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Mechanism of Methotrexate-sensitivity of Choriocarcinoma Cells in Culture

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Four cell lines established from choriocarcinoma were compared for sensitivity to methotrexate (MTX). In this paper, we have compared the relative gene copy number of dihydrofolate reductase (DHFR), the target enzyme of methotrexate (MTX), in order to clarify whether or not amplification of the gene is involved in the relative resistance to MTX observed for one of the cell lines, designated NaUCC-1, which is 4- to 5-fold more resistant to MTX as compared with the other cell lines and exhibits a reduced uptake of [^{3}H]MTX. Neither dot blot nor Southern blot hybridization revealed any significant difference in the gene copy number among the four cell lines. Therefore, the resistance to MTX of the NaUCC-1 line is explained by a reduced uptake of the drug, rather than amplification of the target gene.

Key words: Choriocarcinoma — Methotrexate-sensitivity — Dihydrofolate reductase — Gene amplification

Methotrexate (MTX) has been used as a clinically effective antineoplastic agent against various malignancies. Its target enzyme is dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), required for thymidylic acid synthesis. Thus, inhibition of DHFR by MTX prevents *de novo* synthesis of key precursors for nucleic acids.[1)]

An important problem encountered clinically is the development of resistance of cancer cells to this drug. Three distinct mechanisms have so far been proposed to be responsible for the acquisition of the resistance. One of these is the amplification of the DHFR coding sequence leading to an unusually high level of the enzyme synthesis.[2)] The others involve the impaired uptake of MTX by cells[3,4)] and the occurrence of mutated DHFR which exhibits a low degree of MTX-binding.[5,6)] The amplification of DHFR gene has been best studied in normal and neoplastic cells in culture.[7–11)] The drug resistance has been acquired after exposure of the cells to escalating doses of MTX, and a marked amplification of DHFR gene and an increased DHFR synthesis have been detected in these cells. Furthermore, there is evidence suggesting that the same mechanism is responsible for inducing resistance to MTX during the course of clinical treatment with the drug.[12–15)]

Choriocarcinoma is one of the clinical targets of frequent use of MTX. We have previously established several cell lines from clinical specimens and detected significant differences in MTX-sensitivity from one to another.[16)] In a previous paper, the relative resistance to MTX of one of the cell lines was explained in terms of impaired MTX-uptake. However, it is not clear whether other mechanisms involving gene amplification are also involved in the resistance.

Materials and Methods

Cell Culture Three choriocarcinoma cell lines, designated NaUCC-1, NaUCC-2 and NaUCC-3, were established from human gestational choriocarcinoma. The tumors were cut into 1–2 mm cubes and transplanted subcutaneously into the backs of BALB/c nude mice. After 5 to 8 transplantations in mice, the cells were transferred into Petri dishes and serially passaged in Eagle's MEM (NaUCC-1 and NaUCC-2) or RPMI 1640 medium (NaUCC-3), each supplemented with 10% heat-inactivated fetal calf serum (FCS). BeWo is also a choriocarcinoma cell line kindly provided by R. A. Pattillo[17)] and cultivated in RPMI 1640 medium containing 10% FCS.

NaUCC-1, NaUCC-2 and BeWo were established from patients who had received combination chemotherapy including MTX and then acquired resistance to it. NaUCC-3 was from a patient who had undergone no treatment with MTX or other chemotherapeutic drugs. None of these cell lines had previously been exposed to MTX *in vitro* since their establishment.

Preparation of Cell Lysates for DHFR Assay Cells (10^8) of each cell line grown in plastic Petri dishes were harvested and pelleted by centrifugation at 175*g*. The cells were washed 3 times with Ringer's phosphate glucose solution, then lysed by treating the pellets with 1 ml of hypotonic solution (1m*M* potassium phosphate buffer, pH 7.4) for 1 hr on ice. Complete cell lysis under these conditions was always confirmed microscopically. The lysates were then centrifuged at 15,000 rpm for 30 min and the resulting supernatants were stored at −80° until used for assay of DHFR activity.

