue. Actinomycin D, cisplatin and 5 - FU showed significant cytocidal effects at 1 μg/ml, 10 μg/ml and 50 μg/ml respectively, on cultured cells after 24 h incubation at 37℃. The cytocidal effects increased at a low cell density but reduced at a high cell density in various cell lines. The expression level of one of the ribosomal proteins, nbl (S3a), was measured by northern blot analysis and real time - polymerase chain reaction. The relationship between the nbl mRNA level and drug effects was then investigated for 8 rat and 10 human cell lines. An inverse correlation between chemosensitivity and nbl mRNA level was observed in both rat and human cell lines.

Key Words : Cultured cells, Chemosensitivity, nbl (S3a), Cell density, Alamar Blue

INTRODUCTION

Four therapeutic interventions ; namely surgical operation, radiotherapy, chemotherapy and immunotherapy, as well as their combinations, are used to treat cancer. However, each of these therapeutic methods has advantage and disadvantage. Although current methods of cancer treatment have increased the survival of many cancer patients, new and less toxic treatments are still required. The method of chemotherapy is perhaps the simplest and most applicable for the cancers locating in any place where surgical operations can not be undertaken. However, chemotherapy sometimes induces serious side effects, because the drug treatment affects not only cancers but also normal tissues, particularly actively growing cells. In addition, drugs are not always effective on every cancer. Therefore, chemosensitivity using the cultured tissues or cells derived from patient cancers are often examined before beginning chemotherapy, although this method is not always applicable for all cancers.

In advanced human breast cancer, there is no correlation between p53 or Bcl - 2 protein expression and chemosensitivity, although these proteins are involved in regulating apoptosis[1]. On the other hand, expression levels of p53 and p21 are inversely correlated with inhibitory effects of anti - cancer drugs on gastric and colon cancers[2].

Relationship nbl (S3a) activity and the degree of apoptosis in cultured cells has been reported[3]. The reduction of nbl activity induces apoptosis. These results suggest that there may be a relationship between nbl activity and the drug sensitivity in cultured cells. The existence of this relationship could help us to choose suitable anti - cancer drugs for chemotherapy. To examine this possibility, the present study was initiated, and 8 rat cell lines transformed with virus, virus genes or carcinogens, and 10 human cell lines derived from various cancers were exam-

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ined. Well-established drugs such as actinomycin D, cisplatin[4] and 5-fluorouracil (5-FU)[5,6], were used, because their functions are well characterized. In addition, cisplatin[7,8] and 5-FU[9~11] are clinically used as anti-cancer drugs. The expression level of nbl was estimated by northern blot analysis and real time-polymerase chain reaction (PCR).

MATERIALS AND METHODS

Cell culture

Rat fibroblasts (3Y1-B1-6) were derived from rat fetuses[12], and the cell line was transformed with adenovirus 12 and adenovirus DNA containing the ElA region to generate the cell lines, Ad12-3Y1-Z11[13] and E1A-3Y1-1[14], respectively. Similarly, the 3Y1-B1-6 cell line was transformed with polyoma and Rous sarcoma viruses, and Py-3Y1-S2[13] SR-3Y1-2[13], respectively, were obtained. In addition, the original cell line was also transformed with a chemical carcinogen, nitrosoguanidine, and NG-3Y1-T6R[15], NG-3Y1-T380[15] and NG-3Y1-T15L[15] were established. These cell lines were supplied by the Japanese Cancer Research Bank.

Human urinary bladder carcinoma cell lines, HUB-6 and HUB-15[16], were kindly supplied by Prof. Takahide Kakuya of Utsunomiya University. The KBS-1 cell line, derived from human nasal cancer (unpublished), was kindly supplied by Dr. Shuichi Hashimoto of Meiji Nyuugyo Co., Ltd. Human colon cancer cell lines, CW-2[17] and PMF-1[18], were obtained from the Riken Cell Bank. OSC-19[19], OSC-20[20], SAS-L1[21], and SAS-H1[21], derived from human squamous cell carcinoma were kindly supplied by Prof. Yutaka Imai of Dokkyo University School of Medicine. KA-1 and KA-2 cells were established at Dokkyo University School of Medicine from colon cancers (Akimoto, unpublished); these cells were cultured continuously in Dulbecco's modified Eagle minimum essential medium (DM-MEM) containing 10% fetal bovine serum in plastic culture flasks as the keeping cells.

