Jpn. J. Cancer Res. **89**, 1326–1333, December 1998

EAT/mcl-1, a Member of the *bcl-2* Related Genes, Confers Resistance to Apoptosis Induced by *cis*-Diammine Dichloroplatinum (II) via a p53-Independent Pathway

Takashi Ando,[1,2] Akihiro Umezawa,[1] Atsushi Suzuki,[1] Hajime Okita,[1] Makoto Sano,[1] Yoshiki Hiraoka,[3] Sadakazu Aiso,[3] Takao Saruta[2] and Jun-ichi Hata[1,4]

[1]*Department of Pathology,* [2]*Internal Medicine and* [3]*Anatomy, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582*

EAT/mcl-1 showed increased expression during the differentiation of a multipotent human embryonic carcinoma cell line, NCR-G3, and of myeloblastic cells "ML-1," and has sequence similarity to Bcl-2. In this present study, we determined whether the apoptotic cell death induced by chemotherapeutic agents could be inhibited by EAT/mcl-1, as has been found with Bcl-2. Cells transfected with EAT/mcl-1 showed higher resistance to *cis*-diammine dichloroplatinum (II) (CDDP) and carboplatin compared with the parental line (10)1 and neomycin-resistance gene-transfected clone, (10)1/*neo*. There was, however, no difference in sensitivity to etoposide, *N*,*N*-bis-(2-chloroethyl)-*N′*-(3-hydroxypropyl) phosphordiamidic acid cyclic ester monohydrate, adriamycin or other chemotherapeutic agents tested. DNA fragmentation of the parental cells following treatment with CDDP and carboplatin was observed in a concentration-dependent manner. In contrast, cells transfected with EAT/mcl-1 did not show DNA fragmentation following treatment with the same concentration of these drugs. EAT/mcl-1 was capable of delaying the onset of p53-independent apoptosis, although it could not inhibit apoptosis completely. Since CDDP and carboplatin damage DNA and then activate c-abl and the JNK/SAPK pathway, EAT/mcl-1 may inhibit p53-independent apoptosis through a c-abl/JNK (SAPK)-dependent mechanism. EAT/mcl-1 has functional homology to Bcl-2 in that it can enhance cell viability under conditions which otherwise cause apoptosis and increase resistance to chemotherapeutic agents.

Key words: mcl-1/EAT — Apoptosis — *cis*-Diammine dichloroplatinum (II) — bcl-2 — p53

We previously established a multipotent human embryonic carcinoma (EC) cell line, NCR-G3, to serve as a model system of early human embryogenesis.[1)] NCR-G3, which is derived from a testicular EC, possesses pluripotency in differentiation, and is capable of differentiation to trophectoderm cells upon exposure to retinoic acid (RA) or heat shock.[2,3)] These differentiated cells produce human chorionic gonadotropin, a trophectoderm-specific hormone.[1)] We employed this system to search for molecules essential for EC cell differentiation. *EAT/mcl-1* gene was found to be one such gene; it is up-regulated during the early stage of EC cell differentiation.[3)] *EAT/mcl-1* was originally identified as a gene which is induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in myeloid leukemic cells.[4)] In both cell types, gene expression is increased at the early stages of differentiation upon exposure to inducers.

Since its discovery, EAT/mcl-1 has attracted much interest from various researchers because of its sequence similarity to *bcl-2*. This group of genes differs from many oncogenes in that it can promote cell survival or inhibit apoptosis without promoting cell proliferation.[5)] The ability of Bcl-2 to inhibit apoptosis has been observed in many different cell types, as well as under a variety of conditions, including incubation of sensitive cells with glucocorticoids, removal of required growth factors, or exposure to cytotoxic drugs, γ-radiation or apoptosis-inducing gene products such as c-Myc.[6–8)]

Several other proteins with sequence similarity to Bcl-2 can also influence cell survival. Bcl-xL was isolated by low stringency hybridization using *bcl-2* gene as a probe; Bcl-xL is even more efficacious than Bcl-2 in promoting cell survival under certain apoptosis-inducing conditions.[9)] Another family member, Bax, was discovered as a gene product that can heterodimerize with Bcl-2[10)]; the heterodimer appears to protect cells, whereas Bax homodimers cause accelerated apoptosis.[11)] Related proteins have been identified in other species, all of which have sequence similarity to the carboxyl region of Bcl-2. The Ced-9 protein of *Caenorhabditis elegans* prevents programmed cell death during development of this nematode.[12)] The Epstein-Barr virus protein BHRF1 can also enhance cell viability.[13,14)]

Considering the sequence similarity between EAT/mcl-1 and this Bcl-2 family, it is speculated that EAT/mcl-1 might also influence cell viability. A previous study has

[4] To whom correspondence should be addressed.

shown that apoptosis, induced by c-*myc* overexpression in Chinese Hamster Ovary (CHO) cell line, was delayed by EAT/mcl-1.[15] Apoptosis induced by etoposide was also reported to be inhibited by EAT/mcl-1 in FDP-C1, murine myeloid progenitor cells.[16] However, little is known about the mechanism by which EAT/mcl-1 contributes to apoptosis. Based on the studies so far reported, several apoptotic pathways have been postulated.[17] Although apoptosis and its inhibition by Bcl-2 have been well documented,[18–22] similar mechanisms involving EAT/mcl-1 have not been fully elucidated. In this study, we examined whether EAT/mcl-1 overexpression could affect apoptosis induced by chemotherapeutic agents such as *cis*-diammine dichloroplatinum (II) (CDDP), alkylating agents, antibiotics and topoisomerase inhibitors in (10)1 mouse fibroblast cell line.[23]

