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Molecular characterization of the In Vivo alkylating agent resistant murine EMT-6 mammary carcinoma tumors

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Abstract The expression of several early-response genes and genes associated with malignant disease was assessed in the EMT-6/parent tumor and the EMT-6/CTX and EMT-6/CDDP in vivo resistant tumor lines growing as tumors or as monolayers in culture. In the absence of treatment the levels of mRNA for the genes c-*jun*, c-*fos*, c-*myc*, Ha-*ras* and *p53* were increased in the EMT-6/CTX and EMT-6/CDDP as compared with the EMT-6/parent tumor, whereas the expression of *erb*-2 was similar in all three tumors. Although the cells from each of the three tumors show increased expression of early response genes after exposure to cisplatin (CDDP; 100 μ*M*, 2 h) or 4-Hydroxyperoxycyclophosphamide (4-HC; 100 μ*M*, 2 h) in culture, in mRNA extracted from tumor tissue these changes are absent or very small. Both C-*jun* and *erb*-2 were detectable in liver. There was increased expression of both of these genes in the livers of tumor-bearing animals as compared with non-tumor-bearing animals. The highest expression of both c-*jun* and *erb*-2 occurred in the livers of animals bearing the EMT-6/CDDP tumor. Treatment of the animals with CDDP or cyclophosphamide, in general, resulted in increased expression of both genes at 6 h post treatment. The increased expression of these genes may impart metabolic changes in the tumors and/or hosts that contribute to the resistance of these tumors to specific antitumor alkylating agents.

Key words Antitumor alkylating agents · In vivo drug resistance · C-*jun* expression · *Erb*-2 expression · EMT-6 tumors

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Introduction

The group of drugs classified as the antitumor alkylating agents comprises small chemically reactive molecules that covalently attach to cellular components. The lethal lesion associated with exposure of cells to the antitumor alkylating agents is believed to be bifunctional binding of the drugs to DNA (76–78). The EMT-6 mammary carcinoma sublines resistant to the antitumor alkylating agents cyclophosphamide, thiotepa, cisplatin (CDDP), and carboplatin were produced by repeated exposure of fresh tumor-bearing hosts to each drug [78]. After ten treatments, metastable resistant tumors were produced. Although the tumors were resistant to drug treatment, the tumor cells in monolayer culture were not [78, 79]. As determined by the tumor cell-survival assay from tumors treated in vivo at a level of 1 log (90%) of cell killing, the EMT-6/CDDP tumor is 4-fold resistant to CDDP and the EMT-6/CTX tumor is 3-fold resistant to cyclophosphamide as compared with the EMT-6/parent tumor [78].

Antitumor alkylating agent exposure may be regarded as a stress such as that induced by carcinogens, heat, UV radiation, X-rays or hypoxia [28, 63, 65, 83]. Exposure to an antitumor alkylating agent may induce expression of programmed stress response(s) including induction of early-response genes [2, 7, 19, 21, 35, 36, 45, 51, 63–65, 72] such as *jun*, *fos* and *myc* [1, 5, 10, 12, 16, 18, 21–23, 26, 34, 37, 50, 52–55, 57–59, 61, 67–70, 73, 74, 80–82, 86–88] as well as genes involved in signal transduction such as *ras*, [6, 8, 9, 17, 31, 32, 40, 44, 46, 48, 49, 62, 72, 85] and growth-control genes such as *p53* and *erb-B* [3, 13–15, 24, 25, 29, 41, 42, 60, 66, 71, 84]. Because the EMT6 tumor lines are resistant *in vivo* but not when grown in monolayer culture, the expression of such genes may induce molecular events that aid survival through the action of autocrine or paracrine factors affecting distal normal tissues.

The current study was undertaken to determine whether expression of several genes associated with neoplasia was altered in the EMT-6/CTX or EMT-6/CDDP tumor and cells relative to the EMT-6/parent tumors and cells and whether exposure to alkylating agent stress provoked similar responses in these lines.

Materials and methods

Drugs

cis-Diamminedichloroplatinum(II) Cisplatin (CDDP) was a gift from Dr. Alfred Crosswell, Bristol-Myers-Squibb Co. (Wallingford, Conn.). Cyclophosphamide (CTX) was purchased from Sigma Chemical Co. (St. Louis, Mo). 4-Hydroxyperoxycyclophosphamide (4-HC) was a gift from Dr. J. Pohl, Asta Medica (Frankfurt am Main, Germany).

Tumor System.

The EMT-6/parent mouse mammary carcinoma grown as a solid tumor s.c. in the flanks of female BALB/c mice (Taconic Farms, Germantown, N.Y.) has been used widely in radiobiology and chemotherapy studies. We have established alkylating agent-resistant EMT-6 tumor lines by repeated treatment of tumor-bearing animals with CDDP (20 mg/kg) or CTX (300 mg/kg) injected i.p. 24 h before passage of each tumor line into fresh host animals ten times [78]. The parent tumor line was passaged in the same manner in the absence of drug treatment. The alkylating agent sublines designated: EMT-6/CDDP (resistant to CDDP) and EMT-6/CTX (resistant to cyclophosphamide) were maintained as frozen tumor brei in liquid nitrogen and used for experiments during the second and third tumor passages [78, 79].

