Resistance to anticancer drugs in NIH3T3 cells transfected with c-myc and/or c-H-ras genes

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Summary NIH3T3 cells transfected with c-H-ras and/or c-myc genes were examined for differences in drug sensitivity. The five transfecants used were N8, NIH3T3-3-nm-1, pT22-3-nm-2, pP1-4 and pT22-3. They were transfected with pKOneo alone, pKOneo and c-myc, pKOneo and c-myc plus activated c-H-ras, normal c-H-ras and activated c-H-ras genes, respectively. The IC50 of cisplatin, 4-hydroperoxycyclophosphamide, adriamycin, melphalan, and CPT-11 were significantly higher for NIH3T3-3-nm-1 than for the parental NIH3T3 and N8 cells. Transfection with normal and activated c-H-ras oncogenes only led to increases in the IC50 of alkylating agents. There was no significant difference between the IC50 of N8 and those of NIH3T3 parental cells to any of these anticancer agents. These results strongly suggest that the expression of the c-myc gene plays a role in the acquisition of drug resistance. The c-myc gene may therefore provide us with an important clue in determining the mechanism of drug resistance.

The majority of malignant tumours are theoretically considered to be composed of both drug-sensitive and resistant cells. If chemotherapeutic agents are administered, only the drug-sensitive cells are removed. In addition, cells with acquired resistance develop and after several courses of chemotherapy only resistant cells are present. Accordingly, inherent and acquired resistances are major causes of failure in cancer chemotherapy. In order to elucidate the mechanism of drug resistance, various biochemical and molecular biological investigations have been conducted. Among factors which have been demonstrated to be related to drug resistance are the following: (1) decreased drug uptake, (2) increased drug efflux mediated by P-glycoprotein, (3) increased intracellular detoxification by glutathione, metallothionein, etc., (4) amplification of genes for the key enzymes of drug metabolism, (5) decreased DNA damage and increased repair, (6) activation of oncogenes. The relationship of some genes to drug resistance has been directly demonstrated by transfection studies (Sklar, 1988a,b). Examples of this are the contribution of P-glycoprotein to multidrug resistance (Bell et al., 1985; Ma et al., 1987; Bradley et al., 1989) and the dihydrofolate reductase (DHFR) gene (Schimke, 1984) to methotrexate resistance. However, there is little evidence demonstrating a direct relationship between oncogenes and drug resistance, although some oncogenes have been associated with poor prognosis (Little et al., 1983; Nau et al., 1985; Slamon et al., 1987). In order to study the contributions of oncogenes to drug resistance more directly, we have determined by tetrazolium dye (MTT) assay, the drug sensitivity of cells transfected with the c-myc and/or c-H-ras oncogenes.

Materials and methods

Cell lines

NIH3T3 cells were used as the parental cell line. Transfection with cloned oncogenes was performed by a modified calcium precipitation method. For transfection with the c-myc oncogene we used the recombinant plasmid DNA designated R-myc-27 cloned into the EcoRI PvuII region of pBR322, propagated in Escherichia coli. R-myc-27 contained SacI-EcoRI 4 kb Shiraishi c-myc DNA (partially digested at the 3'-SacI site in the first intron of Shiraishi c-myc DNA) containing the second and third exons that was ligated to the PvuII-SacI 1.0 kb fragment of the Rous sarcoma virus long terminal repeat (RSV-LTR) (Delorbe et al., 1980; Shibuya & Yamaguchi, 1987; Rothberg et al., 1984). The cloned cell line, which was cotransfected with pKOneo plasmid (Fasano et al., 1984) and selected with 400 μg ml−1 of G418 (Sigma, MO), was designated NIH3T3-3-nm-1, pT22-3, the transformant obtained with the activated c-H-ras oncogenes, was obtained by transfection of plasmid PT22 which was the recombinant pBR322 containing activated human c-H-ras gene cloned from T24 bladder carcinoma in BamHI site (Santos et al., 1982; Fasano et al., 1984; Goldfarb et al., 1982; Perucchini et al., 1981). In addition, we obtained two transfected cell lines, pT22-3-nm-2 and pP1-4, which were transfected with activated c-H-ras plus c-myc and normal c-H-ras genes, respectively. N8 was obtained by transfection of pKOneo plasmid only and selected with G418. All the cell lines used in this study were cultured in Dulbecco’s modified Eagle’s medium (DMEM Nissui, Pharmaceutical Co Ltd., Tokyo) containing 10% heat-inactivated calf serum (GIBCO, Grand Island, NY), penicillin (100 μg ml−1) and streptomycin (100 μg ml−1) [c-DMEM] in a highly humidified atmosphere of 5% CO2 plus 95% air at 37°C. Cell line PC-9/CDDP, used as the positive control for Glutathione S-Transferase-π gene expression (Fujiwara et al., 1990), was established by exposure of PC-9, a non-small cell lung cancer cell line, to stepwise increasing concentrations of cisplatin (Hong et al., 1988). K562/ADM, an adriamycin resistant human myelogenous leukaemia cell line, kindly provided by Dr T. Tsuruo, as the positive control for mdr-1 gene expression by Northern blot analysis. PC-9/CDDP cells and K562/ADM cells were cultured in RPMI1640 medium with 10% heat-inactivated fetal bovine serum (FBS, Im- munobio-Biological Laboratories, Fujioka, Japan), penicillin (100 U ml−1) and streptomycin (100 μg ml−1) in a humidified atmosphere of 5% CO2 plus 95% air at 37°C.