MATERIALS AND METHODS

Cell line and culture Mouse p53-deficient (10)1 fibroblast cell line[23] was grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Plasmids A cDNA clone containing the entire EAT/mcl-1 coding region was excised with *Pst*I and *Apa*I from the Bluescript SK(−) vector and blunted. After addition of *Pst*I linker and digestion with *Pst*I, the 2.4-kb fragment obtained was inserted into the *Pst*I site of the pSRα vector. This places EAT/mcl-1 under the SRα promoter. The plasmid pSRα-*neo* was constructed by cloning the neomycin resistance gene-containing fragment into *Eco*RI/*Hin*dIII-cut pBR322-SRα.

DNA transfection and amplification Cells were transfected by the Lipofectin method (Gibco BRL, Gaithersburg, MD) according to the instructions of the manufacturer. (10)1 cells were plated at a density of 2×10^5 cells/60 mm dish and grown overnight. Five micrograms of pSRα-*neo* with or without 0.5 μg of pSRα-EAT/mcl-1 and 10 μg of Lipofectin reagents were added to cells and incubation was continued for 48 h. Cells were grown at a concentration of 600 μg/ml of G418 for approximately 2 weeks.

Immunoblotting assay Immunoblot analysis for EAT/mcl-1 protein was carried out as previously described.[24] Preblocked blots were reacted with rabbit polyclonal antibody against human EAT/mcl-1 (Pharmingen, San Diego, CA) at a 1:2000 dilution in Tris-buffered saline (pH 7.6) with 0.05% Tween 20 (TBS-T) at room temperature for 1 h and then incubated with a 1:2000 dilution of peroxidase-conjugated swine anti-rabbit IgG antibody (Zymed, San Francisco, CA) in TBS-T for 1 h. For immunocytochemistry, we generated a monoclonal antibody, 3A2. The 3A2 monoclonal antibody specifically reacts with human EAT/mcl-1. Immunoblots were developed by using the ECL western blotting detection system (Amersham International, Amersham, UK). Prestained molecular weight markers ("Rainbow" proteins) were obtained from Amersham.

DNA fragmentation in (10)1 cells (10)1 cells (5×10^6 cells) were harvested and suspended in 200 μl of DNA extraction buffer (10 m*M* Tris-HCl [pH 7.4], 10 m*M* EDTA, 0.5% Triton X-100, 0.4 mg/ml RNase A). After incubation at 37°C for 1 h, a 2-μl aliquot of 20 mg/ml proteinase K was added and the mixture was further incubated for an additional hour. Then the sample was analyzed by electrophoresis in 2% agarose gels followed by ethidium bromide staining. The stained gels were photographed under a UV illuminator.

RNA blot analysis RNA was prepared by a standard method.[25] Twenty micrograms of total cellular RNA was denatured with glyoxal, then electrophoresed on a 1.0% agarose gel and blotted onto GeneScreen Plus membranes (NEN Research Products, Boston, MA). The membranes were hybridized with cDNA inserts labeled with [α-^{32}P]-dCTP by the random-primer method at 65°C for 14–16 h in a buffer containing 5× SSPE (1× SSPE is 0.18 mol of NaCl, 10 mmol of NaH_2PO_4/Na_2HPO_4 [pH 7.4], and 1 mmol of EDTA), 5× Denhardt's (1× Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 1% sodium dodecyl sulfate (SDS), and 10 mg/ml poly(A). The blots were washed with 2× SSC (1× SSC is 0.15 mol of NaCl, 50 mmol of NaH_2PO_4, and 5 mmol of EDTA), then with 1× SSC containing 1% SDS at room temperature and 65°C. Final washings were with 0.1× SSC containing 0.1% SDS at 65°C. A 2.4-kb full length cDNA designated as EAT/mcl-1 probe was used as a probe.

RESULTS

Chemotherapy is widely used in cancer treatment and many agents are known to induce apoptosis through p53-dependent pathways.[26–28] Resistance to chemotherapeutic agents has been reported to be acquired by functional mutations of p53 in tumors.[29, 30] Bcl-2 in certain ovarian cell lines expressing mutant p53 was found to further enhance the levels of resistance to CDDP, one of the most commonly used agents in cancer treatment.[31] To determine the role of *EAT/mcl-1* gene in resistance to chemotherapeutic agent-induced cell death via p53-independent pathway(s), we transfected EAT/mcl-1 into (10)1 cells, which do not express p53.[23]

Generation of (10)1 cells expressing EAT/mcl-1 Cells were transfected with pSRα-EAT/mcl-1 in addition to pSRα-*neo* (denoted as EAT/n). The gene was expressed in 10 of 12 G418-resistant clonal cell populations as assessed by RNA blot hybridization to an EAT/mcl-1