Dihydrofolate Reductase Assay This enzyme was assayed by measuring the decrease in absorbance at 340 nm which occurs when reduced nicotinamide adenine dinucleotide phosphate (NADPH) and DHF are converted to nicotinamide adenine dinucleotide phosphate (NADP) and THF, respectively.[18] The reaction mixture contained, in a final volume of 1.0 ml, the following components; 100 m*M* Tris-HCl, pH 7.5, 150m*M* KCl, 10m*M* 2-mercaptoethanol, 0.1m*M* NADPH, 0.1m*M* DHF and 0.1 ml of cell lysates. Absorbance reading was done during at least 10 to 15 min at 30° on a Shimadzu UV 260 spectrophotometer. The mixture without cell lysates was used as a blank control. Purified DHFR from chicken liver (Sigma Chemical Co., USA) was used as a positive control. One unit of the enzyme activity was defined as the activity which reduces 1 μmol of the substrate per min at 30°. Protein determination was done according to the Folin-Lowry method.[19]

Dot Blot Hybridization High-molecular-weight DNAs isolated from cells by the method of Blin and Stafford[20] were digested with *Hin*d III (Takara Shuzo Co., Kyoto) according to the manufacturer's manual. The digested DNA was denatured in 0.2*M* NaOH on ice, and 2 μg of the denatured DNA was spotted on a Gene Screen (NEN, Boston) with the aid of a dot plate DP 96 (Tokyo Roshi, Tokyo). The membrane filter was then neutralized with 2×SSC (standard saline citrate), dried, cut into halves and baked at 90° for 2 hr. One of the filter pieces was hybridized with ^{32}P-nick-translated murine DHFR cDNA (pDHFR 11) probe. The cDNA inserted into pBR322 was kindly provided by Dr. R. T. Schimke (Stanford University). The whole plasmid containing murine DHFR cDNA was used as the hybridization probe. The nucleotide sequence homology between the mouse and the human DHFR reading frame is 89%.[21] ^{32}P-nick translation was done by using a Nick Translation Kit N.5000 (Amersham, UK). The hybridization was done with the heat-denatured cDNA (5×10^7 cpm) for 24 hr at 42° in 50% deionized formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05*M* Tris-HCl (pH 7.5), 1*M* NaCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate, 10% dextran sulfate and 100 μg/ml salmon sperm DNA. Following hybridization, the membranes were washed in 0.3*M* sodium chloride, 0.06*M* Tris-HCl (pH 8.0), 0.002*M* EDTA and 1.0% SDS for 1 hr at 60°. As an internal standard, the other half of the filter was hybridized with ^{32}P-nick-translated human globin cDNA kindly supplied by Drs. S. Weissman and J. Pan (Yale University). Each dot was excised, checked for radioactivity by scintillation counting and reconstructed for exposure to Kodak X-S-1 film. To quantitate the gene copy number, a simulation study was made by mixing the apparopriate amount of DNA of human blast cell leukemia lines, K562 and K562/R4, followed by hybridization with the DHFR and globin probes according to the method of Carmen *et al.*[14] K562/R4 is a highly MTX-resistant cell line, which was developed by exposing the parental K562 cells to sequentially increasing MTX concentrations, and has 200-fold amplification of the DHFR coding sequence.[10] These cell lines were kindly provided by Dr. J. R. Bertiono.

Southern Blot Analysis High-molecular-weight DNA (20 μg) from cells was digested with *Eco* RI, *Hin*d III and *Pst* I as described above and electrophoresed on 0.8% agarose gels in Tris borate buffer as described by Manitatis.[22] After denaturation, the DNA bands were transferred to a Gene Screen, essentially as described by Southern.[23] The blotted membranes were hybridized with ^{32}P-labeled DHFR cDNA and exposed to Kodak X-S-1 film for 2 wk at −80°. Lambda phage DNA fragments prepared by digestion with *Hin*d III were used as size markers.

Results

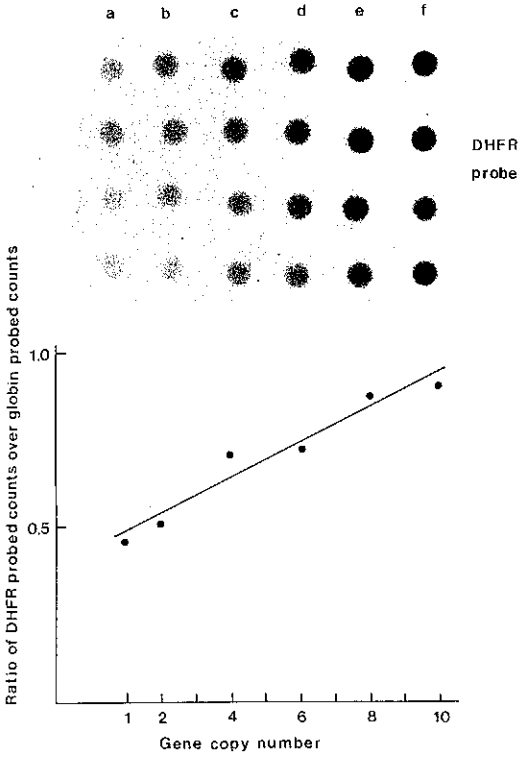
Table I shows the data on the specific activity of DHFR of the four cell lines as well as their MTX-sensitivity and uptake of [^{3}H]-MTX. The cell line NaUCC-1 is about 4- to 5-fold more resistant to MTX as compared with the other three lines, and shows a relatively low level of [^{3}H]MTX uptake. The levels of DHFR specific activity of NaUCC- 1, NaUCC-2 and NaUCC-3 are similar, whereas that of BeWo is relatively high.