Characterisation of cell lines

For the study of doubling times of these transfecants, a single cell suspension containing 5 × 105 cells was placed into 60 mm dishes (Falcon, Becton Dickinson Labware, Oxnard, CA). Cells were counted daily for 6 days and the doubling time of each cell line in its logarithmic phase was calculated. For the evaluation of plating efficiency, single cell suspensions were obtained by trypsinisation and diluted with c-DMEM to the appropriate concentrations with the final cell
numbers of $5 \times 10^5$ well for pT22-3-nm-2, pP1-4 and pT22-3, and $1 \times 10^5$ well for NIH3T3, N8 and NIH3T3-nm-1. One ml quantities of cell suspension in c-DMEM containing 0.06% agar (Difco Laboratories, Detroit, MI) were plated as the bottom layer in 35 mm flat-bottomed wells of a tissue culture multi-well plate (Linbro, Flow Laboratories Inc., McLean, VA). The bottom layer was prepared just before the top layer was added. The top layer contained 0.35% agar in enriched McCoy's 5A medium (Gibco, Grand Island, NY), consisting of 50 ml of heat-inactivated foetal calf serum, 25 ml of heat-inactivated horse serum (GIBCO, NY), 4 ml of 2.2% sodium pyruvate, 4 ml of 200 mM glutamine, 0.8 ml of 2.1% serine and 5 ml of penicillin (100 U ml$^{-1}$) and streptomycin (100 $\mu$g ml$^{-1}$) mixed with 400 ml McCoy's 5A medium. After the top layer was added the plates were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ plus 95% air for 10 days. Each experiment was performed in triplicate and repeated three times. Colonies more than 50 $\mu$m in diameter were counted using an automatic particle counter (CP-2000, Shiraimatsu, Osaka).

Southern blot analysis

Transfectants were tested for the presence of the transfected genes by Southern blot analysis. DNAs were digested with the BamH I or EcoR I restriction endonuclease for c-H-ras and with EcoR I restriction endonuclease for c-myc under the conditions directed by the manufacturer. Digested DNAs were electrophoresed on 0.8% agarose and transferred to nitrocellulose membranes (Southern, 1975). We probed with a 1.5 kg Clal-EcoR I fragment for c-myc, a 3.0 kb Sac I fragment for c-myc and a 3.0 kb Sac I fragment for c-H-ras each labelled with $[^{32}P]$dCTP using the Multiprime DNA Labelling System (Amersham, Japan). Filters hybridised with labelled probes were autoradiographed and developed (Southern, 1975).

Northern blot hybridisation analysis

Total RNA was prepared from the wild type NIH3T3, NIH3T3-nm-1, pT22-3-nm, pP1-4, pT22-3, PC-9/CDDP and K-562/ADM cells by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1986). Approximately 20 $\mu$g of total RNA was electrophoresed and transferred to nitrocellulose filters (Maniatis et al., 1982). We probed with a 0.4 kb Pst I fragment containing 2nd exon of the human c-myc gene (Shibuya & Yamaguchi, 1987), a 3.0 kb Sac I fragment of activated human c-H-ras gene cloned from T24 bladder carcinoma (Viola et al., 1985), gP2 coding for the human Glutathione Transferase (GST)- $\pi$ (Kano et al., 1987) and PMDR-1 coding for the human mdr-1 gene (Roninson et al., 1986). All these probes were labelled with $[^{32}P]$dCTP to a specific activity of $2 \times 10^8$ cpm$^{-1}$ DNA using the Multiprime DNA Labelling System (Amersham, Japan). Hybridisation was carried out for 24 h under stringent conditions [$5 \times$ SSC (SSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 50% formamide, 42°C]. After hybridisation, the filters were washed three times in $0.1 \times$ SSC containing 0.1% sodium dodecyl sulphate at 65°C for 15 min. The filter was autoradiographed at $-70^\circ$C.