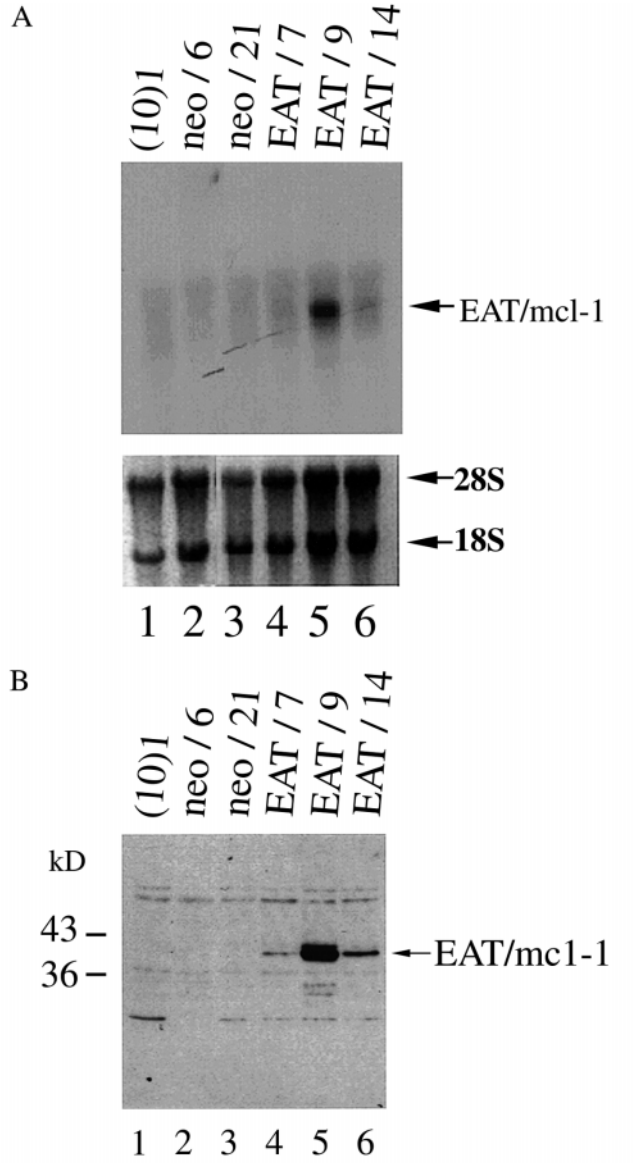

Fig. 1. Expression of EAT/mcl-1 in (10)1, p53-deficient cells. A. Expression of *EAT/mcl-1* gene exogenously transfected in (10)1 cells. Stable transformant (10)1 cells with EAT/mcl-1 were obtained, subcloned and designated as EAT/7, EAT/9, and EAT/14. The neo/6 and neo/21 cells, which were transfected with the neomycin-resistance gene, served as the controls. (10)1 is the parental line and does not express EAT/mcl-1. The position of the specific EAT/mcl-1 transcript is shown by an arrow. General RNA degradation was not observed, since similar amounts of the 28S and 18S rRNAs were recovered from all the cells (bottom). B. Immunoblot analysis of (10)1 cells transfected with the *EAT/mcl-1* gene. Sixty micrograms of total protein from each cell line [(10)1, neo/6, neo/21, EAT/7, EAT/9 and EAT/14, respectively, from left to right] was electrophoresed. The blot was probed with rabbit polyclonal antibody to human EAT/mcl-1. The position of EAT/mcl-1 (predicted molecular weight of 37 kilodaltons) is indicated by an arrow on the right. Estimated molecular size is shown in the left.

cDNA probe. Three cell populations expressing EAT/mcl-1 mRNA and protein at different levels (Fig. 1) were chosen for further analysis. Two clonal populations of control G418-resistant cells transfected with pSRα-*neo* were also chosen.

(10)1 cells and *neo*/n had no detectable EAT/mcl-1 expression at the mRNA level (Fig. 1A). Each EAT/n clonal population expressed the *EAT/mcl-1* gene at different levels; EAT/9 expresses the highest level of EAT/mcl-1 and EAT/7 the lowest, relative to (10)1 rRNA accumulation. Similarly, as evaluated by immunoblot analysis, EAT-transfected cells produced various levels of EAT/mcl-1 (Fig. 1B). The level of EAT/mcl-1 protein was proportional to the transcriptional level found by RNA blot analysis. The exogenously transfected EAT/mcl-1 protein

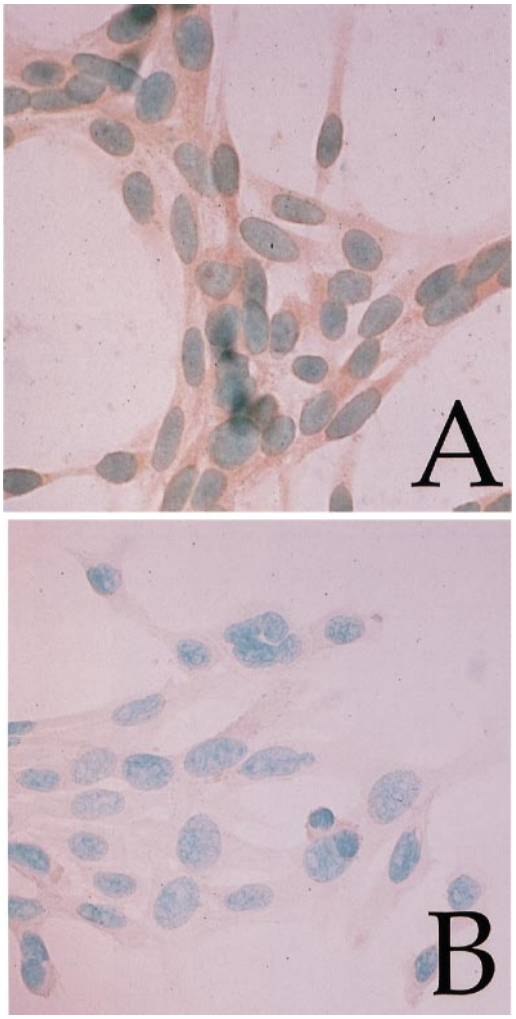

Fig. 2. Immunohistochemical analysis of (10)1 cells transfected with EAT/mcl-1. (10)1 cells (EAT/9) transfected with EAT/mcl-1 show a positive reaction in their cytoplasm (A), while neo/6 cells do not express the EAT/mcl-1 protein (B).

