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The mechanism of differential sensitivity to methotrexate of normal and malignant human epidermal cells*

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Summary. Squamous carcinoma cells are much more sensitive (>10^4 times) to the cytotoxic effects of methotrexate (MTX) and 5-fluorodeoxyuridine (FUDR) than are normal human keratinocytes as measured by cell growth. Among the drugs tested, this phenomenon was found to be specific for MTX and FUDR, since arabinosylcytidine (ARA-C), 13-bis-chloroethylnitrosourea (BCNU), and daunomycin failed to show differences in inhibition between the normal and malignant cell lines. Drug uptake studies did not reveal a significant difference in MTX intracellular levels between malignant and normal epidermal cell lines at 60 min. Thymidine (TdR) salvage was assessed by examining the effects of the presence of 3 μM TdR on MTX-induced cytotoxicity. On the withdrawal of TdR, normal cells demonstrated an increased level of inhibition amounting to 4 orders of magnitude, whereas the squamous-cell carcinoma cells showed no change in sensitivity. Interestingly, the immortal nontumorigenic keratinocyte line (NM-110) was similarly not rescued by the addition of TdR. The high degree of sensitivity to MTX shown by squamous-cell carcinoma (SCC) and NM-110 cells is attributable to a significant diminution of their ability to use exogenous TdR as compared with that of normal keratinocytes and might be indicative of a biochemical change associated with cellular immortality.

Introduction

Methotrexate (MTX) and 5-fluorouracil (5-Fu) are standard and effective agents used in the treatment of human squamous-cell carcinoma. The cytotoxic effects of these drugs have been studied in vitro without the benefit of comparison with their effects on normal cells [5]. This has mainly been due to the problems associated with growth of normal cells in culture. The development of a defined culture medium has offered the possibility of conducting studies that can directly compare the responses of normal and malignant keratinocytes to drugs [9].

Using these culture techniques, Firestone et al. [4] could show that the cytotoxicity of MTX and 5-fluorodeoxyuridine (FUDR) was 3–4 orders of magnitude greater for squamous-cell carcinoma (SCC) cells than for normal human keratinocytes as demonstrated by both cellular growth and DNA synthesis bioassays. We extended their observations by testing the selectivity of three additional chemotherapeutic agents and investigating the possible mechanism of differential sensitivity to MTX.

Materials and methods

Cell lines

Newborn epidermal cells. Newborn epidermal cells (NEC) were established from neonatal foreskins obtained from the Brigham and Women's Hospital (Boston, Mass.) as previously described [9]. The cells were grown in Keratinocyte Growth Media (KGM, Clonetics Corp., Denver, Colo.), which consists of MCDB 153 modified (containing 3 μM thymidine, Tdr) with 5 μg/ml hydrocortisone(HC), 0.8% (v/v) bovine pituitary extract, penicillin (100 IU/ml), streptomycin (100 μg/ml), and epidermal growth factor (EGF) [2].

SCC cells. The SCC-25 and SCC-15 cell lines have been described elsewhere [10]. SCC-25 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and HC (0.4 μg/ml). SCC-15 cells were grown in a 1:1 (v/v) mixture of Ham's F12 and DMEM supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and HC (0.4 μg/ml). Both media contain ca. 3 μM Tdr.

NM-110 cells. The NM-110 cell line was obtained from Dr. H. P. Baden and has been described elsewhere [1]. These cells were established from a pool of apparently normal neonatal forekins. When characterized, the

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Table 1. Comparison of drug effects on the growth of normal and malignant human epidermal cells

Cell Line	ID_{50} (μM)[a]				
	MTX	FUDR	ARA-C	BCNU	Daunomycin
SCC-25	0.02	0.009	0.3	119	0.1
SCC-15	0.08	0.3	7	271	–
NM-110	0.186	1.93	0.186	16.1	0.021
NEC-1	>1,000	200	0.4	32	0.05
NEC-2	>1,000	20	1	77	–

[a] Concentration of drug causing 50% inhibition of growth following a 48-h exposure to drug. All media contained ca. 3 μM Tdr. The results were taken from a respresentative experiment.

cells had been in culture for >1 year and had undergone >400 doublings. Their pattern of growth and differentiation were otherwise similar to that of normal keratinocytes. Screening was negative for SV40, BK, HPV-16, and HPV-18 viruses. In the athymic nude mouse model, the cells were nontumorigenic. Cytogenetically, they displayed trisomy of chromosome 8. Cultures were maintained in KGM +10% FBS.