Drug effects

Actinomycin D, cisplatin and 5-FU were purchased from Invitrogen Corp. (Carlsbad, CA, USA), Bristol Pharmaceutical Co., Ltd. (Tokyo, Japan) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. Trypsinized cells were plated into 96-well culture plates at different cell population densities and cultured for 2-3 days at 37℃, and then further cultured in the presence of the drug for one day. To estimate the number of viable cells, the colorimetric method using Alamar Blue, Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan)[22] was used, according to the protocol recommended by the manufacturer. The cells were incubated with 10% Alamar blue for 3-4 hrs at 37℃, and then the emission at 590 nm based on 544 nm excitation was measured with a Fluoroskan Ascent CF (Labsystems).

Nbl mRNA expression

Cells cultured in 60-mm or 100-mm plastic dishes were harvested, and the total RNA was extracted and purified with ISOGEN purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), according to the method of Chomezynsky and Sacchi[23]. To measure nbl mRNA, northern blot analysis was carried out according to a method described previously[24]. A part of the total RNA (10 μg) extracted was used for northern blot analysis. The probes for hybridization with mRNA on the membrane were prepared from PCR products. PCR primers were as follows : human and rat nbl forward primer ; 5′-GTCTCAAGGGTCGTGTGTTTG-3′, human and rat nbl reverse primer ; 5′-TCTGCACCTCTCGGGTCATGA-3′, human GAPDH forward primer ; 5′-CATGGTCTACATGTTCCAGT-3′, human GAPDH reverse primer ; 5′-GGCTAAGCAGTTGGTGGTGC-3′, rat GAPDH forward primer ; 5′-CATGGTCTACATGTTCCAGT-3′, and rat GAPDH reverse primer ; 5′-GGCTAAGCAGTTGGTGGTGC-3′. These primers were prepared by ESPEC OLIGO SERVICE (Tsukuba, Ibaraki, Japan). PCR products were purified with rapid PCR purification systems, purchased from Marligen Bioscience Inc. (Ijamsville, MD, USA). The products were labeled with alpha ^{32}P-dCTP (3,000 Ci/mmol), purchased from Amersham Life Science (Backinghamshire, England), using Megaprimer™ DNA labeling systems, Amersham Life Science. The amount of the labeled probe on the filter was measured with a BAS2000, Fuji Film, (Tokyo, Japan).

To measure nbl mRNA, real time-PCR was Also carried out with a Gene Amp 5700, Applied Biosystems (Tokyo, Japan). Taqman probes of rat nbl (5′-ACACGACCCTTGAGGCCATCTGATG-3′) were designed and obtained from Applied Biosystems. Other primers were as follows : nbl forward primer, 5′-GCTCCAGCAAT-

GTTCAATATTAGGA-3′ ; nbl reverse primer, 5′-TCATCGTTCTGTAGATCTGCAAGAC-3′.

Cell cycle analysis

To measure the population of the cell cycle, FACS caliber purchased from Becton Dickinson was used. Monolayer cells were trypsinized to obtain single cell suspensions. The cells were then incubated with the Cycle Test™ Plus DNA Reagent (Becton Dickinson, San Jose, CA, USA) according to the protocol recommended by the manufactures, and the cell cycle profile was analyzed, using a FACS calibur (Becton Dickinson).

RESULTS

Effects of cell density on the cytocidal effects of Actinomycin D

A preliminary study suggested that the sensitivity of cultured cells to actinomycin D depended on the cell densities. To confirm this, the cytocidal effects of actinomycin D was investigated at three different cell densities. At 1.0 μg/ml, the effects increased as the cell population density decreased (Fig. 1). In the experiments using a very high cell density, no significant cytocidal effect was observed (data not shown). Thus, the sensitivity of cultured cells to actinomycin D depended on the cell population density.

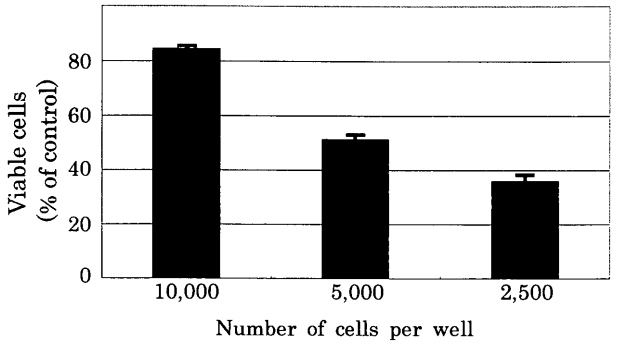

Fig. 1 Cell density effects on Actinomycin D sensitivity in cultured cells. KBS-1 cells were plated into 96-well plates. The following inoculum sizes were used ; 10,000, 5,000 and 2,500 cells/well. Cells were cultured for 2 days and then treated with Actinomycin D at 1.0 μg/ml for 24 hrs. The number of viable cells was estimated by the Alamar Blue assay. The value is the mean ± S.D. of 8 wells (see Materials and Methods).