RNA isolation and Northern-blot analysis.

RNA was isolated from exponentially growing EMT-6/parent, EMT-6/CDDP, and EMT-6/CTX early-passage cell monolayers without drug exposure or at various time points (0.5, 1, 3, 6 h.) after a 2 h. exposure to CDDP (100 μM) or 4-HC (100 μM). RNA was also isolated from tumor tissue of animals bearing EMT-6/parent, EMT-6/CDDP, and EMT-6 CTX tumors on day 8 after tumor cell implantation, when the tumors were about 100 mm^3 in volume, either with drug treatment or at various time points (1 or 6 h) after administration of CDDP (20 mg/kg, i.p.) or CTX (300 mg/kg, i.p.) to the animals. Finally, RNA was isolated from tumors and livers of animals bearing EMT-6/parent, EMT-6/CDDP, and EMT-6/CTX tumors at 4 or 8 days after tumor cell implantation without drug treatment or at 6 h after administration of CDDP (20 mg/kg, i.p.) or CTX (300 mg/kg, i.p.).

Total cellular RNA was isolated according to the method of Chomczynski. RNA was quantitated by absorbance at 260 nm and by comparison with known concentrations of yeast RNA standard that were electrophoresed through an agarose gel and stained with ethidium bromide. Total cellular RNA was electrophoresed through a 1% agarose:6% formaldehyde gel at 50 V overnight at 4°C. RNA was transferred onto a nitrocellulose membrane in 20 X SSC [1 X SSC = 0.15 M NaCl, 0.0125 M sodium citrate, (pH 7.0)] for 20 h. The filters were air-dried and baked in vacuo for 2 h. at 80°C. Pre-hybridization was done at 37°C for 16 h. in a solution containing 50% formamide, 5 X SSC, 5 X Denhardt's solution, 25 mM KPO_4 (pH 7.4) and 50 µg salmon-sperm DNA/ml. Hybridization was performed at 42°C in the same solution with the addition of 10% dextran sulfate and 10^6 cpm/ml of DNA probes labeled by random priming with [γ-^{32}P]-dATP or end labeling with [γ-^{32}P]-dATP. Filters were washed at room temperature for 5 min in a solution containing 1 X SSC and 0.1% sodium dodecyl sulfate (SDS). This was followed by two washes for 30 min each at 50°C in a solution containing 0.5 X SSC, 0.1% SDS and one wash for 30 min. at 50°C in 0.2 X SSC, 0.1% SDS. Filters were exposed to Kodak XAR film with intensifying screen at -80°C.

DNA Probes.

DNA probes were made by uniformly labeling specific gene fragments with [γ-^{32}P]-dCTP (NEN; specific activity, 3000 Ci/nmol), using the random primer protocol of Feinberg and Vogelstein. Unincorporated nucleotides were removed by Sephadex G-50 filtration and specific activity was determined by trichloroacetic acid precipitation. The specific activity of labeled fragments was typically $0.7–1.0 \times 10^9$ cpm/µg. Gene fragments used in hybridization studies were as follows:

1. v-Ha-*ras*–this probe was a 2.2 kb (*Bam*H1-*Eco*R1) fragment from clone HB-1.1 of *Escherichia coli*, purchased from ATCC (Rockville, Md.).
2. C-*jun*–this probe was a 2.6-kb (*Eco*R1) insert from clone JAC.1 of *E. coli* HB101 containing the plasmid pGEM2, purchased from ATCC (Rockville, Md.).
3. C-*fos*–this probe was a 1.3 kb (*Bgl*11-*Pvu*11) fragment from clone pfos-1 of *E. coli* MC1061 containing the plasmid pBR322 purchased from ATCC (Rockville, Md.) [79].
4. C-*myc*–this probe was a 1.5-kb (*Pst* 1) insert from clone pMyc3Pst of *E. coli* HB101 containing the plasmid pBR322, purchased from ATCC (Rockville, Md.).
5. *p53*–the *p53* probe was a 350-bp (*Xho*I-*Pvu*III) fragment from the murine p53 cDNA clone p53-71 obtained from the laboratory of Dr. Nelson Fausto, Brown University and originally described by Oren et al.
6. GAPDH–this probe was isolated from the *Pst*I-*Rsa*I fragment of HcGAP3, a partial cDNA clone representing 0.7 kb of the human glyceraldehyde 3-phosphate dehydrogenase gene described by Chatterjee et al. [11].
7. *Erb*-2–this probe was a 40 base oligonucleotide corresponding to exon 4 of the human c-*erb* B2/*neu* gene, purchased from Oncogene Science (Uniondale, N.Y.).