is localized in the cytoplasm, suggesting that exogenous EAT/mcl-1 may also be localized in the same site (Fig. 2). There was no remarkable morphological difference between the parental cell line and transfected clones as observed by phase contrast microscopy.

Apoptotic cell death was induced by CDDP in a time-dependent and concentration-dependent manner To investigate the response of (10)1 to chemotherapeutic agents, we first incubated (10)1 with several concentrations of CDDP and estimated the cell viability at several time points. Cell death of (10)1 was induced by CDDP in a time-dependent manner, and also in a concentration-dependent manner. As the concentration of the drugs was increased, the cell started to die at an early time-point after treatment. The appearance of dying cells was compatible with the specific features of apoptosis (Fig. 3). The fragmentation of genomic DNA into oligo-nucleosomal-sized units was also detected (Fig. 4).

Altered resistance of transfected cells to chemotherapeutic agents (10)1, *neo*/n, and EAT/n clonal populations were tested for relative viability in the presence of several chemotherapeutic agents including CDDP, carboplatin, methotrexate (MTX), etoposide (VEP), mitomycin (MMC), adriamycin (ADM), cyclophosphamide (CPA) and dexamethasone (DEX). EAT/9 and EAT/14 cells showed significant resistance to the toxic effects of CDDP compared with *neo*/n cells, while EAT/7 cells did not show significant resistance (Fig. 5A). The difference between control cells and EAT/n cells was most distinct with CDDP and carboplatin (Fig. 5B). The cell death of transfected EAT/9 and EAT/14 cells was delayed approximately 48 to 72 h compared with that of *neo*/n cells. In contrast to CDDP and carboplatin, the sensitivity of EAT/n cells upon exposure to MTX and VEP was similar to that of 10(1) and 10(1)/neo cells; resistance to apoptosis was not detected with these agents (Fig. 5B). Similarly,

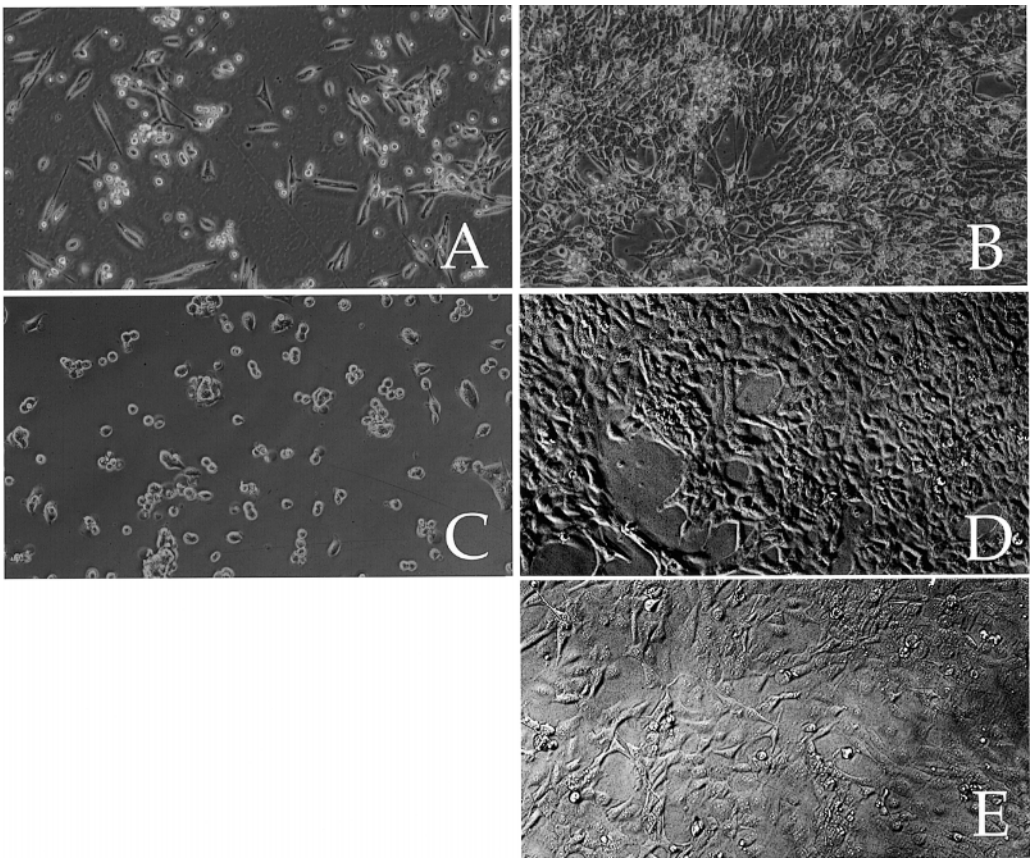

Fig. 3. EAT/mcl-1 confers CDDP resistance in (10)1 cells, which are p53-deficient. EAT/9 (B and D) cells showed increased resistance to apoptosis induced by CDDP as compared to neo/6 (A and C) control cells. Cells were exposed to 5 μg/ml (upper column, A and B) and 1.5 μg/ml (C, D and E) of CDDP for 24 h. The low cell number of neo/6 as compared to EAT/9 may be due in part to the fact that neo/6 cells treated with CDDP became round in shape and detached from the culture dish. EAT/14 (E) showed similar morphology to EAT/9.