Dose response effects of drugs on cells

Cultured PMF cells were incubated with actinomycin D at different concentrations. Actinomycin D showed significant cytocidal effects at 1 μg/ml, and similar effect were observed even at 0.5 μg/ml and even at 0.1 μg/ml (Fig. 2, upper panel). In other cells, the concentration of Actinomycin D needed to demonstrate a significant effect was 1.0 μg/ml. Therefore, 1.0 μg/ml of Actinomycin D was used throughout subsequent experiments.

Cisplatin, showed obvious cytocidal effects at 10 μg/ml, whereas at 1 μg/ml it showed no significant effect (Fig. 2, middle panel). Even though 5 μg/ml of cisplatin showed significant effects on some cell lines, it was not sufficient for the other. Therefore, the concentration of cisplatin was kept at 10 μg/ml throughout subsequent experiments.

Cytocidal effects of 5-FU were also investigated at different concentrations. This drug showed no significant effect even at 10～25 μg/ml while at 50 μg/ml it showed cytocidal effects (Fig. 2, lower panel). To obtain sufficient cytocidal effects, the concentration of 5-FU was kept at 50μg/ml.

Effects of cell density on drug sensitivity of various cells

In various cell lines, the drug effects were examined at three different cell densities (Fig. 3). The largest effects of actinomycin D and cisplatin were observed in Ad-3Y1-Z11 cells. The effect of 5-FU was almost the same in 8 rat cell lines. All three drugs, showed their largest cytocidal effects at the lowest cell population density in almost all rat cell lines (Fig. 3). On the other hand, the smallest effect was observed at the highest cell density.

The cytocidal effects of three drugs differed among 10 human cell lines. The three drugs also showed their largest cytocidal effects at the lowest cell population density in 10 human cell lines, while the smallest effect was observed at the highest cell population density (Fig. 4).

Effects of Alamar Blue concentration

When the concentration of the substrate, Alamar Blue, was insufficient, a cell number reduced by drugs cannot be detected because the remaining live cells are sufficient for complete consumption of the substrate. In fact, no significant effect of the drugs was observed at the highest cell population density. To determine whether this small effect at the highest cell population density is due to an insufficiency of substrate, the amount of the substrate was

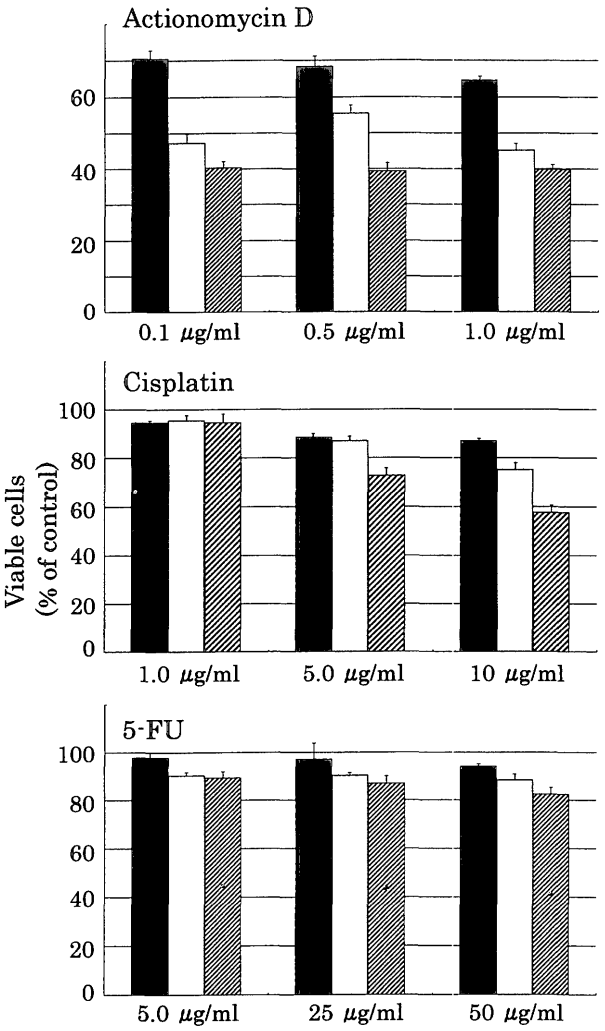

Fig. 2 Dose-response effects of the drugs on cultured cells. PMF cells were plated onto a 96-well plate. Three inoculum sizes were used ; 10,000 (solid bar), 5,000 (gray bar) and 2,500 (hatched bar) cells/well. Cells were cultured for 2 days and then treated with each drug at different concentrations for 24 hrs. The value is the mean ± S.D. of 8 wells.