Densitometric Analysis.

Densitometric analysis of x-ray film images of the northern blots was carried out using a scanner (Hewlett-Packard Scanjet Plus Scanner). Peak area was determined using ImageQuant Software (version 3.15).

Results

Several characteristics of the EMT-6 tumors are shown in Table 1. All of these tumors are aneuploid with modal chromosome numbers ranging from 56 to 68. Normal diploid mouse cells have 40 chromosomes. The percentage of cells in the S phase is similar for the EMT/6 parent and the alkylating agent-resistant tumors. The EMT-6/parent and the in vivo alkylating agent-resistant tumors EMT-6/CTX and EMT-6/CDDP were grown to 100 mm^3 in female BALB/c mice. When these tumors were excised and total tissue

Table 1. DNA and cell cycle analysis of the EMT-6/parent, EMT-6/CTX, and EMT-6/CDDP tumors[a]

Tumor	DNA Index	% S phase	Modal Chromosome Number
EMT-6/parent	1.61	28.7	62–63
EMT-6/CTX	1.68	29.3	68
EMT-6/CDDP	1.65	24.6	56

[a] Normal diploid cells in mice have 40 chromosomes (20 pairs)

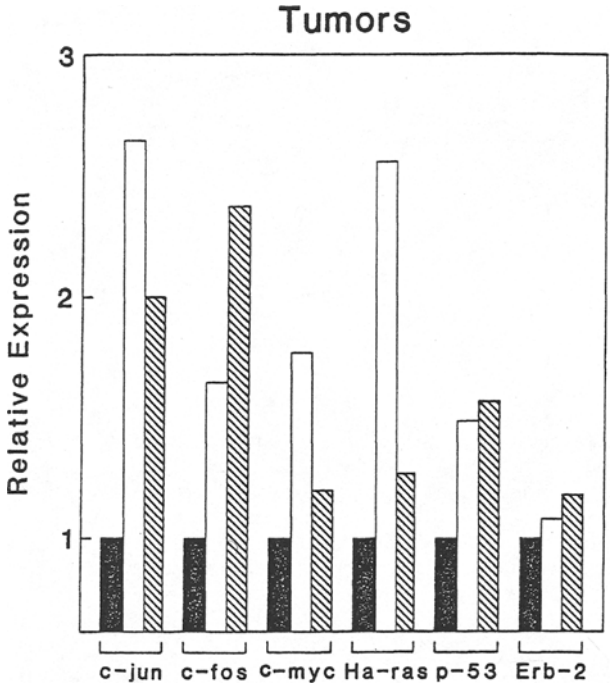

Fig. 1. Relative expression of c-*jun*, c-*fos*, c-*myc*, Ha-*ras*, *p*-53 and erb-2 mRNA in the EMT-6/parent (■), EMT-6/CTX (□) and EMT-6/CDDP (▨) tumors as determined by Northern blot analysis. The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

RNA was analyzed for expression of the early-response genes c-*jun*, c-*fos*, and c-*myc*, increased expression of each of these three messages was found in the EMT-6/CTX tumors and increased expression of c-*jun* and c-*fos* was found in the EMT-6/CDDP tumors relative to the EMT-6/parent tumor (Fig. 1). The expression of Ha-*ras* was higher in the EMT-6/CTX tumors than in the EMT-6/parent or EMT-6/CDDP tumors. The expression of *p53* was modestly elevated in both of the resistant tumors as compared with the parent tumor, and the expression of *erb*-2 was the same in all three tumors.

When grown in monolayer culture the EMT-6/CDDP cells had a lower expression of c-*jun* than did the EMT-6/parent or EMT-6/CTX cells. Stressing the cells by exposure to 4-HC (100 μ*M*, 2 h) or CDDP (100 μ*M*, 2 h) resulted in a marked elevation in c-*jun* expression in the EMT-6/parent cells and an even more robust response in the EMT-6/CTX cells, which peaked at 0.5–1 h after drug exposure (Fig. 2). There was no change in the expression of c-*jun* in the EMT-6/CDDP cells after exposure to the same drug treatments. Very small increases in c-*jun* expression that were not significant were observed in tumor tissues after treatment of the tumor-bearing animals with CTX (300 mg/kg) or CDDP (20 mg/kg).

The EMT-6/CDDP cells in monolayer culture had a 3-fold higher expression of c-*fos* mRNA than did the EMT-6/parent and EMT-6/CTX cells. The expression of c-*fos* markedly increased in all three cell lines after exposure to 4-HC and did not return to baseline at 6 h after drug exposure Fig. 3. Only the EMT-6/CDDP cells had a similarly robust increase in c-*fos* expression after exposure to CDDP. *In vivo*, the EMT-6/CDDP tumor showed increased expression of c-*fos* after treatment of tumor-bearing animals with CTX or CDDP, where there was no change in the expression of c-*fos* in the EMT-6/parent or EMT-6/CTX tumors after the same treatments.

