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Selection of Radioresistant Cells by Vitamin A Deficiency in a Small Cell Lung Cancer Cell Line

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Radiation sensitivity of a human small cell lung cancer cell line, Lu-134-B cells, cultured in serum-supplemented medium and of cells transferred to and cultured in delipidized serum-supplemented (vitamin A-deficient) medium was studied. The cells cultured in serum-supplemented medium showed the phenotype of classic small cell lung cancer sensitive to radiation, while cells transferred to delipidized serum-supplemented medium showed partial squamous cell differentiation and became resistant to radiation. These results suggest that some small cell lung cancer cells *in vitro* change their morphology and radiosensitivity depending on the culture conditions. The change in radiosensitivity was reproducible, and was not reversible by culture of the radioresistant cells in delipidized serum-supplemented medium with addition of retinoic acid (vitamin A-sufficient medium) for two months, although squamous cells disappeared. Acquisition of radioresistancy was considered to occur as the result of clonal selective growth in delipidized medium of a minor cell population in the original cell culture, based on a study of chromosome number. It was also found that there was no association of *myc*-family oncogenes with the changes of radiosensitivity in this cell line.

Key words: Small cell lung carcinoma — Culture — Chromosome — Radiosensitivity — Vitamin A

SCLC is a highly malignant human cancer[1-3] and occasionally reveals partial squamous or glandular cell differentiation.[4,5] Morphological change of SCLC to squamous or glandular cells, associated with decreased response to chemotherapy and radiotherapy and shorter survival time, has been observed in patients who received intensive chemotherapy and/or radiotherapy, and eventually were autopsied.[6-8] Cellular morphological changes have also been observed *in vitro* after long-term culture, along with changes in biochemical and biological properties of the tumor cells, such as an increase in radioresistance.[9-11] Some variant SCLC cell lines contain amplified c-*myc*,[12] and transfection of c-*myc* gene to a classic SCLC cell line has also been reported to change the cells to a variant cell type.[13] The mechanisms by which tumors become resistant to chemotherapy or radiotherapy must be clarified. We have reported a cellular morphological change of human SCLC cell line, Lu-134-B, to squamous cells and *vice versa* when the culture medium was changed from vitamin A-sufficient to vitamin A-deficient medium.[14] In order to characterize these cells further, we investigated radiosensitivity, chromosome numbers and *myc*-family oncogene amplification and expression of Lu-134-B cells cultured in SSM (vitamin A-sufficient medium), in DL-SSM (vitamin A-deficient medium) and in DL-SSM with retinoic acid after having been cultured in DL-SSM. It was found that the cells cultured in DL-SSM which changed in morphology from small cells to combined small cells and squamous cells (4%) became radioresistant with changes in modal chromosome numbers but without any change in copy number of *myc*-family oncogenes or c-*myc* mRNA expression. Culture of these cells in DL-SSM with retinoic acid resulted in disappearance of the squamous component but the radioresistancy and the modal number of chromosomes remained unchanged.

The abbreviations used are: SCLC, small cell lung cancer; FCS, fetal calf serum; SSM, serum-supplemented medium (RPMI 1640 and 10% FCS); DL-FCS, delipidized FCS; DL-SSM, delipidized serum-supplemented medium (RPMI 1640 and 10% DL-FCS); AADC, aromatic L-amino acid decarboxylase, NSE, neuron-specific enolase; CK-BB, creatine kinase, brain isoenzyme; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline (137 m*M* NaCl, 3 m*M* KCl and 10m*M* phosphate buffer pH 7.0); TCA, trichloroacetic acid.