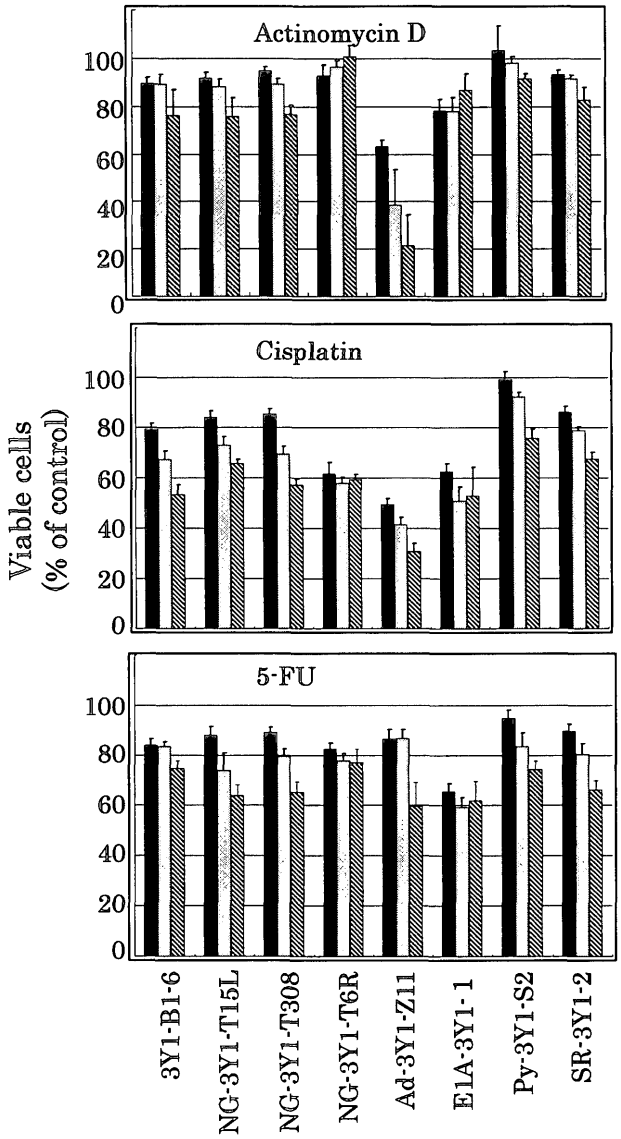

Fig. 3 Drug sensitivities of various rat cell lines at three different cell densities. Cells were inoculated at 10,000 (solid bar), 5,000 (gray bar) and 2,500 (hatched bar) cells/well in a 96-well plate and cultured for 2-3 days. Next, cells were treated with each drug for 24 hrs and then incubated with Alamar blue for 3-4 hrs at 37 The value is the mean ± S.D. of 8 wells.

increased by two-fold. However, no significant effect of the Alamar Blue concentration was observed (Fig. 5). Thus, the concentration of Alamar Blue used in the study was appropriate for cell number assay using the 96-well plate.

Cell cycle

Cell population density should affect cell cycle progression. To investigate this relationship, the cell cycle was analyzed by FACS. Two cell lines, 3Y1-B1-6 and PMF, were cultured at different cell population densities, and then a single cell suspension was prepared by treating the monolayer cells with trypsin. Both cell lines showed typical cell cycle patterns as shown in Fig. 6. The main cell population was in G_1/G_0 phase in both cell lines, even at different cell densities.

Quantitative data are shown in Table 1. At a high cell density, the population of the G_1/G_0 phase larger than that at a low cell density, while the populations of the G_2/M and S phases decreased in both cell lines. These results indicate that DNA synthesis is partially inhibited at