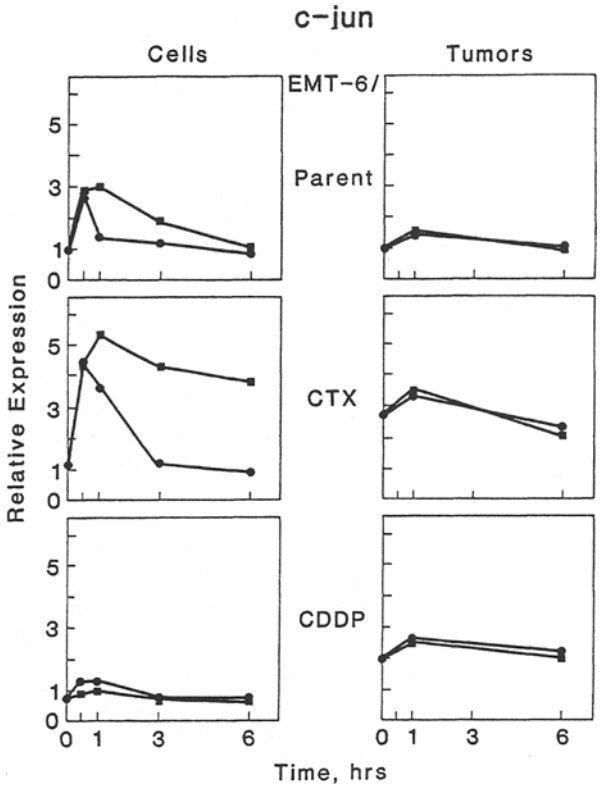

Fig. 2 Relative expression of c-*jun* mRNA in EMT-6/parent, EMT-6/CTX and EMT-6/CDDP cells grown as monolayers or tumors at various time points after treatment with: cells, 4-HC (100 μ*M*, (●) or CDDP (100 μ*M*) (■); tumor bearing animals, CTX (300 mg/kg) (●) or CDDP (20 mg/kg) (■). The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

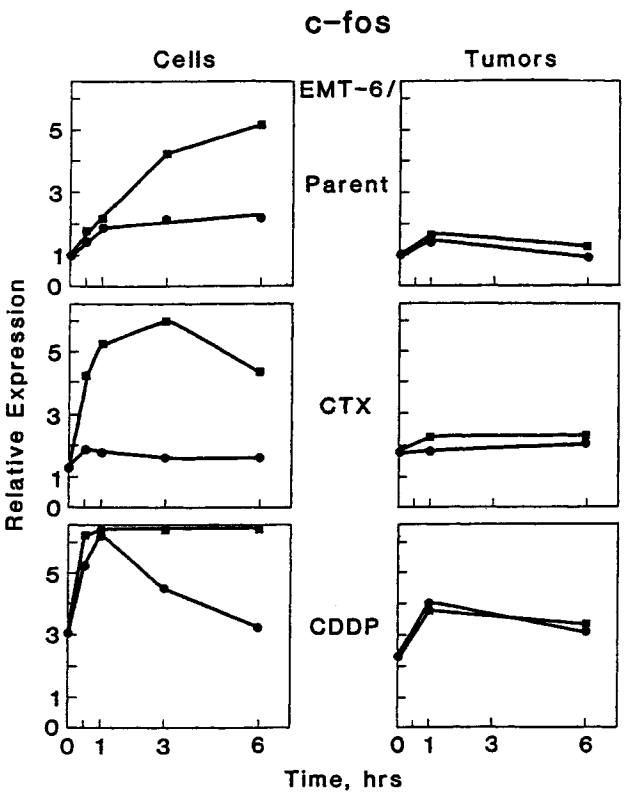

Fig. 3 Relative expression of c-*fos* mRNA in EMT-6/parent, EMT-6/CTX and EMT-6/CDDP cells grown as monolayers or tumors at various time points after treatment with: cells, 4-HC (100 μ*M*, ●) or CDDP (100 μ*M*, ■); tumor bearing animals, CTX (300 mg/kg, ●) or CDDP (20 mg/kg) (■). The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