Table I. Comparison of DHFR Specific Activity and the Response to MTX among Four Choriocarcinoma Cell Lines

Cell line	DHFR activity (mU/mg protein)	Sensitivity to MTX (ED50, μg/ml)[a]	[^{3}H]MTX uptake (cpm/10^6 cells, 2 hr incubation)
NaUCC-1	0.250 ± 0.015	8.0×10^{-3}	1430 ± 361
NaUCC-2	0.169 ± 0.029	2.0×10^{-3}	4409 ± 345
NaUCC-3	0.243 ± 0.124	1.5×10^{-3}	2722 ± 690
BeWo	0.513 ± 0.097	2.0×10^{-3}	2825 ± 64

a) ED50: 50% effective dose, the concentration of MTX required to inhibit 50% of control cell growth.

Figure 1 shows the dot blot hybridization of murine DHFR cDNA probe to mixtures of K562 and K562/R4 DNA. The mixtures were prepared to contain one to ten copies of DHFR gene in 2 μg of DNA, assuming that 200-fold amplification has occurred in K562/R4 cells. The graph is constructed by plotting the ratio of DHFR probed counts over globin probed counts versus the calculated gene copy number in the mixtures. The results indicate that the system is sensitive enough to detect an increase of gene copy number of at least severalfold.



The results of dot blot hybridization with the radiolabeled murine DHFR cDNA probe are compared among the four choriocarcinoma cell lines in Exp.1 in Fig.2. The intensity of hybridization did not differ significantly from one to another under conditions where the same level of hybridization occurred in these cells with a control probe of human globin cDNA. The use of globin cDNA probe as an internal standard eliminated possible errors in DNA concentrations applied to the Gene Screen. The ratios of average value of DHFR probed counts over the average value of globin probed counts also showed no cell-dependent differences (not shown). These results indicate that there is no marked difference in DHFR gene copy number between the cell lines from MTX-treated (NaUCC-1, NaUCC-2 and BeWo) and untreated (NaUCC-3) patients. This was confirmed by another comparison including two additional control cells, K562 and normal villi obtained at therapeutic abortion (Exp. 2 in Fig. 2).

Fig. 1. Gene copy simulation by mixing the appropriate amounts of DNA from K562 and K562/R4. The autoradiograph is the dot blot hybridization by murine DHFR cDNA probe to (a) K562, one gene copy, (b) two gene copies, (c) four gene copies, (d) six gene copies, (e) eight gene copies and (f) ten gene copies. The standard curve was constructed by plotting the ratio of DHFR probed counts over globin probed counts.

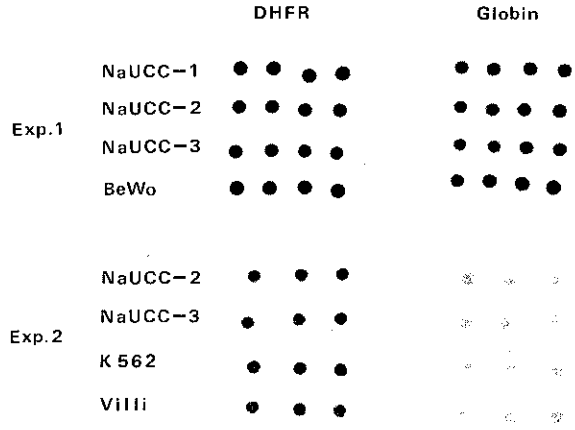


Fig. 2. Dot blot hybridization of choriocarcinoma cell lines, normal villi and K562 cells. DNA (2 μg) from each specimen was spotted and hybridized with radiolabeled DHFR cDNA probe and globin cDNA probe.