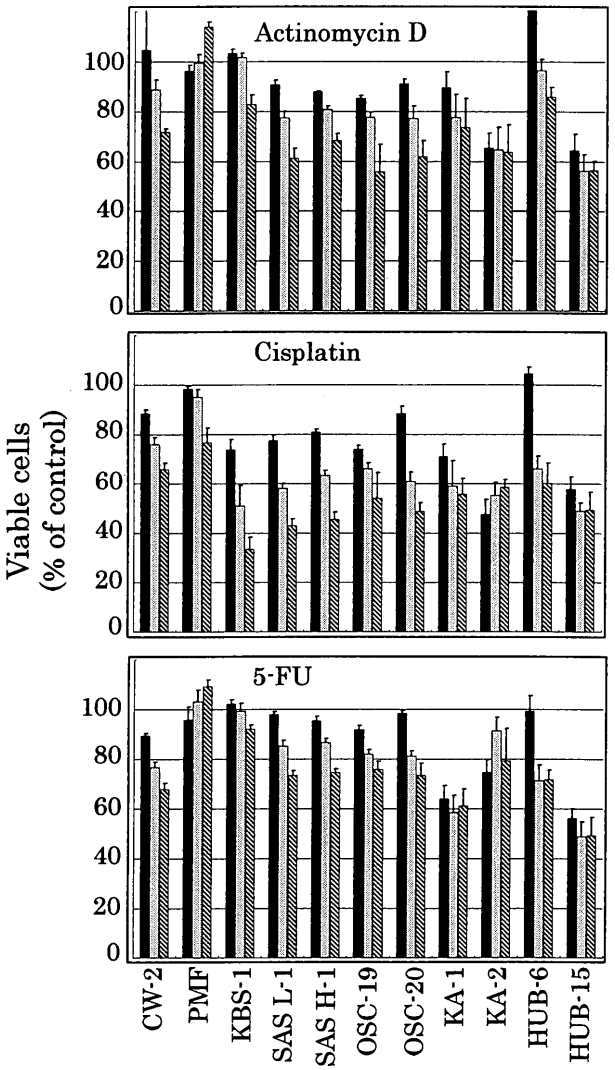

Fig. 4 Drug sensitivities of various human cell lines at three different cell densities. See legend for Fig. 3.

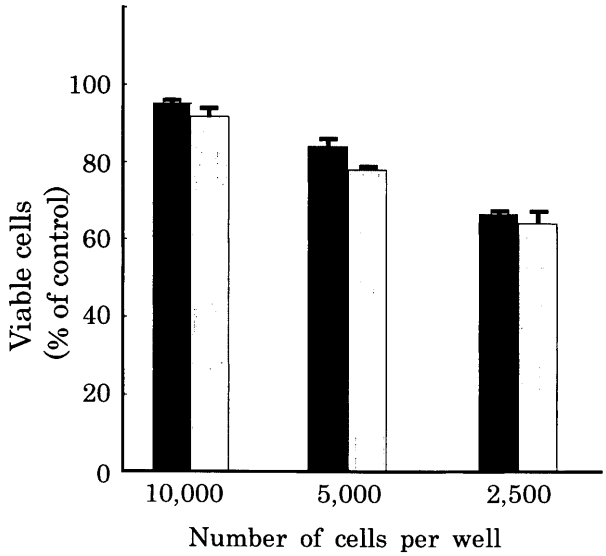

Fig. 5 Effect of Alamar blue concentration on cell assay. Cells were inoculated at three different cell densities ; 10,000, 5,000 and 2,500 cells/well in a 96-well plate, and further cultured for 2 days. Cells treated with Actinomycin D at 1.0 μg/ml for 24 hrs were incubated with Alamar blue at two different concentrations, 10 μl/100 μl (solid bar) and 20 μl/100 μl (gray bar), for 4 hrs at 37℃. The value is the mean ± S.D. of 8 wells.

a high cell density ; and that this inhibitory effect induces a decrease in the population of the G_2/M phase.

Northern blot analysis

Fig. 7-A shows the result of northern blot analysis of the rat cell lines.

Densities of the nbl bands differed among the cell lines, whereas those of

GAPDH bands were relatively constant. Similar results were obtained from human cell lines (Fig. 7-B). In many cell lines, nbl mRNA levels increased at high cell population densities, although some cell lines showed an opposite result.

To normalize the results of nbl mRNA levels, radioactivity corresponding to the nbl band was divided by the radioactivity correspondings to the GAPDH band of the same cell line. Then, each value was divided with a value of the standard cell lines, such as 3Y1-B1-6 or CW-2 (Table 2 and 3). Thus, nbl mRNA levels in different cell lines were compared with those of the standard cell lines. Nbl mRNA levels determined by real time-PCR is also shown in Table 3.

Relationship between nbl activity and drug sensitivity

The mRNA of nbl was measured by northern blot analysis as shown in Fig. 7, and Tables 2 and 3, while the drug effects were measured by Alamar Blue assay based on mitochondrial activity (Figs. 3 and 4). In rat cells, a weak relationship between nbl mRNA levels and chemosensitivity for actinomycin D and cisplatin was observed at different cell population densities, although no statistically significant relationship was obtained (Fig. 8). On the other hand, chemosensitivity of rat cells for 5-FU was independent of nbl mRNA levels at low or middle cell population densities, while a very weak relationship was apparently observed at a high cell population density. Similarly, human cell lines were also examined