Test for sensitivity of NIH3T3 cells and transfectants

Cells of each clone were plated in 60 mm petri dishes (Corning Glass Works, Corning, NY) with 5 ml of DMEM containing 10% calf serum (GIBCO) and 0.27 g of glutamine (Nissui, Japan) for the determination of plating efficiency. Foci were counted at confluent growth. The MTT assay (Mosmann, 1983) was used to test for sensitivity to cisplatin (CDDP, Bristol-Myers K.K., Tokyo), adriamycin (ADR, Kyowa Hakko Kogyo Co Ltd, Tokyo), 4-hydroperoxy cyclophosphamide (hCPA, a metabolite of cyclophosphamide, Shionogi Co Ltd, Osaka), etoposide (VP-16, Bristol-Myers K.K., Tokyo), (4S)-4, 11-diethyl-4-hydroxy-9-[4-piperidinopiperidino]carboxyloxy]-1H-pyrazol[3',4': 6, 7] indolizino[1,2-b] quinoline-3, 14(4H, 12H)-dione hydrochloride trihydrate (CPT-11 Yakult Co Ltd, Tokyo) and melphalan (Sumitomo Chemical Co Ltd, Tokyo). In the case of NIH3T3, N8 and NIH3T3-nm-1 (Table I), $1 \times 10^5$ cells/well with 180 ml of culture medium were plated in a culture plate (Falcon 3072 96-well tissue culture plate), and for pT22-3-nm-2, pP1-4 and pT22-3, $5 \times 10^5$ cells/well were plated with 180 ml of medium in the culture plate. Twenty $\mu$l of drug solution was added to each well. After 4 days of incubation at 37°C, the plate was centrifuged at 1,200 r.p.m. for 5 min and the medium was aspirated from the wells as completely as possible. To each well 200 $\mu$l of dimethyl sulfoxide (Wako Pure Chemical Industries Ltd., Osaka) was added. The plates were then agitated on a plate shaker for 5 min and the optical density was read using a Titerette Multispec MCC plate reader (Flow Laboratories). The absorbance for wells containing drug to that of the control well.

Results

Characteristics of transfectants

Morphologically the c-H-ras transfectants (pT22-3, pT22-3-nm-2 and pP1-4) were transfected but NIH3T3-nm-1, transfected with c-myc alone, was not. N8, transfected with pKOneo plasmid, was also not transformed. NIH3T3, N8 and NIH3T3-nm-1 did not produce any foci, although pT22-3-nm-2, pP1-4 and pT22-3 did. In agreement with this finding, in the study of plating efficiency by the soft agar double layer method (Table I), pT22-3-nm-2, pP1-4 and pT22-3 produced colonies although NIH3T3, N8 and NIH3T3-nm-1 did not. Doubling times of these transfectants were in the range 15 to 23 h.

Figure 1 shows the presence of the c-myc gene in NIH3T3-nm-1 and pT22-3-nm-2 cells transfected with this gene, and the c-H-ras gene in pP1-4, pT22-3 and pT22-3-nm-2 cells transfected with this gene as determined by Southern blot analysis. Figure 2 shows the results of Northern blot analysis of NIH3T3 cells and the four transfectants. NIH3T3-nm-1 and pT22-3-nm-2 expressed the c-myc gene and pT22-3-nm-2, pP1-4 and pT22-3 expressed the c-H-ras gene. The right hand side of Figure 2 shows the expression of $\beta$-actin gene mRNA among the cells lines. Figure 3 demonstrates the expressions of GST-$\pi$ and mdr-1 genes in parental NIH3T3 cells and transfectants together with the positive controls. PC-9/CDDP

Table I Characteristics of the cells

| Cells          | Oncogenes | Characteristics | Plating efficiency (%) | Doubling time (h) |
|----------------|-----------|-----------------|------------------------|-------------------|
| NIH3T3         |           |                 |                        |                   |
| N8             |           |                 | 22                     | 23                |
| N8             | c-myc     |                 | 0                      | 23                |
| N8             | activated c-H-ras |             | 21                     | 15                |
| pP1-4          | normal c-H-ras |               | 20                     | 15                |
| pT22-3         | activated c-H-ras |             | 17                     | 18                |