MATERIALS AND METHODS

Cell line The cell line used was Lu-134-B established from a xenotransplanted tumor in a nude mouse, which had originated from a primary focus of SCLC as described elsewhere.[15] These cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. These cells show morphological features of small cell carcinoma (intermediate cell type) with high levels of AADC, NSE and CK-BB, and contain dense cored granules ultrastructurally.[15] They showed partial morphological change to squamous cells and *vice versa* without significant changes in the above enzyme activities when the medium was changed from SSM to DL-SSM.[14]

Irradiation experiments Three kinds of cells were used for the present experiments; Lu-134-B cells "in SSM," Lu-134-B cells "in DL-SSM" and Lu-134-B cells "in DL-SSM plus retinoic acid." Lu-134-B cells "in SSM" were of the original cell line, the cells having been cultured in SSM for 4 years; Lu-134-B cells cultured "in DL-SSM" were the cells cultured in SSM and then in DL-SSM for at least eight passages, which changed their morphology to squamous cells in part; and Lu-134-B cells cultured "in DL-SSM plus retinoic acid" were the cells to which retinoic acid at a final concentration of 10^{-7} *M* (dissolved in DMSO) was added after they had been cultured in DL-SSM, showing a reversal of morphology to small cells after more than four passages. DL-FCS was prepared by the method of Rothblat *et al.*[16] RPMI 1640 was purchased from Nissui Co., Ltd., Tokyo, and FCS from Gibco Lab., Grand Island, N.Y. Cells at the logarithmic phase of growth were pipetted gently and divided equally into five flasks. Cells suspended in 5 ml of each medium were irradiated at doses of 0, 3.0, 4.5, 6.0 and 8.0 Gy from ^{60}Co (0.6 Gy/min) at room temperature, then pipetted gently, and one ml of each cell suspension was transferred to new flasks containing 4 ml of fresh medium and cultured in a CO_2 incubator.

Growth curves Every seventh day, 5 ml of fresh medium was added to each flask, then the cell suspension was gently pipetted 2 or 3 times using pipettes with a large hole and 5 ml was transferred to a test tube. Cells in test tubes were washed twice with PBS and centrifuged, and the supernatants were decanted to obtain pellets. These cell pellets were stored at −20°C until use. When cells did not grow significantly, the flask was kept standing for about 5 min and after the cells had sedimented, 2.5 ml of supernatant was replaced by 2.5 ml of fresh medium, and culture was continued in a CO_2 incubator. Cell growth was determined by measuring the DNA contents of cell pellets. Cell pellets were washed twice with 5% TCA and then incubated at 90°C for 10 min in 5% TCA to hydrolyze DNA. The supernatant after centrifugation was used for analysis of the DNA content by Burton's method.[17]

Chromosome numbers Chromosome numbers of Lu-134-B cells cultured in SSM, DL-SSM and DL-SSM plus retinoic acid after culture in DL-SSM for more than 8 weeks were counted. Chromosome numbers were also counted in Lu-134-B cells every 14th day after a change of the culture medium from SSM to DL-SSM. Cells in the logarithmic growth phase were arrested at metaphase by the addition of colcemid (0.1 μg/ml) for 2 h. Cells were then collected by centrifugation, swollen in 0.075 *M* KCl, fixed, spread on slide glass, and stained with Giemsa's solution. Cells in metaphase were photographed and the chromosome numbers of more than 80 cells in each sample were counted.

DNA and RNA analyses Approximately 10 μg of DNA prepared by proteinase K digestion and phenol-chloroform extraction[18] was digested with *Eco*RI restriction endonuclease, fractionated on 0.8% agarose and transferred to nitrocellulose filters. The filters were hybridized under stringent conditions with c-*myc*, N-*myc* and L-*myc* probes. Poly(A)$^+$RNA (2 μg) prepared by guanidine thiocyanate/cesium chloride gradient centrifugation and oligo(dT)-cellulose affinity chromatography was denatured and electrophoresed on 1% agarose/formaldehyde gel and transferred to nitrocellulose filters.[19] The filters were then hybridized with probes. Probes used for evaluation of the *myc* family oncogenes included a 1.8 kbp *Sma*I-*Eco*RI for L-*myc*, a 1.0 kbp *Eco*RI-*Bam*HI fragment for N-*myc*, and a 1.5 kbp *Cla*I-*Eco*RI fragment for c-*myc*.[19] The probe also included a 2.0 kbp *Pst*I-*Pst*I fragment for β-actin.[20]

