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Increased Levels of Mitochondrial DNA in an Etoposide-resistant Human Monocytic Leukaemia Cell Line (THP-1/E)

Yutaka Tokue, Yasuo Saijo, Ken Satoh and Masakichi Motomiya

Electron microscopic observations of THP-1/E (an etoposide-resistant human monocytic leukaemia cell line) showed a remarkable change of mitochondrial structure. Mitochondria were swollen and cristae were relatively intact. There was no difference in the activity of cytochrome oxidase, an enzyme which contains three subunits coded by mitochondrial DNA (mtDNA) between THP-1/E and THP-1 (the parent cell of THP-1/E). No measurable quantitative change of mitochondrial RNA was observed, but the level of mtDNA in THP-1/E was increased by a factor of about 4 compared with that of mtDNA in THP-1. These results suggest that, on acquisition of resistance to etoposide, some factors affect mitochondria, change its morphology and amplify its DNA.
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INTRODUCTION

UNDER PATHOLOGICAL conditions, mitochondria show changes in size, shape and number of cristae [1]. These ultrastructural changes are associated with biochemical alterations as exemplified by defects in the pathway of substrate oxidation and ion transport systems, deficiency in enzyme levels or cytochrome content. Whether the mitochondrial abnormalities are a cause or consequence of a pathological condition cannot always be easily determined.

Mammalian mitochondrial DNA (mtDNA) is a closed circular double-stranded molecule consisting of 16 kilobase pairs. It codes for two ribosomal RNA genes, 22 transfer RNA genes, and 13 protein-coding genes such as cytochrome b, subunits of cytochrome oxidase, ATPase and complex I of the respiratory chain [2, 3]. Unlike nuclear DNA, mtDNA is not in association with histones, and its repair mechanism has not yet been elucidated [4].

We have recently established an etoposide-resistant leukaemia

cell line (THP-1/E) by continuous exposure of THP-1 cells to etoposide [5]. Prior reports have indicated that mtDNA may be an important target of several known mutagens, carcinogens and antineoplastic agents [6–10] as evidenced by frequent alterations of mitochondria in carcinoma cells [11]. To the authors' knowledge, however, few papers have been published which have dealt with mitochondrial changes in cells which are resistant to antineoplastic agents [12]. Therefore, we have studied morphology and enzyme activity and have quantitated RNA and DNA in mitochondria of this etoposide-resistant cell line.

MATERIALS AND METHODS

Cells

THP-1 human monocytic leukaemia cells [13] were maintained in suspension culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. An etoposide-resistant subline (THP-1/E) was established from THP-1 human monocytic leukaemia cells by subculture in stepwise increasing concentrations of etoposide [5]. All cells were maintained at 37°C in a 5% CO_2 atmosphere.

Electron microscopy

For ultrastructural studies, 10^7 cells were washed twice with 0.1 mol/l sodium cacodylate buffer (pH 7.2), fixed with 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.2) for 1 h at 4°C, and postfixed in 1% OsO_4 in the same buffer for 1 h. Dehydration in graded concentrations of ethanol and propylene oxide and Epon embedding were performed by the conventional method. Thin sections were stained with both uranyl acetate and lead citrate, and examined in a Hitachi H-600 electron microscope at 75 KV.

Determination of cytochrome oxidase activity

Cultured cells were centrifuged, washed twice with 0.25 mol/l sucrose in 10 mmol/l Tris-HCl (pH 7.4) and resuspended in the same buffer. The cell suspension was homogenised in a Dounce homogeniser, and the homogenate thus prepared used as the crude enzyme. The cytochrome oxidase was measured in an incubation medium consisting of 0.2 ml 0.1 mol/l potassium phosphate buffer (pH 7.0) and 0.14 ml of 1% ferrocytochrome c (type III, Sigma). A blank cuvette was oxidised with 0.02 ml 0.1 mol/l potassium ferricyanide. Absorbance at 550 nm was read for 5 min.

The concentration of protein was determined by the method of Bradford using bovine serum albumin as a standard [14].

Preparation of RNA and northern blot analysis

RNA was extracted by the guanidine isothiocyanate/cesium chloride method [15]. RNA was separated by electrophoresis in a 1% agarose gel containing 2 mol/l formaldehyde and was transferred to a nitrocellulose membrane. Dot blots were carried out using a Minifold (Schleicher & Schuell). A 4.8 kb fragment of mtDNA, after digestion with *Eco*R I, was used as a probe. Hybridisation and washing were carried out as described previously [15].