*NIH3T3-nm-1; *pT22-3-nm-2.
Drug sensitivity of transfectants

Figures 4 and 5 show representative response curves for each cell line exposed to hCPA and melphalan. The cell lines transfected with the c-myc oncogene are more resistant than the parental NIH3T3 and N8 cells to these two agents. Each curve was obtained from the average of at least three independent experiments and the IC₅₀ (the concentration of drug which reduces the cell growth to 50% of control) for each cell line was calculated as the average of at least three independently-obtained IC₅₀s. Table II summarises the IC₅₀s of various drugs for all the cell lines. N8, transfected with pKOneo only, showed no significant difference in IC₅₀s compared with those of NIH3T3 cells to any of the anticancer agents. The cell lines transfected with the c-myc gene (NIH3T3-nm-1 and pT22-3-nm-2) were significantly more resistant than in the NIH3T3 and N8 cell line to cisplatin, melphalan, adriamycin, 4-hydroperoxycyclophosphamide, and CPT-11. With VP-16 there was no significant difference in IC₅₀s between any of the transfectants and NIH3T3 cells, but a trend towards resistance was obtained. The cell lines transfected with c-H-ras oncogene (pPl-4 and pT22-3) showed no significant difference in IC₅₀s compared with NIH3T3 and N8 cells to cisplatin and topoisomerase.

![Figure 1: Southern blot analysis of NIH3T3 cells and transfectants.](image1)

![Figure 2: Northern blot analysis of NIH3T3 cells and transfectants.](image2)

![Figure 3: Expression of Glutathione S-Transferase-π gene and mdr-1 gene by Northern blot analysis.](image3)

![Figure 4: Sensitivity of NIH3T3 cells and transfectants to 4-hydroperoxycyclophosphamide.](image4)

![Figure 5: Sensitivity of NIH3T3 cells and transfectants to melphalan.](image5)

and K562/ADM as positive controls showed the expressions of the GST-π (at 0.8 kb) and mdr-1 (at 4.5 kb) genes, respectively. In contrast NIH3T3 cells and transfectants showed negligible expressions of both GST-π and mdr-1 genes.
inhibitors (adriamycin, VP-16 and CPT-11). With alkylating agents (hCPA and melphalan) the transfectants with c-H-ras had higher IC50 values than those of the parental and N8 cells, although that for melphalan in pT22-3 was not significantly different. From these results it is concluded that (1) c-myc oncogenes increased the intrinsic resistance to the anticancer agents, cisplatin, 4-hydroperoxycyclophosphamide, adriamycin, melphalan and CPT-11 and (2) c-H-ras oncogenes increased resistance to alkylating agents only (3) G418 selection did not lead to the isolation of resistant clones which had activated various stress response genes.

Discussion

The MTT assay has the advantage of short incubation time and simplicity. The assay can reflect both cytotoxic and growth inhibitory effect of anticancer agents, but, with longer cell doubling times, it mainly reflects cytotoxic effect. It is therefore important to determine the plated cell numbers and the incubation time in order to achieve an optimal evaluation of drug sensitivity. Following determination that each cell line could maintain linearity in its growth curve and yield accurate reproducibility of optical density, we decided to use a 4-day incubation period and different plating cell numbers, as described in Materials and methods.

All transfectants used in this study were obtained from the same parental NIH3T3 cells. Cells transfectd with the c-myc oncogene acquired resistance to anticancer agents in that IC50 values for all the drugs except VP-16 in nm-1 and nm-2 were significantly higher than those in parental NIH3T3 and N8 cells. Transfection of activated c-H-ras did not affect drug sensitivity in nm-2 considering that IC50 values of pT22-3, transfected with activated c-H-ras, were not higher than those of NIH3T3 and N8 cells except for hCPA. In the two c-myc expressing lines (nm-1 and nm-2) there is a large difference in the amount of c-myc mRNA expressed, but no difference in the degree of drug resistance. One possible explanation for this observation is that the cotransfection of activated c-H-ras gene in nm-2 has some influence on drug sensitivity in this line. Another possibility is that there is a threshold in the amount of c-myc gene expression which influences drug sensitivity and that subclone variation does not always give rise to the difference in drug resistance among transfectants. We obtained another c-myc transfected cell line (nm-9) by the same procedure and this showed about the same IC50 values to CDDP, hCPA, VP-16 and CPT-11 as those for nm-1. Moreover the IC50 value of nm-9 for melphalan was 2.3 times higher than that of nm-1 and 9.8 times higher than that of the parental cells. These findings confirm that independently isolated c-myc subclones show the acquisition of resistance to CDDP, hCPA, VP-16 and CPT-11 compared with the parental and PkOneo transfected cells. Further study is necessary to examine the correlation between drug resistance and the expression of c-myc oncogene by using not cotransfectants but a range of transfectants with different amounts of c-myc oncogene alone.