RESULTS

Figures 1-A, -B and -C show growth curves of Lu-134-B cells "in SSM," "in DL-SSM" and "in DL-SSM plus retinoic acid," after irradiation at doses of 0, 3.0, 4.5, 6.0 and 8.0 Gy. Lu-134-B cells "in SSM" were radiosensitive, and cell growth was suppressed after irradiation of 3.0 Gy (Fig. 1-A). When the cells were irradiated with 8.0 Gy, they appeared eradicated at 20 days but regrowth was noted 80 days after irradiation. On the other hand, Lu-134-B cells "in DL-SSM" grew well even after irradiation of 8.0 Gy (Fig. 1-B) . We repeated this irradiation experiment three times and obtained reproducible results. The growth of Lu-134-B cells "in DL-SSM plus retinoic acid" did not change significantly as compared to that of the cells "in DL-SSM" (Fig. 1-C). Figure 2 shows dose-response curves of Lu-134-B cells "in SSM," "in DL-SSM" and "in DL-SSM plus retinoic acid," showing a significant difference in radiosensitivity between Lu-134-B cells cultured "in SSM" and "in DL-SSM" or "in DL-SSM plus retinoic acid." Figure 3 shows phase-contrast photomicrographs of the cells at irradiation. Cells were irradiated after pipetting to small cell aggregates. This shows that the cell aggregates irradiated were of almost the same size between Lu-134-B cells cultured in SSM (Fig. 3-A) and in DL-SSM (Fig. 3-B).

Figure 4 shows histograms of chromosome numbers of Lu-134-B cells cultured in SSM (Fig. 4-A), in DL-SSM for 8 weeks (Fig. 4-B), and in DL-SSM with retinoic acid at the final concentration of 10^{-7} *M* for 6 weeks (Fig. 4-C). The modal peak of chromosome number changed from 85 to 63 after the change of the culture medium from SSM to DL-SSM and almost no cells with a chromosome number of 85 remained. It was also found that the modal chromosome number changed gradually from 85 to 63 after the medium change (data not shown). The

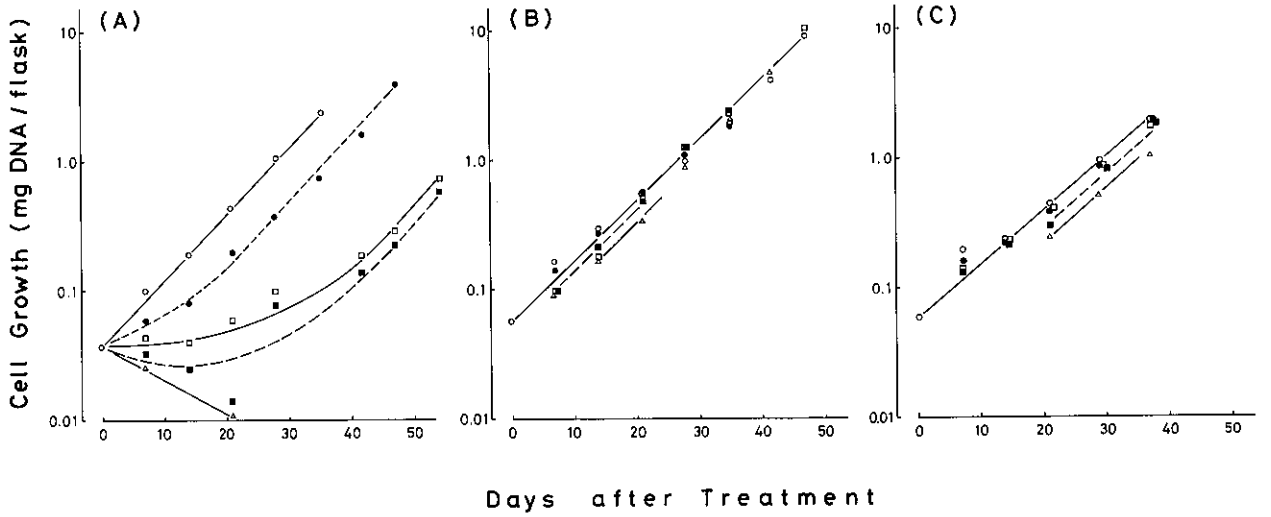

Fig. 1. Growth curves following irradiation of Lu-134-B cells cultured in SSM (A), cells transferred to and cultured in DL-SSM for 8 weeks (B) and cells cultured with added retinoic acid (final concentration of 10^{-7} *M*) for 6 weeks after culture in DL-SSM for 8 weeks (C). Radiation doses: control (○), 3.0 Gy (●), 4.5 Gy (□), 6.0 Gy (■) and 8.0 GY (△). Lu-134-B cells cultured in SSM are radiosensitive, while cells cultured in DL-SSM and cells cultured in DL-SSM with retinoic acid are radioresistant.