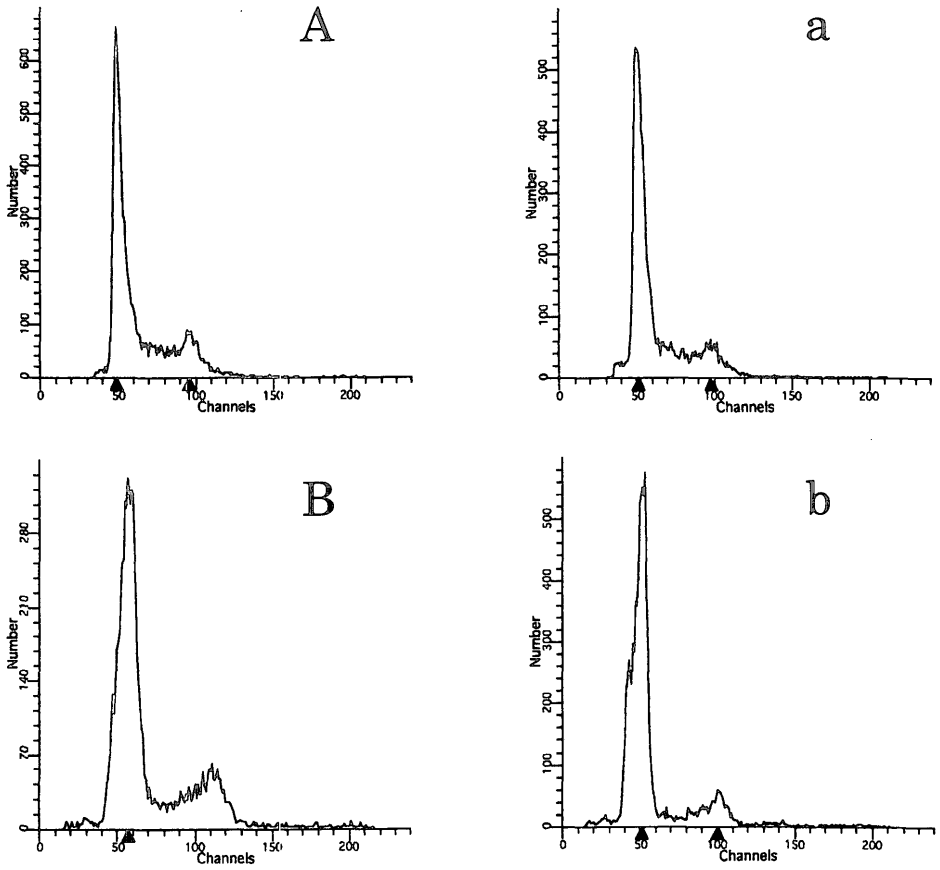

Fig. 6 Flow cytometry of cultured cells. Cultured Rat 3Y1 - B1 - 6, and human PMF cells were inoculated into 30 - mm culture dishes (A and B) and 100 - mm culture dishes (a and b) at the same cell number. Cells were cultured until complete monolayers were formed in 30 - mm dishes, while the cells in 100 - mm dishes had incomplete monolayers. The cell numbers were described in the legends of Table 1.

Table 1 Cell cycle analysis by FACS.

Cell line	Cell density	G_1/G_0	G_2/M	S	G_2/G_1
3Y1 - B1 - 6	Low	70.10	10.46	19.44	2.00
3Y1 - B1 - 6	High	86.89	5.46	7.64	1.98
PMF	Low	54.59	2.93	42.48	2.05
PMF	High	64.73	1.61	33.65	2.03

These values were automatically calculated by the FACS machine. The cell population densities of 3Y1 - B1 - 6 at low and high densities were 3.9×10^4 cells/cm^2 and 8.4×10^4 cells/cm^2, respectively, and those of PMF were 6.3×10^4 cells/cm^2 and 15.9×10^4 cells/cm^2, respectively.

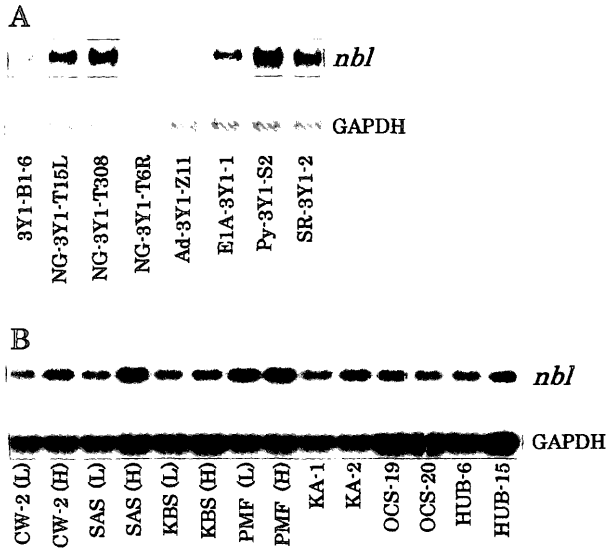

Fig. 7 Northern blot analyses of nbl and GAPDH mRNA. Cells noted as (L) and (H) were inoculated into 100 - mm and 30 - mm dishes, respectively, at the same cell number. Other cells were cultured in 25 - cm^2 culture flasks. A ; rat cells, B ; human cells.