Isolation of DNA and Southern blot analysis

High molecular weight DNA was extracted from THP-1 or THP-1/E cells as described previously [15]. DNA was digested for 4 h with *Eco*R I. The sample digested as above was electrophoresed on an 0.8% agarose gel and transferred to a nitrocellulose membrane by the method of Southern [16]. Prehybridisation was done for 6 h at 65°C in a solution containing 5 × sodium saline citrate, 5 × Denhardt's solution, 0.1% sodium dodecyl sulphate, salmon sperm DNA (0.25 mg/ml) and 50% formamide. Hybridisation with the ^{32}P-labelled probe (10^7 cpm) was carried out overnight at 65°C in 10 ml of the prehybridisation mixture.

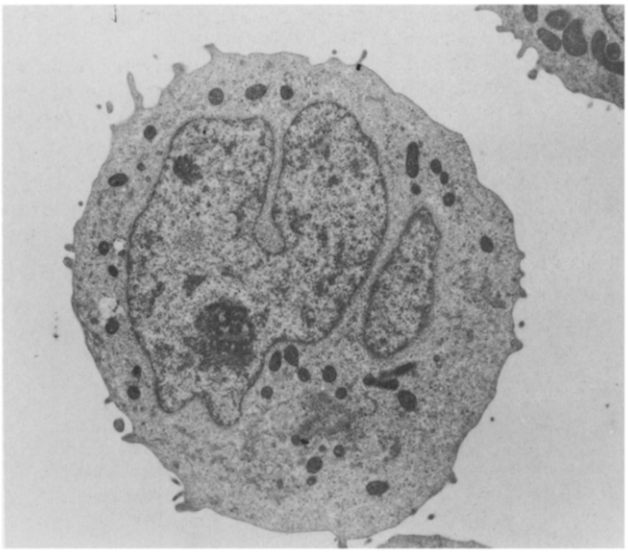

Fig. 1. Ultrastructure of a THP-1 cell: it has a deep-folded nuclei with well-defined nucleolus and the mitochondria are characterized by smooth, ovoid contours and regularly-spaced cristae. The density of mitochondrial matrix exceeds that of the surrounding cytoplasm (× 5000).

Probes

Bovine mtDNA [2] has about 70% homology in sequence with human mtDNA [3], and can be used as a sensitive and specific probe for hybridisation to human mtDNA. Circular mtDNA was isolated from bovine liver as described by Backer and Weinstein [7]. The bovine mtDNA was digested with *Eco*R I and 7.3 kb, 4.8 kb and 4.2 kb fragments were obtained, purified on agarose gel and labelled with [^{32}P]-dCTP. A cDNA containing the complete human α_1-antitrypsin sequence was used as a nuclear DNA probe [17].

RESULTS

Electron microscopy

The nuclei of THP-1 cells had deep folds and a well-defined nucleolus. Mitochondria had smooth, ovoid contours and regularly-spaced cristae spanning the entire width of the organellae. The density of the mitochondrial matrix exceeded that of the surrounding cytoplasm (Fig. 1).

In THP-1/E cells, most mitochondria were enlarged and swollen, and cristae were relatively intact in the low-density mitochondrial matrix (Fig. 2). Such changes were still evident even after 2-month culture of THP-1/E cells in etoposide-free medium.

Cytochrome oxidase

We measured the activity of cytochrome oxidase which contained three subunits coded by mtDNA, as shown in Fig. 3. The activity of cytochrome oxidase in THP-1 was 36.1 (S.D.