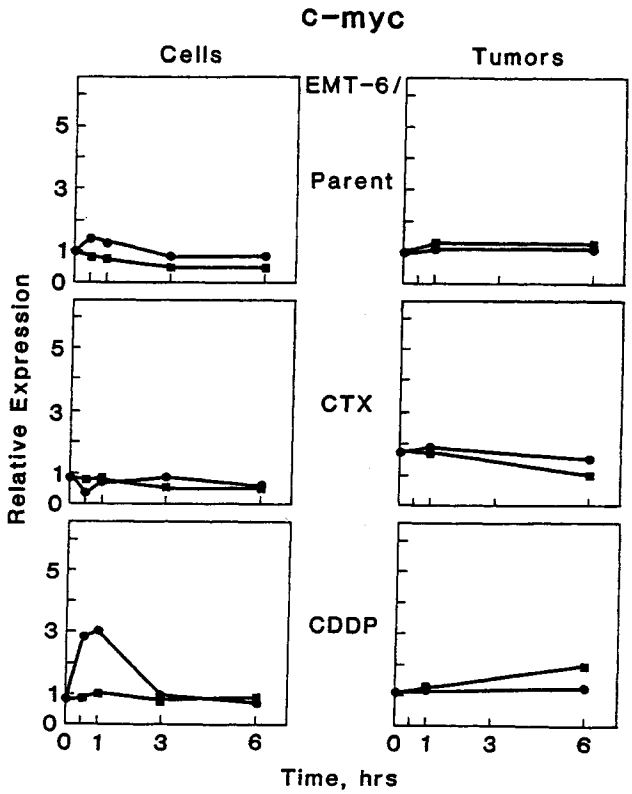

Fig. 4 Relative expression of c-*myc* mRNA in EMT-6/parent, EMT-6/CTX and EMT-6/CDDP cells grown as monolayer or tumors at various time points after treatment with: cells, 4-HC (100 μ*M*, ●) or CDDP (100 μ*M*, ■); tumor bearing animals, CTX (300 mg/kg) (●) or CDDP (20 mg/kg, ■). The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

There was no detectable change in the expression of c-*myc* in the EMT-6/parent or EMT-6/CTX cell monolayers or tumors in response to treatment with either antitumor alkylating agent (Fig. 4). The EMT-6/CDDP cells showed an early increase in c-*myc* mRNA after exposure to 4-HC that returned to baseline by 3 h after drug exposure. No change in c-*myc* expression was seen after exposure of the EMT-6/CDDP cells to CDDP or treatment of EMT-6/CDDP tumor-bearing animals with CTX or CDDP.

There was no major change in the expression of Ha-*ras* mRNA in response to treatment in either the monolayer cultures or the tumors, although a linear increase in Ha-*ras* expression was noted for up to 6 h. in EMT-6/parent tumors treated with 100 μ*M* CDDP (Fig. 5). Although small in magnitude, there was increased expression of *p53* mRNA in the EMT-6/CTX tumors treated with CDDP and the EMT-6/CDDP tumors treated with CTX and CDDP; however, no change in *p*53 expression was seen in the cells grown as monolayers after exposure of the cells to 4-HC or CDDP (Fig. 6). There was no change in the expression of *erb*-2 mRNA in the monolayer cultures or the tumors after treatment with the antitumor alkylating agents (Fig. 7).

Expression of c-*jun* and of *erb*-2 mRNA was detected in the livers of non-tumor-bearing female BALB/c mice and in the livers of animals bearing the EMT-6/parent, EMT-6/CTX, and EMT-6/CDDP tumors. The expression of c-*myc*, c-*fos*, Ha-*ras*, and *p53* was not detectable in RNA isolated from the livers of either tumor-bearing or non-tumor-bearing animals. The presence of the EMT-6/parent or EMT-6/CDDP tumor and, to a lesser degree, of the EMT-6/CTX tumor in the animals resulted in an increased expression of c-*jun* in the livers on day 4 following tumor cell implantation, which decreased in animals bearing the EMT-6/parent or EMT-6/CDDP tumor by day 8 yet was higher than in non-tumor-bearing animals Fig. 8. Treatment of the tumor-bearing animals with CTX or CDDP on day 8 resulted in increased expression of c-*jun* in the livers of animals bearing the EMT-6/parent tumor but did so only after treatment with CTX in the livers of animals bearing either resistant tumor. *Erb*-2 was also increased in expression in the livers of tumor-bearing animals as compared with non-tumor-bearing animals. A marked increase in *erb*-2 expression was found on day 4 in the livers of animals bearing the EMT-6/CDDP tumors, and this expression increased further on day 8.