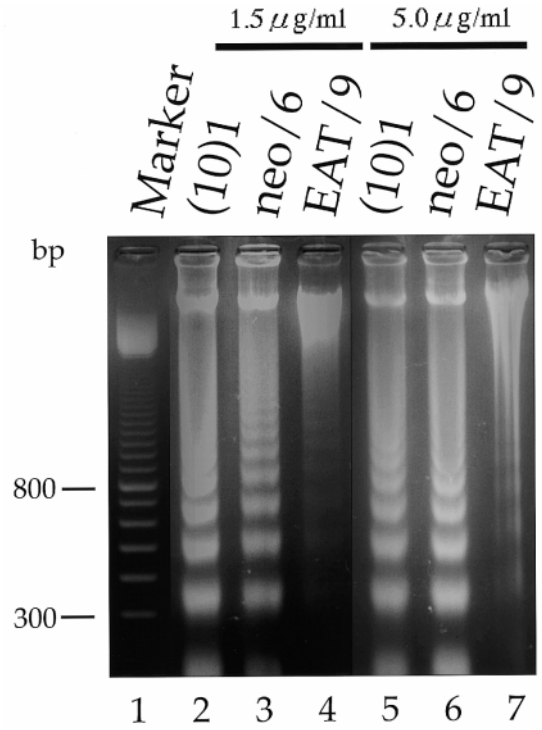

Fig. 4. DNA fragmentation of apoptotic cells. Cells were treated with 1.5 μg/ml and 5 μg/ml of CDDP for 72 h. After treatment, genomic DNA was isolated and electrophoresed in 2% agarose gel. A distinct nucleosomal ladder of 200 bp was observed in neo/6 control cells and (10)1 parental cells. Genomic DNA from EAT/9 showed slight DNA fragmentation, although the fragmentation was apparently less than that of neo/6 cells. As a size marker, a 100 bp ladder is shown in the left lane.

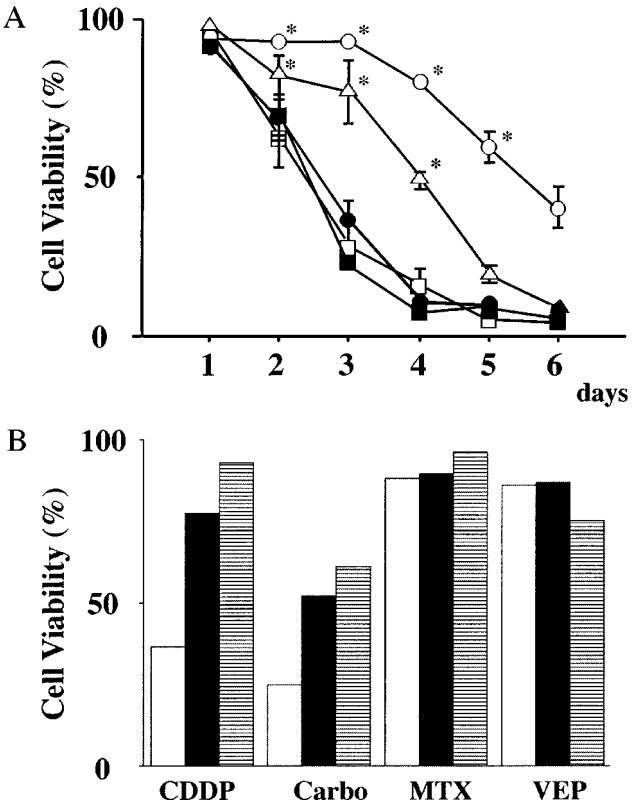

Fig. 5. EAT/mcl-1 confers CDDP resistance in (10)1 cells, which are p53-deficient. A. The kinetics of cell death in cells transfected with neomycin or EAT/mcl-1. Each cell was exposed to 1.5 μg/ml of CDDP for the indicated number of days. Cell viability was determined in terms of loss of membrane integrity as measured by trypan blue dye. Each point is the mean and standard error from triplicate experiments in different dishes. More than 200 cells were counted in each experiment. EAT/9 and EAT/14 cells showed significant resistance to CDDP, compared with neo/6 and neo/21 control cells (* $P<0.01$). ○ EAT/14, △ EAT/9, □ EAT/7, ■ neo/21, ● neo/6. B. The relative survival of cells transfected with neomycin or EAT/mcl-1. Each cell line was exposed to 1.5 μg/ml of CDDP for 3 days, 90 μg/ml of carboplatin for 2 days, 300 μg/ml of MTX for 2 days and 100 μg/ml of VEP for 1 day. □ neo/6, ■ EAT/9, ▥ EAT/14.