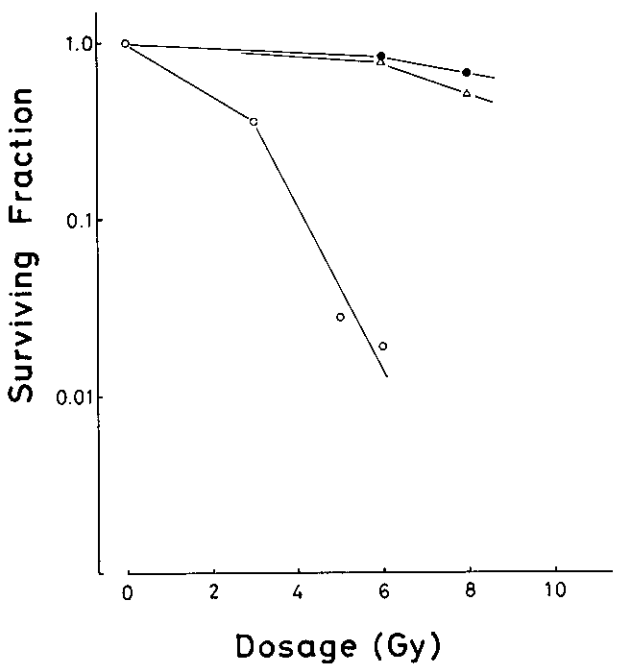

Fig. 2. Radiation dose-response curves for Lu-134-B cells cultured in SSM (○), in DL-SSM (●) and in DL-SSM with retinoic acid at a final concentration of 10^{-7} *M* (△). Curves were constructed by calculating surviving fraction values from back extrapolation to day 0 from the exponential regrowth portions of the multiple growth curves shown in Fig. 1. Lu-134-B cells cultured in DL-SSM and in DL-SSM with retinoic acid show greatly decreased cell kill as compared to cells cultured in SSM.

modal peak of chromosome number of the cells cultured in DL-SSM plus retinoic acid was the same as that of cells cultured in DL-SSM.

In order to know if the change of radiation sensitivity in Lu-134-B cells is associated with changes at the level of the mRNA expression of *myc* family oncogenes, we investigated amplifications and expressions of *myc*-family oncogenes in these cells. Lu-134-B cells showed neither amplification nor rearrangement of c-*myc*, L-*myc* and N-*myc* oncogenes on Southern blot analysis (data not shown). The mRNA expression of *myc* family oncogenes in these cells was examined by Northern blot analysis, and a low level of c-*myc* mRNA expression was detected, but neither L-myc nor N-myc was expressed in these cells. As shown in Fig. 5, the level of c-*myc* mRNA expression was almost the same in both Lu-134-B cells "in SSM" and "in DL-SSM."

DISCUSSION

It was shown that the human SCLC cell line Lu-134-B changed from radiosensitive to radioresistant when the culture medium was changed from SSM to DL-SSM, with a partial change of morphology from small cell to squamous cell. This is the first report to describe a change in radiosensitivity of an SCLC cell line after a change in culture conditions. Deficiency of vitamin A was supposed to be a factor modifying the radiosensitivity of the SCLC line. Alternatively, it was considered that selective growth might have caused these changes. Lu-134-B cells