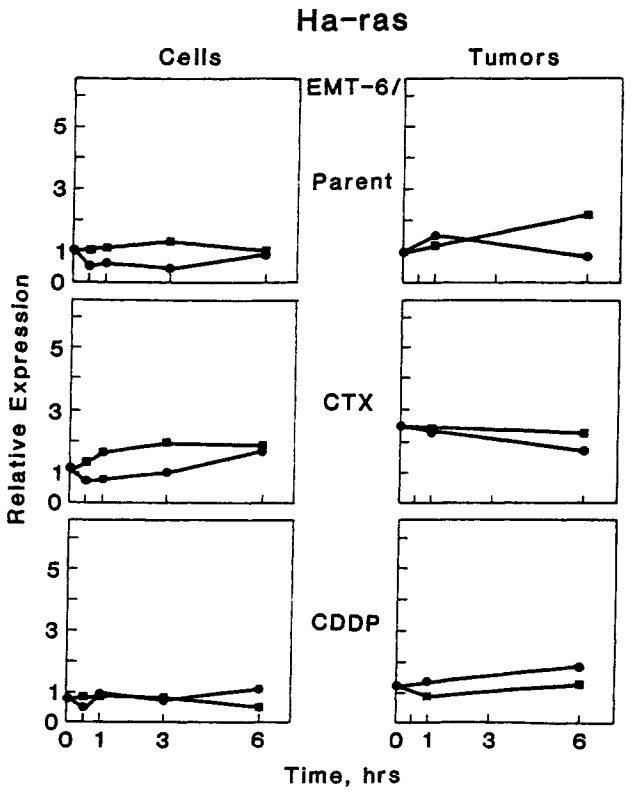

Fig. 5 Relative expression of Ha-*ras* mRNA in EMT-6/parent, EMT-6/CTX and EMT-6/CDDP cells grown as monolayers or tumors at various time points after treatment with: cells, 4-HC (100 μ*M*, ●) or CDDP (100 μ*M*, ■); tumor bearing animals, CTX (300 mg/kg, ●) or CDDP (20 mg/kg, ■). The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

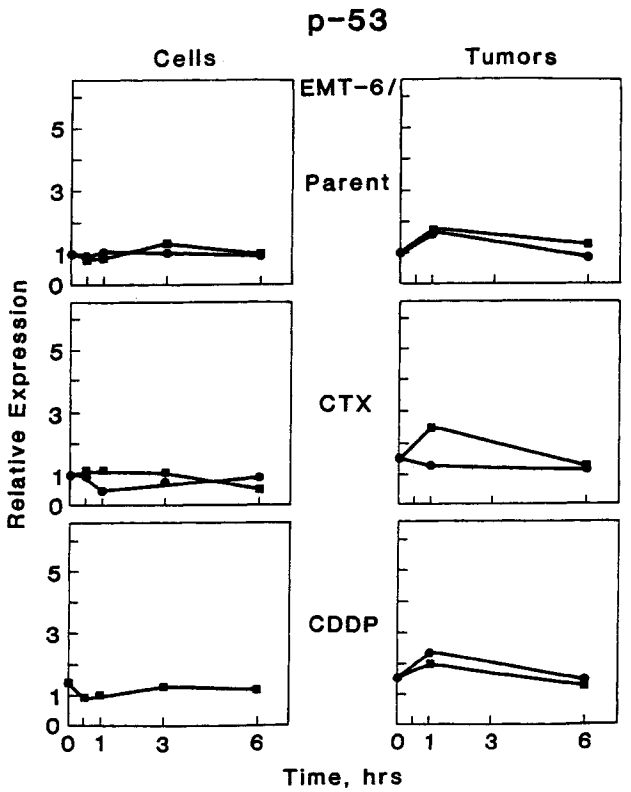

Fig. 6 Relative expression of *p*53 mRNA in EMT-6/parent, EMT-6/CTX and EMT-6/CDDP cells grown as monolayers or tumors at various time points after treatment with: cells, 4-HC (100 μ*M*, ●) or CDDP (100 μ*M*, ■); tumor bearing animals, CTX (300 mg/kg, ●) or CDDP (20 mg/kg, ■). The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

Although the expression of *erb*-2 increased from day 4 to day 8 in the livers of animals bearing either of the resistant tumors, there was a decrease in *erb*-2 expression in the livers of animals bearing the EMT-6/Parent tumor. At 6 h after treatment of the tumor-bearing animals with CTX or CDDP the expression of *erb*-2 was increased in the livers of animals bearing the EMT-6/parent tumor, showed no change in the livers of animals bearing the EMT-6/CTX tumors, and was decreased in the livers of animals bearing the EMT-6/CDDP tumors. The expression of GAPDH was constant throughout both the in vitro and in vivo treatments (Fig. 1–8).

Discussion

The notion of in vivo resistance acknowledges that tumors do not exist in isolation from the host and, consequently, that mechanisms of resistance to drugs as well as other therapeutic modalities that involve tumor/host interactions may occur. One possibility is that tumors that are resistant can evoke a more rapid or more robust response to stresses such as exposure to an antitumor alkylating agent. The EMT-6 in vivo-resistant tumor lines were developed by repeated exposure to a single dose of a specific antitumor alkylating agent at 2- to 3-week intervals. This study was conducted to determine if the resistant tumors existed in an active stress-response metabolic state in the absence of treatment and/or if the response of the resistant tumors to the stress of alkylating agent exposure differed from that of the parent tumor.