EAT/n cells did not exhibit resistance to treatment with MMC, ADM, CPA and DEX (data not shown).

While expression of endogenous EAT/mcl-1 was not detected in (10)1 and (10)1/neo cells, the levels of EAT/mcl-1 transcripts varied in transfected (10)1/EAT cell lines (Fig. 1). Despite this variation, protection (in terms of DNA fragmentation and cell viability) was apparent in EAT/9 and EAT/14 cells. EAT/7 cells without expression of transfected EAT/mcl-1 were indistinguishable from mock-transfected cells. The lack of an expression level-dependent relationship between EAT/mcl-1 transcripts and the ability to delay the onset of apoptosis suggests that these transfectants contained levels of EAT/mcl-1 above the threshold for protection from apoptosis, as previously reported.[15]

Since a survival role for EAT/mcl-1 in the regulation of apoptosis has been postulated,[4, 15] overexpression of EAT/mcl-1 in (10)1 cells may have increased the ability of those cells to form colonies. If so, EAT/n cells would exhibit increased plating efficiency in comparison with *neo*/n and (10)1 controls. An association between plating efficiency in the absence of these agents and EAT/mcl-1 expression was not observed (data not shown). Furthermore, the growth rate of cells expressing EAT/mcl-1 was not significantly different from that of the control cells, nor did G418 exposure affect the growth of G418-resistant cells.

A major concern is whether the resistance to CDDP-induced apoptosis as conferred by EAT/mcl-1 allowed the

cells to survive and then regrow, or whether cell death was merely delayed. We examined the viability of CDDP-treated (10)1 cells at several time points (Fig. 5A). When (10)1 cells were treated with CDDP, EAT/9 and EAT/14 clones were more resistant than the controls. The survival fractions of drug-treated EAT/9 and EAT/14 clones were always higher than those of (10)1 or *neo*/n, but cell death was apparent at later time points.

DISCUSSION

Does EAT/mcl-1 modulate apoptosis via a p53-independent pathway? Considering the sequence similarity between EAT/mcl-1 and Bcl-2,[3, 32] and the fact that other family members have been reported to influence cell viability, we speculated that EAT/mcl-1 might influence apoptotic pathways and contribute to differentiation through modulation of cell viability. The present studies show that EAT/mcl-1 delayed cell death induced by CDDP and carboplatin in this system. Since drug resistance can be partly attributed to decreased cellular susceptibility to apoptosis,[33–38] these data, therefore, support the idea that EAT/mcl-1 contributes to cell survival.

Since (10)1 cells used in this study lack the p53 protein, apoptotic events occur independently of p53. p53 is reported to be involved in the activation of apoptosis, but p53-independent apoptotic pathways also exist.[39, 40] Although p53 has been shown to induce cell cycle arrest and gene expression (*p21*/*waf-1*/*cdi-1*), the mechanism underlying the p53-independent apoptotic pathway involving EAT/mcl-1 remains unclear.

The inhibition of apoptosis by EAT/mcl-1 may be directly correlated with the mechanism of CDDP-induced cell death.[37, 41, 42] The inhibition of DNA repair by adduction of CDDP to DNA is probably the major mechanism contributing to apoptosis. Following such DNA damage by CDDP, c-abl is involved and then the JNK (SAPK) pathway is activated.[43–45] Since EAT/mcl-1 inhibits release of cytochrome *c* from mitochondria, EAT/mcl-1 may function downstream of c-abl/JNK (SAPK) and therefore inhibit apoptosis induced by CDDP or carboplatin. The other possibility is that EAT/mcl-1 affects the accumulation of CDDP, and thus changes the chemosensitivity.[46] EAT/mcl-1 may induce or interact with an efflux pump for these drugs.

Relatively weak modification of apoptosis by EAT/mcl-1 may be attributed to lack of p53 Since the ratio of bcl-2:bax plays an important role in the induction of apoptosis,[17] the possibility of transactivation of other *bcl-2* gene family members by p53 must be taken into consideration. Since (10)1 cells do not possess wild-type p53, the ratio of molecules which determine the induction of apoptosis may be disturbed. This may lead to the slight modification of apoptotic cell death observed with EAT/mcl-1. If this is the case, we cannot completely eliminate the possibility that failure of EAT/mcl-1 to inhibit apoptosis induced by CPA, ADM and VEP is limited in the cells used in this study. Inhibition of apoptosis by bcl-2 depends on the cells used. In p53-deficient lymphoma cells, bcl-2 significantly inhibits apoptosis by VEP, CDDP and MMC.[47] In contrast, bcl-2 does not prevent apoptosis by CDDP, VEP and MTX in lung small cell carcinoma cells.[48, 49]

Clinical implications of EAT/mcl-1 Our data provide further evidence of the ability of this protein to confer a survival advantage upon tumor cells.[43, 50] We may consider a scenario *in vivo* in which a mixed population of EAT/mcl-1 positive and negative tumor cells are differentially susceptible to chemotherapy, and in which the more susceptible EAT/mcl-1 negative tumor cells are more rapidly cleared out, resulting in an initial response to chemotherapy, while a resistant EAT/mcl-1 positive cell population develops which may be associated with subsequent relapse.