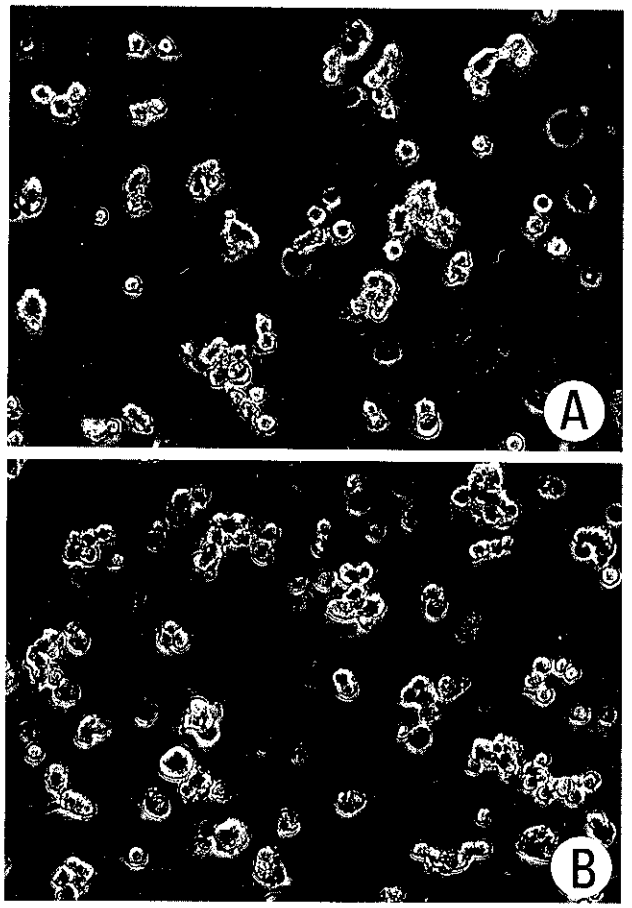

Fig. 3. Phase-contrast photomicrographs of Lu-134-B cells cultured in SSM (A) and DL-SSM (B) at irradiation. Cells were pipetted gently to form small cell aggregates. The sizes of cell aggregates were almost the same in both cell lines. ×90.

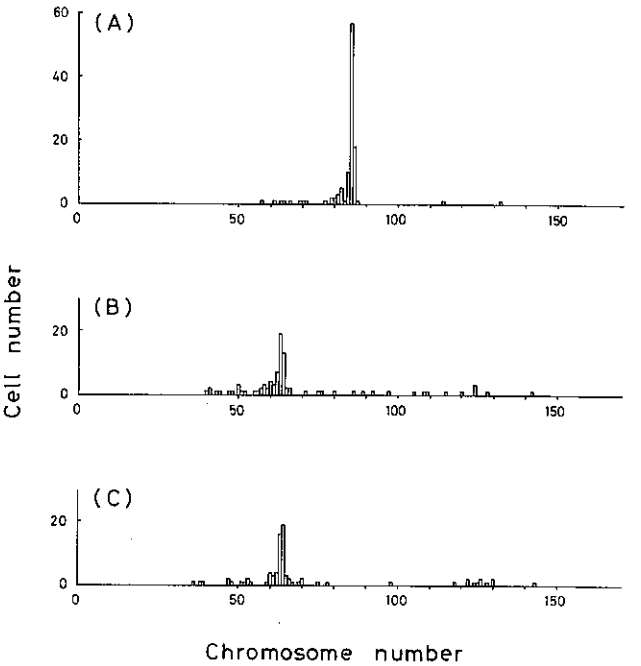

Fig. 4. Chromosomal histograms of Lu-134-B cells cultured in SSM (A), in DL-SSM for 8 weeks (B) and in DL-SSM with retinoic acid (final concentration of 10^{-7} *M*) for 6 weeks after culture in DL-SSM for 8 weeks (C).

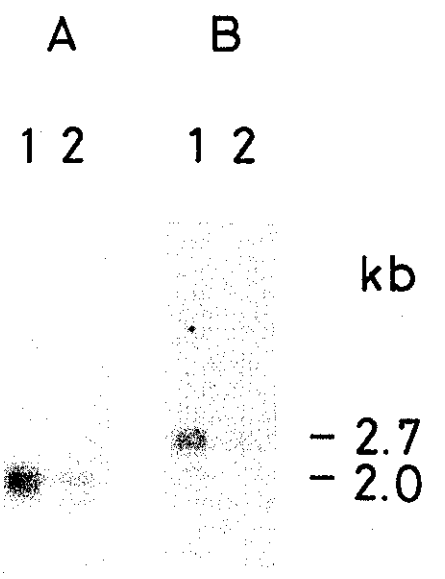

Fig. 5. mRNA expression of β-actin (A) and c-*myc* (B) genes in Lu-134-B cells "in SSM" (1) and "in DL-SSM" (2). The level of c-*myc* mRNA expression relative to that of β-actin mRNA expression was almost the same in both cell lines.