Jun, *fos* and *myc* are nuclear protooncogene products whose protein products link extracellular signals with changes in gene expression [1, 12, 26, 52, 54, 55, 57, 59, 61, 73, 82]. The proto-oncogene *jun* product has the characteristics of the transcription factor activator protein-1 (AP-1). The products of the *fos* and *myc* proto-oncogenes are involved in transcriptional transregulation [4, 10, 22, 23, 50, 53, 58, 74, 86, 88]. The protooncogenes c-*fos* and c-*jun* can function cooperatively as inducible-transcription factors in signal transduction processes. Their protein products, Fos and Jun, can form a heterodimeric complex that interacts with the DNA regulatory element at the AP-1

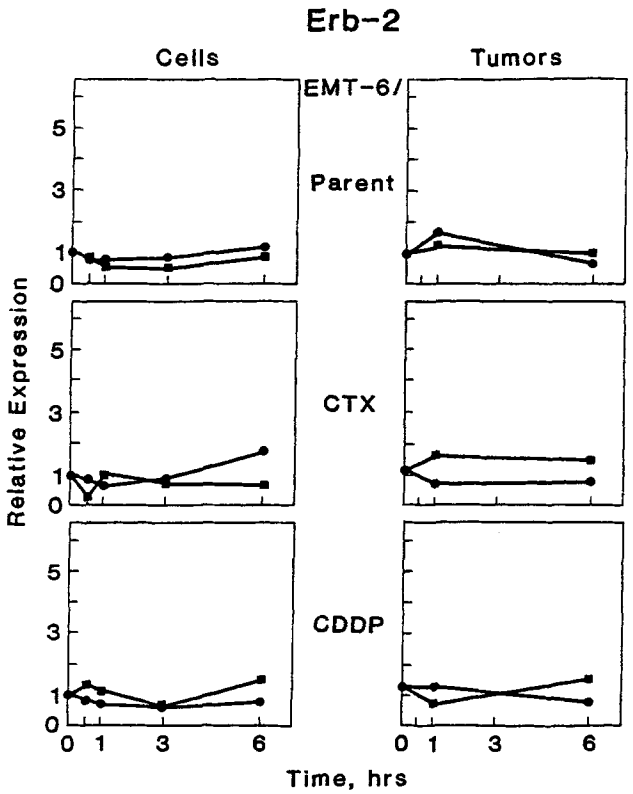

Fig. 7 Relative expression of *erb*-2 mRNA in EMT-6/parent, EMT-6/CTX and EMT-6/CDDP cells grown as monolayers or tumors at various time points after treatment with: cells, 4-HC (100 μM, ●) or CDDP (100 μM, ■); tumor bearing animals, CTX (300 mg/kg, ●) or CDDP (20 mg/kg, ■). The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

binding site. The *myc* protooncogene encodes two nuclear phosphoproteins, p62 and p64, involved in control of cellular proliferation and differentiation [4, 16, 18, 22, 50, 53, 70, 74, 80, 81, 88]. Each of these nuclear protooncogenes has been implicated in transformation and tumorigenesis in various cellular systems [19, 22, 23, 27, 34, 35, 69, 83]. The expression of these nuclear protooncogenes has also been associated with progressive clinical disease [18, 50, 81, 88]. The *p53* tumor-suppressor gene encodes a 53-kDa nuclear phosphoprotein involved in the control of cell proliferation [13, 29, 60, 71]. Mutations in the *p53* gene are frequently found in human tumors [13, 14, 24, 25, 29, 41, 42, 71, 84].

The *ras* oncogene encodes a signal-transducing G protein located on the inner surface of the cellular plasma membrane [6, 17, 31, 33, 44, 46, 48, 56, 62, 85]. Extracellular signals are received by transmembrane receptor protein(s) which directly or indirectly induce changes in the *ras* protein exchanging GDP for GTP. This change in the *ras* protein initiates a cascade of response(s) to the extracellular factor in the cell [48, 62]. Point mutations in the *ras* gene have been associated with uncontrolled cell growth and malignancy [17, 46, 56]. The *erb*-2 (*neu*) oncogene encodes a trans-membrane protein related to epidermal growth factor receptor [30, 38, 39, 43, 47, 66]. Amplification of the gene and overexpression have been described in aggressive types of breast cancer [30, 38, 39, 43, 47].

In cell culture it has been demonstrated that exposure of cells to DNA-damaging agents, including: X-rays, heat shock, monofunctional alkylating agents, CDDP, nitrogen mustard, UV radiation, hydrogen peroxide, Adriamycin, 4-HC, melphalan, etoposide, and 5-fluorouracil can induce expression of c-*jun*, c-*fos*, and c-*myc* as an early response [7, 16, 20, 21, 28, 51, 68, 70, 72, 74, 80] and that *p53* expression may be required