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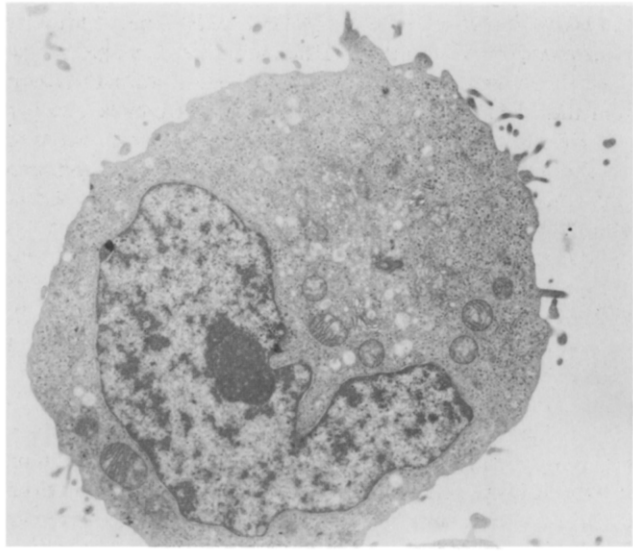

Fig. 2. Ultrastructure of a THP-1/E cell. Most of the mitochondria are enlarged and swollen, and cristae are relatively intact in a low density mitochondrial matrix (× 5000).

13.0) and that in THP-1/E 34.1 (11.3) nmol/min/mg protein, respectively. No significant difference was observed between the two cell lines.

Northern blot and dot blot analysis of mRNA

Figure 4a shows the results of northern blot analysis. Equal amounts (10 μg) of total RNA from THP-1 and THP-1/E cells were electrophoresed. MtDNA isolated from bovine liver was digested with *Eco*R I and a 4.8 kb fragment was used as a probe. Two major bands (mRNA species of 1.7 kb and 0.7 kb) were detected both in THP-1 and THP-1/E cell lines. No difference in size of mRNA species was observed between the two cell lines.

Figure 4b shows the results of dot blot analysis which was carried out with a 2-fold dilution series. DNA probe was the same as that used for northern blot. No quantitative change was observed between the two cell lines.

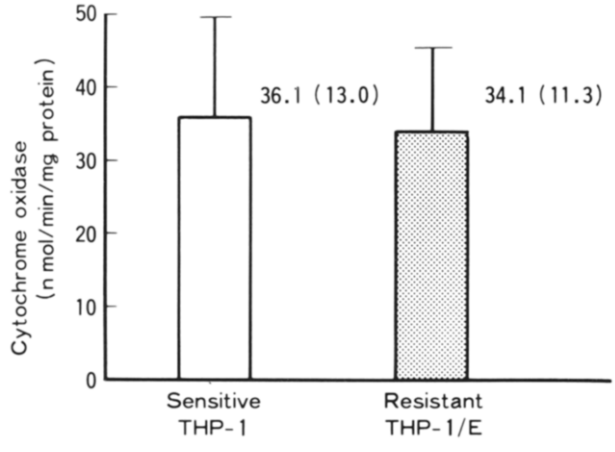

Fig. 3. Activity of cytochrome oxidase in THP-1 and THP-1/E cell lines. Seven independent determinations of THP-1 and THP-1/E were made. No significant difference was observed between these two cell lines. Mean (S.D.).

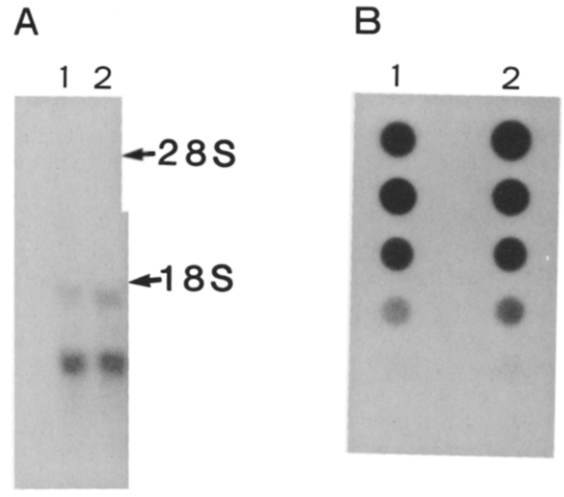

Fig. 4. (A) Northern blot analysis of RNA. Arrows indicate the position of 28S and 18S rRNA in this gel. (B) Dot blot analysis was carried out with a 2-fold dilution series. The same probe as that used for northern blots was used. Lane 1, THP-1/E; 2, THP-1.