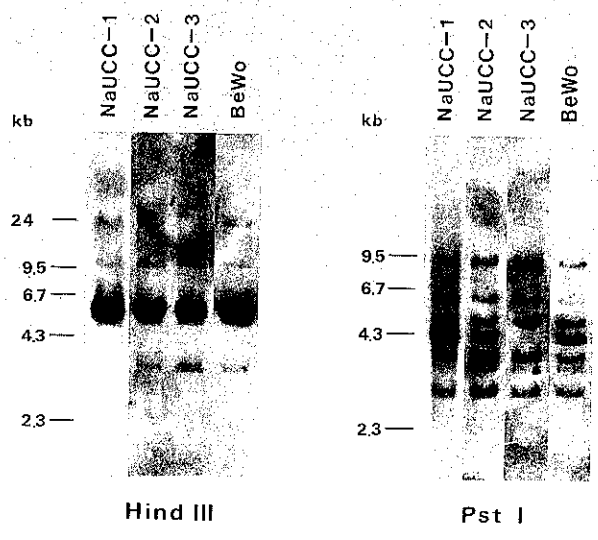


Fig. 3. Southern blot hybridization. DNA (20 μg) from each cell line was digested with *Hind* III and *Pst* I, separated on agarose gels, transferred onto a Gene Screen and hybridized with radiolabeled DHFR cDNA probe. Lambda phage DNA fragments prepared by digestion with *Hind* III were used as size markers.

The restriction pattern of the DHFR gene in these cell lines was examined with 3 different restriction endonucleases as described in "Materials and Methods." The digestion patterns with *Hind* III and *Pst* I are shown in Fig. 3. In *Hind* III or *Eco* RI (not shown) digestion, all cell lines gave essentially identical hybridization patterns, and no marked increse in the intensity was observed for any band. In *Pst* I digestion, NaUCC-1, NaUCC-2 and BeWo gave hybridization patterns almost identical to each other but different from that of NaUCC-3. The major band of 4.3 kb obtained for the former three cells by *Pst* I digestion was deleted in NaUCC-3 cells. Besides, a minor band of 7.1 kb was absent in NaUCC-3 cells, though it was very faintly but consistently detected in the other cells. Overall, however, there appears again to be no significant amplification of the DHFR gene in any cell line.

Discussion

We have previously reported that the NaUCC-1 cell line is about 4 -to 5-fold more resistant to MTX, as compared with the other three cell lines, and shows the lowest level of [^{3}H]MTX uptake of the cell lines. In the

present study, no evidence has been obtained by dot blot or Southern blot hybridization analyses that the DHFR gene is amplified in the NaUCC-1 cells. It has been also demonstrated that DHFR specific activity is not elevatd in these cells. Therefore, the relative resistance to MTX of these cells appears to be explained by a reduced uptake of the drug rather than amplification of the target gene, as has been demonstrated to be the case in other drug-resistant cells. The mechanism of impaired transport remains to be elucidated. Several recent reports have described the P-glycoprotein, which could be involved in impaired drug transport.[24, 25] However, the possibility has not yet been ruled out that DHFR of NaUCC-1 cells is altered by mutations so as to show reduced affinity to MTX. The NaUCC-3 line was found to show some difference in restriction endonuclease fragment length. Since this line was derived from a patient who had not received MTX and showed usual sensitivity to the drug, the significance of this difference is not clear.

Our present results do not necessarily indicate that DHFR gene amplification does not occur in a clinical setting. Amplified copies of the DHFR gene are sometimes stable and sometimes unstable. In the case of stably amplified genes, they are present on the homogeneously staining region (HSR), whereas in the case of unstably amplified genes, they are on double minute chromosomes (DMs). If the cells are cultivated for a long period in the absence of MTX, there is frequent loss of even stably amplified genes.[26] NaUCC-1, NaUCC-2 and BeWo derived from patients treated with MTX were cultivated without MTX for more than several years. Therfore our results do not rule out the possibility of *in vivo* gene amplification.

A few DMs are present in each NaUCC-1 cell (unpublished results). Since there is no amplification of the DHFR gene in this cell line, these DMs presumably do not encode the DHFR sequence.

The methods employed here can not detect possible heterogeneity in a cell population. Thus in order to confirm our results, an *in situ* hybridization analysis is necessary.

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