cultured in SSM have a modal chromosome number of 85 and very few cells have a chromosome number of 63. When the culture medium was changed to DL-SSM, cells with a chromosome number of 85 gradually disappeared and cells with a chromosome number of 63 increased. When the medium was further changed to DL-SSM plus retinoic acid, however, the modal chromosome number of 63 stayed unchanged for 1 year. Lu-134-B cells cultured in SSM were radiosensitive and cells after a change of their culture medium to DL-SSM were radioresistant, while cells further changed to DL-SSM plus retinoic acid remained radioresistant. Therefore, it is supposed that Lu-134-B cells cultured in SSM are composed of many radiosensitive cells that have a chromosome number of around 85 and a few radioresistant cells that have a chromosome number of around 63. When the medium was changed to DL-SSM, growth of cells with a chromo-

some number of 85 might have been suppressed and the growth of cells with a chromosome number of 63 might have been accelerated. After eight weeks, there remained almost no cells with a chromosome number of 85, and overall radioresistance had been acquired. It is desirable to perform these experiments using cloned cells, but cloning of Lu-134-B cells is very difficult because they grow in floating aggregates. The radioresistance of Lu-134-B cells cultured in DL-SSM may be caused by the difference of medium at the time of irradiation. Therefore, Lu-134-B cells cultured in SSM were irradiated in SSM and DL-SSM, and then both groups of cells were cultured in SSM. The results indicated that both groups of cells were radiosensitive, with the same growth curves. Furthermore, the medium of Lu-134-B cells which had been cultured in DL-SSM for more than 8 weeks was changed to SSM, then culture was performed for a further 8 weeks and the cells were irradiated. These cells were also radioresistant, as were the cells cultured and irradiated in DL-SSM (data not shown). These experiments showed that the difference in radiosensitivity between the cells cultured in SSM and DL-SSM was due to the difference in the nature of these cells and not due to the difference in the nature of the media at the time of irradiation. Because both cell lines grew as floating aggregates of almost the same size and were irradiated after gentle pipetting to small cellular aggregates of almost the same size (up to 10 cells/aggregate), the cells irradiated are likely to have been similarly oxygenated. As to the cell cycle, there appeared to be no marked difference at the time of irradiation, since both cell lines grew at almost the same speed as indicated by the growth curves. However, the mechanisms underlying the differences in radiation response of these cells must be defined further by studying various aspects such as oxygen environment, cell cycle, DNA break, DNA repair and/or genome size.

SCLC has been reported to change its morphology not only to squamous cells but also to large cells or glandular cells.[6-8] Factors that cause these changes have not yet been identified. If any conditions that reverse these changes were found, SCLC cells could possibly be successfully eradicated by radiotherapy and chemotherapy. The radiosensitivity of Lu-134-B cells in DL-SSM did not change significantly with the addition of retinoic acid but the morphology reverted from combined SCLC and squamous cells to SCLC cells. This phenomenon can be explained by the assumptions that cells with a modal chromosome number of 63 are capable of becoming squamous in the absence of retinoic acid, and that the cells with a modal number of 85 are eradicated by deletion of vitamin A from the medium.

The facts that there was no amplification of *myc* family oncogenes, nor any change in the level of c-*myc* mRNA expression before and after the change of culture condition, indicate the absence of involvement of *myc* family oncogenes in the radiosensitivity of this cell line. Almost no acceleration of growth speed and no significant decrease in AADC activity (from 2840 to 2510 pmol/min/mg protein[14]) were observed when the culture medium was changed from SSM to DL-SSM. These findings indicate that Lu-134-B cells possess features of both classic SCLC in terms of enzyme activity and c-*myc* oncogene-amplified and variant SCLC in terms of radiosensitivity.

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