Table 2 nbl mRNA levels in various human cell lines compared with CW-2 cell line.

Cell line	Experiment # 1	Experiment # 2	Experiment # 3	Aver. ± S.D.
CW-2	1	1	1	1
PMF	1.38	1.26	1.82	1.49 ± 0.29
KBS-1	0.60	0.63	0.60	0.61 ± 0.02
SAS L-1	0.93	0.85	1.89	1.22 ± 0.58
SAS H-1	1.16	0.87	1.92	1.32 ± 0.54
OCS-19	0.87	0.69	1.53	1.03 ± 0.44
OCS-20	0.74	0.71	1.56	1.01 ± 0.48
KA-1′	1.16	0.98	2.14	1.43 ± 0.62
KA-2	1.44	1.09	1.55	1.36 ± 0.24
HUB-6	1.02	0.57		0.80
HUB-15	0.81	0.42		0.61

The radioactivity corresponding to nbl mRNA was divided by that of GAPDH, and then each value was divided by the value of CW-2 cells.

Table 3 nbl mRNA levels in various rat cell lines compared with the original cell line.

Cell line	Experiment # 1	Experiment # 2	RT-PCR	Aver. ± S.D.
3Y1-B1-6	1	1	1	1
NG-3Y1-T15L	1.26	1.27	1.19	1.24 ± 0.04
NG-3Y1-T308	1.51	1.22	2.58	1.77 ± 0.72
NG-3Y1-T6R	0.78	1.06	1.12	0.99 ± 0.18
Ad-3Y1-Z11	0.58	0.70	0.91	0.73 ± 0.17
E1A-3Y1-	0.83	1.02	0.86	0.90 ± 0.10
Py-3Y1-2	1.34	0.70	0.84	0.96 ± 0.34
SR-3Y1-2	1.17	0.48	1.59	1.08 ± 0.56

The data presented # 1 and # 2 were obtained by northern blotting analysis. The value of nbl mRNA /GAPDH mRNA of each cell line was divided by the value of 3Y1-B1-6 cells.

(Fig. 9). The cytocidal effects of actinomycin D, cisplatin and 5-FU on human cell lines decreased with increasing nbl mRNA levels at any cell density. An inverse relationship between nbl mRNA levels and chemosensitivity for these drugs was weakly observed at different cell population densities, although no statistically significant relationship was obtained.

DISCUSSION

Apoptosis is induced, when high nbl activity is reduced by actinomycin D in HL-60 cells[3]. In this case, not only the high nbl activity at the initial stage, but also a reduction of nbl activity by a drug are necessary to induce apoptosis. On the other hand, in the present study, more cell death was induced more dramatically at lower nbl mRNA levels (Fig. 7). Nbl mRNA levels depended on the cell population density and was relatively high at high cell population densities in many cell lines ; whereas the cytocidal effects of the drugs examined here disappeared at high cell population densities (Figs. 3 and 4). Thus, it seems that cells expressing the lower levels of nbl mRNA levels are the more sensitive to drugs such as actinomycin

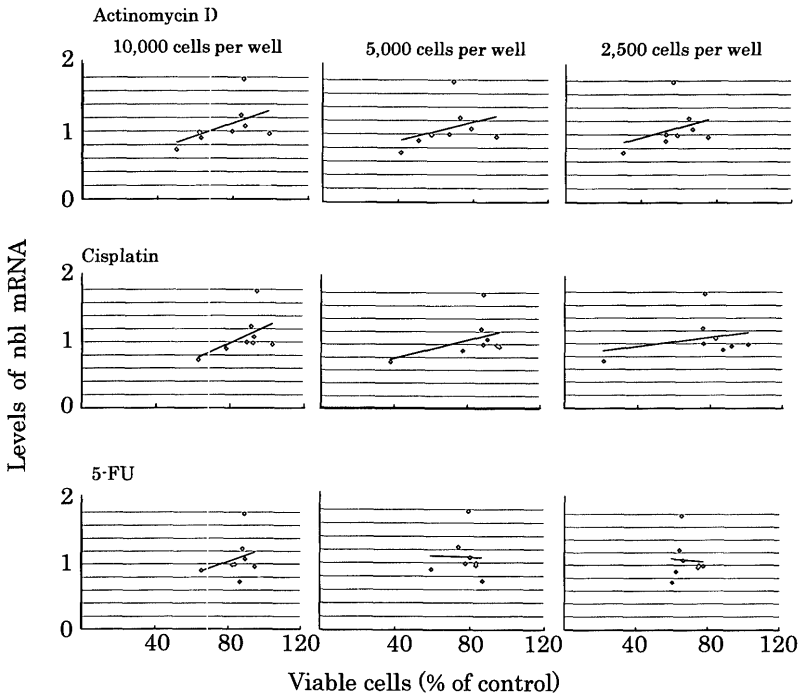

Fig. 8 Relationships between the ratio of nbl mRNA/GAPDH mRNA and drug sensitivity at different cell densities in various rat cell lines.