ACKNOWLEDGMENTS

The authors thank J. Ozawa, K. Kido, S. Kusakari, H. Abe and M. Takahashi for their technical assistance, K. Takeichi for the photograph, and Drs. T. Yamada, F. Urano, and A. Hashiguchi for helpful discussions. This work was supported by a grant from the Ministry of Education, Science, Sports and Culture to J. H. and A. U., by a Keio University Special Grant-in-Aid for Innovative Collaborative Research Project to J. H. and A. U., by Keio Gijuku Fukuzawa Memorial Funds for the Advancement of Education and Research from Keio University to H. O., and by a National Grant-in-Aid for the Establishment of a High-Tech Research Center at Private Universities.

(Received June 11, 1998/Revised August 19, 1998/2nd Revised September 11, 1998/Accepted September 18, 1998)

REFERENCES

1) Hata, J.-I., Fujimoto, J., Ishii, E., Umezawa, A., Kokai, Y., Matsubayashi, Y., Abe, H., Kusakari, S., Kikuchi, H., Yamada, T. and Maruyama, T. Differentiation of human germ cell tumor cells *in vivo* and *in vitro*. *Acta Histochem. Cytochem.*, **25**, 563–576 (1992).
2) Maruyama, T., Umezawa, A., Kusakari, S., Kikuchi, H., Nozaki, M. and Hata, J.-I. Heat shock induces differentiation of human embryonal carcinoma cells into trophectoderm lineages. *Exp. Cell Res.*, **224**, 123–127 (1996).
3) Umezawa, A., Maruyama, T., Inazawa, J., Imai, S.-I., Takano, T. and Hata, J.-I. Induction of mcl1/EAT, bcl-2 related gene, by retinoic acid or heat shock in the human

embryonal carcinoma cells, NCR-G3. *Cell Struct. Funct.*, **21**, 143–150 (1996).
4) Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P. and Craig, R. W. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc. Natl. Acad. Sci. USA*, **90**, 3516–3520 (1993).
5) Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334–336 (1990).
6) Tsujimoto, Y. Stress-resistance conferred by high level of bcl-2 alpha protein in human B lymphoid cell. *Oncogene*, **4**, 1331–1336 (1989).
7) Siegel, R. J., Bueso, R. C., Cohen, C. and Koss, M. Pulmonary blastoma with germ cell (yolk sac) differentiation: report of two cases. *Mod. Pathol.*, **4**, 566–570 (1991).
8) Bisonette, R. P., Echeverri, F., Mahboubi, A. and Green, D. R. Apoptotic cell death induced by c-*myc* is inhibited by *bcl-2*. *Nature*, **359**, 552–554 (1992).
9) Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Linsten, T., Turka, L. A., Mao, X., Nunez, G. and Thompson, C. B. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597–608 (1993).
10) Oltavi, Z. N., Milliman, C. L. and Korsmeyer, S. J. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–619 (1993).
11) Yin, X. M., Oltavi, Z. N. and Korsmeyer, S. J. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature*, **369**, 321–323 (1994).
12) Hengartner, M. O., Ellis, R. E. and Horvitz, H. R. *Caenorhabditis elegans* gene ced-9 protects cells from programmed cell death. *Nature*, **356**, 494–499 (1992).
13) Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G. and Rickinson, A. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cell from programmed cell death. *Proc. Natl. Acad. Sci. USA*, **90**, 8479–8483 (1993).
14) Tarodi, B., Subramanian, T. and Chinnadurai, G. Epstein-Barr virus BHRF1 protein protects against cell death induced by DNA-damaging agents and heterologous viral infection. *Virology*, **201**, 404–407 (1994).
15) Reynolds, J. E., Yang, T., Quian, L., Jenkinson, J. D., Zhou, P., Eastman, A. and Craig, R. W. Mcl-1 a member of the Bcl-2 family, delays apoptosis induced by c-myc overexpression in Chinese hamster ovary cells. *Cancer Res.*, **54**, 6348–6352 (1994).
16) Zhou, P., Qian, L., Kozopas, K. M. and Craig, R. W. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. *Blood*, **89**, 630–642 (1997).
17) Reed, C. J. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematol. Oncol. Clin. North Am.*, **9**, 451–473 (1995).
18) Mack, R. B., Katz, S. M. and Amenta, P. S. Choriocarcinoma of the kidney. *J. Am. Osteopath. Assoc.*, **92**, 799–802 (1992).
19) Miyashita, T. and Reed, J. C. *bcl-2* gene transfer increases relative resistance of S49.1 and WEH17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.*, **52**, 5407–5411 (1992).
20) Miyashita, T. and Reed, C. J. bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*, **81**, 151–157 (1993).
21) Lotem, J. and Sachs, L. Regulation of bcl-2, bcl-Xl and bax in the control of apoptosis by hematopoietic cytokines and dexamethasone. *Cell Growth Differ.*, **6**, 647–653 (1995).
22) Weller, M., Malipiero, U., Aguzzi, A., Reed, C. J. and Fontana, A. Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. *J. Clin. Invest.*, **95**, 2633–2643 (1995).
23) Harvey, D. M. and Levine, A. J. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes Dev.*, **5**, 2375–2385 (1991).
24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
25) Umezawa, A., Tachibana, K., Harigaya, K., Kusakari, S., Kato, S., Watanabe, Y. and Takano, T. Colony-stimulating factor 1 is downregulated during the adipocyte differentiation of H-1/A marrow stromal cells and induced by cachectin/tumor necrosis factor. *Mol. Cell. Biol.*, **11**, 920–927 (1991).
26) Clarke, A., Purdie, C., Harrison, D., Morris, R., Bird, C., Hooper, M. and Wyllie, A. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849–852 (1993).
27) Lowe, S., Rulley, H., Jacks, T. and Housman, E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, **74**, 957–967 (1993).
28) Lowe, S., Schmitt, E., Smith, S., Osborne, B. and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847–849 (1993).
29) Lee, J. and Bernstein, A. p53 mutations increase resistance to ionizing radiation. *Proc. Natl. Acad. Sci. USA*, **90**, 5742–5746 (1993).
30) Sachs, L. and Lotem, J. Control of programmed cell death in normal and leukemic cells: new implications for therapy. *Blood*, **82**, 15–21 (1993).
31) Eliopoulos, A., Kerr, D., Herod, J., Hodgkins, L., Krajewski, S., Reed, J. and Young, L. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene*, **11**, 1217–1228 (1995).
32) Xiao-Ming, Y., Zoltan, N. O. and Stanley, J. K. BH1 and BH2 domains of Bcl-2 are required for inhibition of apo-