Southern blot analysis

Total cellular DNA extracted from THP-1 or THP-1/E cells was digested with *Eco*R I, which cleaves human mtDNA at three different sites. Equal amounts (10 μg) of total DNA were electrophoresed. Southern hybridisation was performed, using as probes radio labelled 16.3 kb bovine mtDNA (7.3 + 4.8 + 4.2 kb) or complete human α_1-antitrypsin sequence (Fig. 5). A single major band of 10 kb for α_1-antitrypsin DNA was detected in both THP-1 and THP-1/E cell lines. The two cell lines exhibited similar levels of hybridisation. In contrast, more mtDNA hybridised with DNA from THP-1/E than with that from THP-1 cell lines. Densitometric analysis of autoradiograms

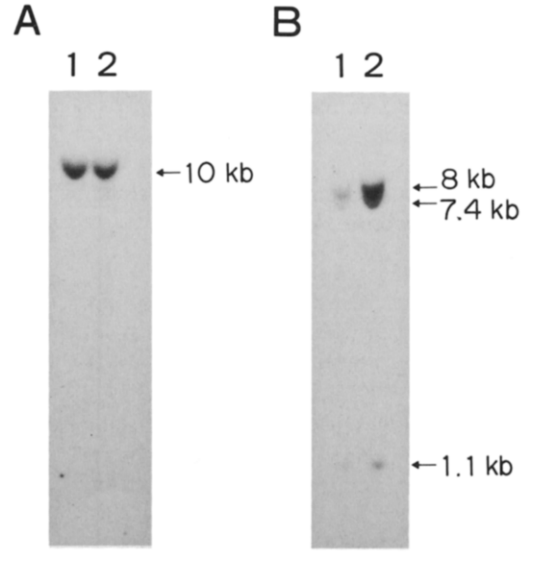

Fig. 5. Southern blot analysis of α_1-antitrypsin and mtDNA. (A) The membrane was hybridised with radiolabelled complete human α_1-antitrypsin sequence. A single major band of 10 kb was detected in both THP-1 and THP-1/E cells. Two lanes exhibited similar levels of hybridisation. (B) The membrane was hybridised with radiolabelled bovine mtDNA probe. Three bands (8 kb, 7.4 kb and 1.1 kb) were detected in two cell lines, but the level of mtDNA in THP-1/E was increased by a factor of about 4 as compared with that of mtDNA in THP-1. Lane 1, THP-1; 2, THP-1/E.

(three independent determinations) indicated a difference of a factor of 3.97 (0.66) in the level of hybridisation between THP-1/E and THP-1 cell-lines (data not shown). Three bands (8 kb, 7.4 kb and 1.1 kb fragments) were detected, but no difference in their sizes was observed between the above two cell lines.

DISCUSSION

Ultrastructural studies of THP-1/E, an etoposide-resistant human monocytic leukaemia cell line, revealed changes of mitochondrial structure which were irreversible even after removal of etoposide. Similar changes in mitochondria have also been observed after treatment with ethidium bromide or cloramphenicol [18]. With the latter two reagents, however, the structural changes such as swelling and loss of cristae were reversible after removal of the drug. After treatment with ethidium bromide or chloramphenicol, the activity of cytochrome oxidase was decreased to a level of about one half the control value. Ditercalinium, a bifunctional intercalating molecular with antitumour activity, also causes swelling and loss of cristae in mitochondria. After treatment with ditercalinium, cytochrome oxidase activity decreased exponentially, and mtDNA became lost in the absence of detectable nuclear DNA alterations [10]. In contrast, our study found no difference in the activity of cytochrome oxidase between THP-1 and THP-1/E.

Tapiero *et al.* [12] have reported that Friend leukaemia cells resistant to doxorubicin are crossresistant to rhodamine 123. When cells resistant to doxorubicin were treated with rhodamine 123, mitochondria remained intact. However, when the doxorubicin-resistant cells were treated with verapamil plus rhodamine 123, the intracellular levels of rhodamine 123 increased, mitochondria became swollen and cristae became disorganised.

Our previous studies on drug uptake and efflux revealed no difference between THP-1 and THP-1/E in either kinetics or steady-state level of etoposide. There was no difference in the effect of verapamil on cellular uptake of etoposide between the above two cell lines [19]. These observations suggest that the mechanism of resistance to etoposide in THP-1/E may be different from that of resistance of Friend leukaemia cells to doxorubicin.