In order to determine how we can overcome resistance to anticancer agents, the genetic basis of such resistance is the most urgent issue that needs addressing (Anonymous, 1987; Busch, 1987). With regard to myc family genes, amplification of the N-myc oncogene is associated with rapid progression in neuroblastoma (Seeger et al., 1985).

Additionally small cell lung cancer patients have a shorter survival period if their cells show c-myc gene amplification, and c-myc amplification is associated with a more virulent variant type of small cell lung cancer cell line (Johnson et al., 1987b). Such cell lines transfected with c-myc oncogene show morphological changes in culture (Johnson et al., 1986) and have shorter doubling times (Gazdar et al., 1985). These observations therefore suggest that myc family genes are associated with some biological characteristics of malignant tumours, but do not directly prove that these genes contribute to the resistance to anticancer drugs. In the present study we have clearly demonstrated that transfection with the c-myc gene is associated with increased resistance to cisplatin, adriamycin, melphalan, CPT-11 and 4-hydroperoxycyclophosphamide.

This is the first report showing that c-myc oncogene can increase resistance to anticancer agents and there have been no reports to elucidate the mechanism of increase in drug resistance by c-myc oncogenes. It has been reported that transfection with the c-H-ras gene could increase the resistance to cisplatin and radiation in cell lines (Sklar, 1988a,b). On the other hand Toffoli et al. (1989) reported that transfection with H-ras did not induce resistance to CDDP, VP-16, Mitomycin C on adriamycin. In the present study we have demonstrated that the cell lines transfected with the c-H-ras gene increased resistance to some alkylating agents, but not to cisplatin, VP-16 and adriamycin. Although we have no data for Mitomycin C, our findings are not always inconsistent with the results of Toffoli. Previous findings have suggested that a mechanism of resistance to anticancer agents might be a decrease in intracellular accumulation of drugs. On the other hand, as in v-H-ras oncogene-transfected cells (Burt et al., 1988), c-myc oncogenes might increase the expression of other genes known to be involved in drug resistance. There have been some reports that the overexpression of the GDT-1 gene is associated with the acquisition of resistance to cisplatin (Nakagawa et al., 1988) and adriamycin (Cowan, 1986). Our results in Table II show that nm-1 transfected with c-myc oncogene acquired a multidrug resistance phenotype. However, no apparent expression of GST-1 and mdr-I genes could be found in NIH3T3 cells or any of the transfected cells. These results therefore indicate that resistance to anticancer agents in c-myc transfected cells cannot be explained by mdr-1 and GST-1 gene expression. The c-myc transfected cell lines acquired higher resistance to alkylating agents than to other drugs (Table II, Figures 4 and 5). Considering that the target of alkylating agents is mainly DNA in the cell nucleus and that c-myc protein is primarily located in the nucleus, we are planning to study the accumulation of anticancer agents and examine DNA damage and repair in c-myc transfected cells as possible mechanisms of resistance.

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### Table II

| Cells      | CDDP | hCPA  | Melphalan | ADR | VP-16 | CPT-11 |
|------------|------|-------|-----------|-----|-------|--------|
| NIH3T3     | 0.21 ± 0.03a | 0.34 ± 0.02 | 0.95 ± 0.05 | 0.21 ± 0.03 | 0.06 ± 0.01 | 9.64 ± 0.26 |
| N8         | 0.26 ± 0.09 | 0.59 ± 0.15 | 1.20 ± 0.43 | 0.22 ± 0.06 | 0.04 ± 0.03 | 9.09 ± 0.82 |
| mm-1        | 0.44 ± 0.03f | 1.00 ± 0.18f | 4.63 ± 0.91f | 0.34 ± 0.03f | 0.07 ± 0.01f | 16.2 ± 3.25f |
| mm-2        | 0.34 ± 0.04 | 1.11 ± 0.37 | 8.60 ± 0.20f | 0.46 ± 0.05g | 0.21 ± 0.02a | 21.2 ± 0.72 |
| pT22-3     | 0.26 ± 0.02 | 0.60 ± 0.06f | 15.40 ± 1.54b | 0.12 ± 0.02 | 0.06 ± 0.01 | 9.07 ± 1.51 |
| spT22-3    | 0.11 ± 0.01f | 0.71 ± 0.03 | 2.67 ± 0.61 | 0.07 ± 0.01 | 0.04 ± 0.00 | 7.07 ± 0.67 |

a,b,c IC50 values are obtained as the concentrations which inhibit control cell growth by 50%; *Mean ± s.d. Values were calculated from the results obtained by at least three independent experiments; **N8 cell line was obtained by the transfection of pKOneo plasmid alone to NIH3T3 cells; 1987a, 1987b).
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