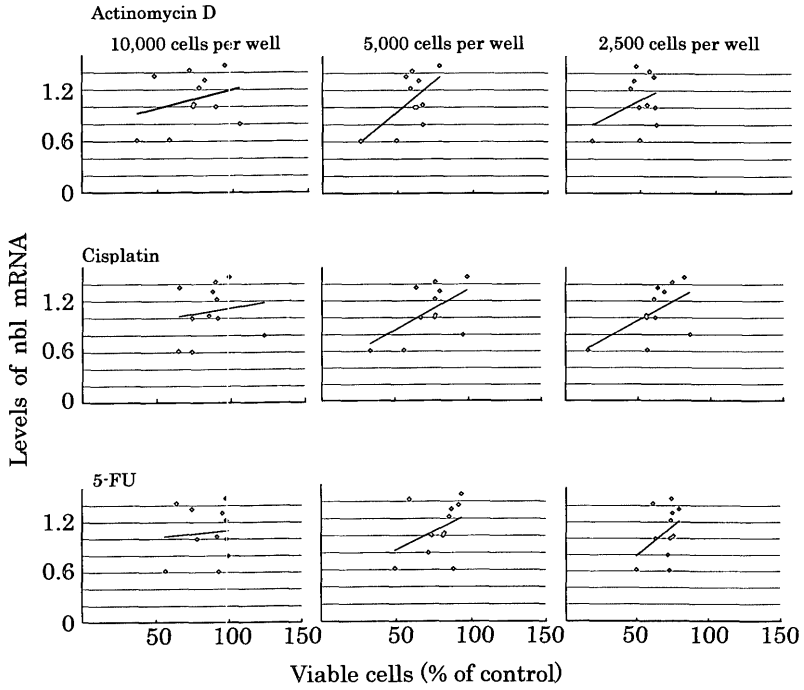

Fig. 9 Relationships between the ratio of nbl mRNA/GAPDH mRNA and drug sensitivity at different cell densities in various human cell lines.

D, cisplatin and 5 - FU.

Cisplatin and 5 - FU are well - known anti - cancer drugs that inhibit DNA Synthesis, while actinomycin D is known as a drug that inhibits transcription and induces apoptosis in cultured cells[25~28]. This mechanism of actinomycin D is based on the reduction of nbl activity induced by the inhibition of nbl mRNA synthesis. In the present study, these drugs showed similar cytocidal effects on tested cul-

tured cells, although their mechanisms of action were different. Thus, cisplatin and 5-FU might have reduced nbl activity in cultured cells.

In the present study, the cytocidal effects of the drugs were shown to be decreased with increasing cell population densities. When the cell cycle progression is almost stopped at a high cell population density, it is reasonable that the cytocidal effects of the drugs which inhibit DNA synthesis can not be observed, because the resting G_0 cells do not go though DNA synthesis. However, FACS analysis showed that the main populations of cells were in G_1/G_0 and S phases, even at a high cell density. These results suggest that the decrease in the cytocidal effect of the drugs at a high cell density seems to be due to factors other rather than DNA synthesis inhibition. Similarly, it was reported that cancer cells in the cell cycle are more rapidly killed by anti-cancer drugs than cells in a resting phase[29].

The rat cell lines examined in the present study have been used to investigate whether polyanions inhibit cell growth in vitro[30], and cell growth of some cell lines was indeed strongly inhibited. When the present results are compared with earlier ones, there is no relationship between nbl activity and the effects of polyanions. On the other hand, we observed a relationship between nbl mRNA levels and drug sensitivity. These differences might be based on the difference in the active sites of the compounds. Anti-cancer drugs such as actinomycin D, cisplatin and 5-FU affect DNA or RNA syntheses, while polyanions seem to affect the plasma membrane.

At present, we have no clinical data that could indicate a relationship between nbl mRNA levels and the chemotherapeutic effects on cancer. This study suggests that cancer cells that express low nbl activity are sensitive to drugs such as actinomycin D, cisplatin and 5-FU. Thus, chemotherapy seems to be more applicable to cancers with low levels of nbl mRNA, but not to cancers with high nbl mRNA levels. In addition, chemotherapy seems to be more appropriate for cancers consisting of a relatively small number of cells. The present results suggest that further, more precise, investigations on the relationship between nbl activity and the chemosensitivity of cancers may be helpful for determining the treatment strategy in each cancer patient.

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