The mechanism of action of etoposide appears to involve production of single-strand and double-strand breaks in DNA [20–22]. The etoposide-induced DNA cleavage is caused by an interaction of etoposide with topoisomerase II [23]. However, controversy still exists as to the mechanism of resistance to etoposide, presumably because the cell lines used and the patterns of crossresistance were different from experiment to experiment. Thus reduced drug accumulation or altered membrane permeability has been observed in the resistant cell lines of mouse tumour [24], while no decrease or minimal change has been reported in drug-resistant Chinese hamster cells [25] and in a multidrug-resistant human tumour cell line [26]. To the authors' knowledge, few papers have been published which have dealt with a relationship between mitochondria and etoposide resistance.

On the other hand, amplification of a number of genes has been reported in somatic mammalian cells, including amplification of the dihydrofolate reductase (DHFR) gene in association with the acquisition of resistance to methotrexate [27, 28]. Overproduction of DHFR, the target protein of methotrexate, is the result of dose-related amplification of the DHFR gene. Glaichenhaus *et al.* [29] observed that the levels of mitochondrial gene expression increased in rat fibroblast cells immortalised or transformed by viral and cellular oncogenes. Changes in the expression of the mitochondrial genome did not appear to result from the difference in the number of mtDNA molecules per cell. Heerdt *et al.* [30] reported that the level of expression of cytochrome oxidase subunit 3 decreased in colon adenomas and carcinomas in comparison with that of normal mucosa and returned to higher levels when the colonic adenocarcinoma cell line was induced to differentiate with sodium butyrate. These changes were not associated with alterations in the number of mtDNA. In the reports of Glaichenhaus *et al.* and Heerdt *et al.*, increased gene expression was the major change and the level of DNA remained unchanged.

The data presented in this report demonstrate that in THP-1/E cell lines amplification of mtDNA is a major change which may not necessarily parallel the levels of mRNA or enzyme activity. It is possible in THP-1/E cells that transcription from mtDNA is inhibited by continuous exposure to etoposide and/or that the changes of displacement-loop region of mtDNA, which has evolved as a control site for both replication and transcription, induce amplification of mtDNA, but leave the rate of transcription unchanged. Another possibility is a change of turnover rate of mRNA. Despite an increased production of RNA, the level of mRNA may not change due to a faster rate of RNA degradation. However, it is also possible that amplification of mtDNA is the result of defensive reaction of cells placed under disadvantage. Whether such mitochondrial changes are a cause or a consequence of acquisition of resistance to etoposide remains to be solved.

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Serial Serum MCA Measurements in the Follow-up of Breast Cancer Patients

Ofer Merimsky, Moshe Inbar, Mara Hareuveni, Bruria Witenberg, Yoram Wolman and Samario Chaitchik

Mucin-like carcinoma-associated antigen (MCA) was serially assayed in 58 women with histologically proven breast cancer after their treatment for primary disease. MCA sensitivity and specificity were 87.5% and 76.9%, respectively, and the positive predictive value 82.4%. 10 patients had elevated MCA and no evidence of disease (NED) during their follow-up, of whom 4 finally developed overt metastases. The 4 had a mean (S.D.) MCA value of 46.48 (18.26) U/ml during the lead time, versus 18.76 (2.69) U/ml in the other 6, who are still at high risk for developing overt metastases. Raised levels of MCA in patients with NED create a dilemma of "treat" versus "wait and see". Early treatment of patients with serially uprising MCA levels should be evaluated in a prospective randomised study to assess its ability to prevent or delay the development of overt metastatic disease and influence survival.
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INTRODUCTION

THE USE of tumour markers for the diagnosis, assessment and follow-up of breast cancer patients has until recently been disappointing. In order to predict early clinical recurrence, a reliable marker of high sensitivity and specificity, which could detect small tumour burdens, is required. The widely used carcinoembryonic antigen (CEA) has been proven to be of little value for predicting clinical recurrence and has successfully monitored only 60% of patients with metastatic disease during treatment, as the others did not show elevated serum CEA [1].

Mucin-like carcinoma-associated antigen (MCA) is a high molecular weight glycoprotein, produced by oestrogen-dependent and oestrogen-independent mammary carcinoma cells. It is also produced (to a much lesser extent) by several other normal tissues such as breast ducts and renal distal tubules [2]. MCA levels are not elevated in over 95% of patients with localised