ptosis and heterodimerization with Bax. *Nature*, **369**, 321–323 (1994).
33) Dive, C. and Hickman, J. Drug-target interactions: only the first step in the commitment to a programmed cell death? *Br. J. Cancer*, **64**, 192–196 (1991).
34) Dive, C. and Wyllie, A. Apoptosis and cancer chemotherapy. *In* "Cancer Chemotherapy," ed. J. Hickman and T. Tritton, pp. 21–56 (1993). Blackwell Scientific Publications, Oxford.
35) Eastman, A. and Barry, M. Apoptosis as a target for cancer chemotherapy. *In* "New Molecular Targets for Cancer Chemotherapy," ed. D. Kerr and P. Workman, pp. 143–158 (1994). CRC Press, London.
36) Fritsche, M., Haessler, C. and Brandner, G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene*, **8**, 307–318 (1993).
37) Eastman, A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells*, **2**, 275–280 (1990).
38) Kondo, S., Barna, P. B., Morimura, T., Takeuchi, J., Yuan, J., Akbasak, A. and Barnett, H. G. Interleukin-1 beta-converting enzyme mediates cisplatin-induced apoptosis in malignant glioma cells. *Cancer Res.*, **55**, 6166–6171 (1995).
39) Fernandez-Sarabia, M. J. and Bischoff, J. R. Bcl-2 associates with the *ras*-related protein R-*ras* p23. *Nature*, **366**, 274–275 (1993).
40) Merlo, G. R., Basolo, F., Fiore, L., Duboc, L. and Hynes, N. E. p53-dependent and p53-independent activation of apoptosis in mammary epithelial cells reveals a survival function of EGF and insulin. *J. Cell Biol.*, **128**, 1185–1196 (1995).
41) Chen, Z., Naito, M., Mashima, T. and Tsuruo, T. Activation of actin-cleavable interleukin 1 beta-converting enzyme (ICE) family protease CPP-32 during chemotherapeutic agent-induced apoptosis in ovarian carcinoma cells. *Cancer Res.*, **56**, 5224–5229 (1996).
42) Zamble, D. and Lippard, S. Cisplatin and DNA repair in cancer chemotherapy. *Trends Biochem. Sci.*, **20**, 435–439 (1995).
43) Seimiya, H., Mashima, T., Toho, M. and Tsuruo, T. c-Jun NH2-terminal kinase-mediated activation of interleukin-1 beta converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. *J. Biol. Chem.*, **272**, 4631–4636 (1997).
44) Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M., Gjerset, R. and Mercola, D. The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J. Biol. Chem.*, **272**, 14041–14044 (1997).
45) Kharbanda, S., Ren, R., Pandey, P., Shafman, T., Feller, S., Weichselbaum, R. and Kufe, D. Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature*, **376**, 785–788 (1995).
46) Chen, Z. S., Mutoh, M., Sumizawa, T., Furukawa, T., Haraguchi, M., Tani, A., Saijo, N., Kondo, T. and Akiyama, S. An active efflux system for heavy metals in cisplatin-resistant human KB carcinoma cells. *Exp. Cell Res.*, **240**, 312–320 (1998).
47) Strasser, A., Harris, A., Jacks, T. and Cory, S. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibited by Bcl-2. *Cell*, **79**, 329–339 (1994).
48) Okamoto-Kubo, S., Nishio, K., Heike, Y., Yoshida, M. and Saijo, N. Apoptosis induced by etoposide in small-cell lung cancer cell lines. *Cancer Chemother. Pharmacol.*, **33**, 385–390 (1994).
49) Ohmori, T., Podack, E. R., Nishio, K., Takahashi, M., Miyahara, Y., Takeda, Y., Kubota, N., Funayama, Y., Ogasawara, H., Ohira, T., Ohta, S. and Saijo, N. Apoptosis of lung cancer cells caused by some anti-cancer agents (MMC, CPT-11, ADM) is inhibited by bcl-2. *Biochem. Biophys. Res. Commun.*, **192**, 30–36 (1993).
50) Tsuruo, T., Hamilton, T. C., Louie, K. G., Behrens, B. C., Young, R. C. and Ozols, R. F. Collateral susceptibility of adriamycin-, melphalan- and cisplatin-resistant human ovarian tumor cells to bleomycin. *Jpn. J. Cancer Res.*, **77**, 941–945 (1986).