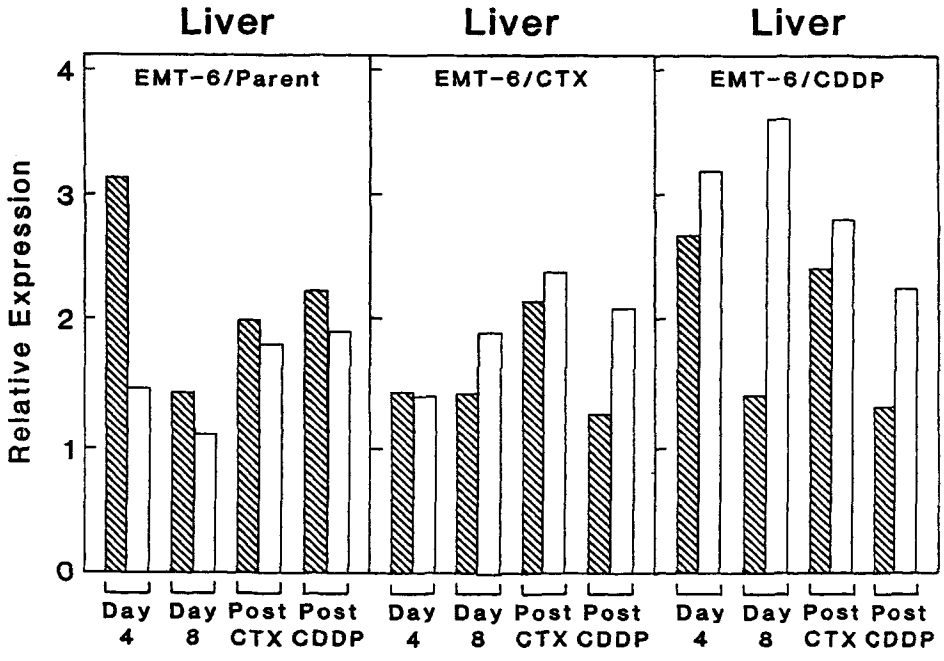

Fig. 8 Relative expression of c-*jun* (▨) and erb-2 (□) mRNA in the livers of animals bearing the EMT-6/parent, EMT-6/CTX or EMT-6/CDDP tumors at 4 or 8 days post tumor cell implantation or 6 h after treatment of tumor-bearing animals with CTX (300 mg/kg) or CDDP (20 mg/kg). The data are presented relative to the expression of c-*jun* and erb-2 mRNA levels (set equal to 1) in the livers of non-tumor bearing animals. The expression of GAPDH was used as a standard for densitometric analysis of the autoradiograph.

during apoptosis [42]. As early as 1987 it was reported that normal skin fibroblasts from patients with Li-Fraumeni syndrome known to be radiation-resistant over-expressed c-*myc*. Overexpression of *ras* oncogenes has been associated with resistance to ionizing radiation and CDDP [21, 31, 44, 45, 51, 72], as has overexpression of *fos*. Overexpression of H-*ras* and that of *myc* oncogenes act synergistically in producing resistance to ionizing radiation [41, 45]. Recently it has been shown that expression of mutant variants of *p53* oncogene can increase cellular resistance to ionizing radiation [41]. Little is known about the mechanism(s) responsible for the changes in cellular response to the stress of ionizing radiation in these systems.

The EMT-6 in vivo alkylating agent-resistant tumors exhibited overexpression of *jun*, *fos*, *myc*, *ras* and *p53* mRNA in the absence of treatment. In response to stress in culture, the EMT-6/parent as well as the two alkylating agent-resistant lines showed marked increases in the expression of *fos* and the EMT-6/parent and EMT-6/CTX lines had increased expression of *jun*. In vivo early–response increases in the expression of these genes were small or not detectable, except that *fos* expression increased after drug administration in animals bearing the EMT-6/CDDP tumor.

The differences between the responses of the tumors/cells to the stress of alkylating agent exposure in vivo and in vitro may have been due to the difference between the drug concentrations used for the exposures (that for cell culture being higher), to the inclusion of normal as well as tumor cells in the in vivo samples or to a difference in the response of the cells to the alkylating agent stress due to environmental factors. It is clear that the presence of a tumor in a host can alter the metabolic status of the host. The presence of the EMT-6/CDDP or EMT-6/CTX tumor in BALB/c mice altered the pharmacokinetics of CDDP and CTX in the animals and altered the response of bone marrow granulocyte/macrophage colony-forming units (CFU-GM) to these cytotoxic drugs [78, 79]. The liver is critically involved in the metabolic disposition of xenobiotics and may therefore be involved in the mechanism(s) of in vivo resistance. The changes observed in c-*jun* and *erb*-2 expression in the livers of tumor-bearing animals are an indication of a change in the metabolic status of that tissue in the tumor-bearing animals and represent an area warranting further study.

A heightened ability to respond to stress, chemical or physical, coupled with a signal to proliferate may be a model for malignant disease, when the stress is induced by an anticancer agent, drug resistance results. Therefore, the increased expression of these genes may impart metabolic changes in the tumor and/or host that contribute to the resistance of these tumors to specific antitumor alkylating agents.

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