Materials

Radiolabeled [5-^{3} H]-deoxythymidine and [5-^{3} H]-deoxycytidine were obtained from New England Nuclear (Boston, Mass). Unlabeled nucleosides were obtained from P-L Biochemicals (Milwaukee, Wis.); MTX, FUDR, and daunomycin were purchased from Sigma Chemical Co. (St. Louis, Mo.); arabinosylcytidine (ARA-C) was supplied by The Upjohn Co. (Kalamazoo, Mitch.); 13-bis-chloroethylnitrosourea (BCNU) was obtained from Bristol Laboratories (Evansville, Ind.); and DFBS was purchased from Gibco.

Methods

Growth assays. Cellular growth was assayed as previously described [13]. Exponentially growing cells were harvested and subcultured in Linbro multiwell tissue-culture trays. The drug was added to cells in the log phase of growth at 48 h after plating (72 h for SCC-15 and NM-110) and the cells were continuously exposed to this single dose for 48 h. Triplicate cultures were harvested and the cells were counted in a Model Z Coulter counter. All growth assays were performed in duplicate.

Macromolecular assays. Exponentially growing cells were harvested and subcultured in Linbro multiwell tissue-culture trays for 72 h to establish cells in the log phase of growth. Cells were preincubated with varying concentrations of drug for 18 h prior to the addition of [^{3} H]-deoxycytidine ([^{3} H]-Cdr). After incubation with [^{3} H]-Cdr (2 μCi, 25.7 Ci/mmol) for 1 h at 37° C, the medium was aspirated and the cell monolayers were washed with 0.9% saline, following which 0.5 ml 10% trichloroacetic acid (TCA) was added. The precipitate was washed three times with 0.9% saline and the DNA was solubilized overnight with 0.5 ml 1*N* KOH at 4° C. Aliquots of DNA solution were prepared for scintillation counting as previously described [14]. Each determination was carried out in triplicate and repeated several times.

MTX transport. MTX uptake studies were performed on cells in the logarithmic phase of growth at 72 h after plating in Linbro multiwell tissue-culture trays. The cell monolayers were initially washed three times with Hank's Balanced Salt Solution (HBSS) at 37° C. The [^{3} H]-MTX (spec. act., 37.1 Ci/mmol) was diluted 3-fold with unlabeled MTX to a final concentration of 1 μM and subsequently added to the cells, which were then incubated for 10, 20, 40, and 60 min. The monolayers were next washed ten times with 2 ml cold 0.9% saline, after which they were ultrasonicated in 1 ml triple-distilled water (Artec Sonic Dismembrator, setting 2.5 for three 20-s bursts). The recovered intracellular MTX was aliquoted and prepared for scintillation counting as previously described [13]. Aliquots for each determination were assayed for protein content using the method described by Lowry et al. [8]. All assays were performed in triplicate and repeated, with similar results being obtained.

Results

Table 1 shows the ID_{50} value (the concentration of drug required to inhibit the growth of 50% of the cell population) obtained for two different SCC cell lines and three keratinocyte lines at 48 h after continuous incubation with various concentrations of MTX, FUDR, ARA-C, BCNU, and daunomycin. As originally noted by Firestone et al. [4], SCC lines are 3–4 orders of magnitude more sensitive to MTX and FUDR than are NEC cells. This differential sensitivity is specific for these two drugs and was confirmed by the results we obtained using ARA-C, BCNU, and daunomycin.

The results of MTX transport studies are shown in Fig. 1, which compares the intracellular levels of MTX over a 60-min period. These transport studies were carried out using an extracellular MTX concentration of 1 μM. At the end of 60 min, the intracellular drug concentration in the two normal and two malignant epidermal cell lines were similar, ranging from 4.3 to 5.6 pmol/mg protein.

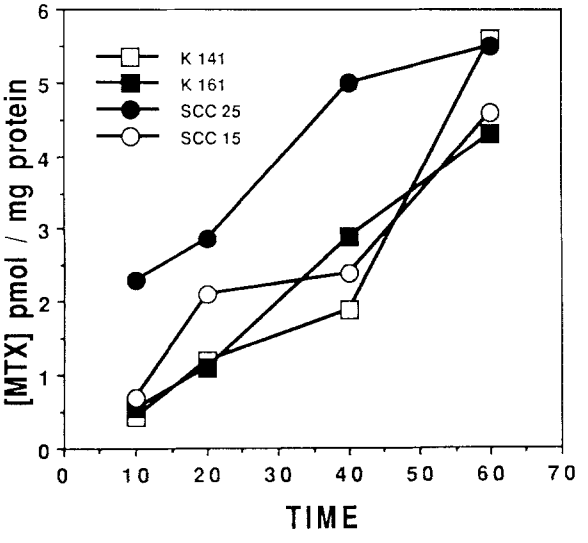

Fig. 1. Methotrexate (*MTX*) transport in normal and malignant keratinocytes. The transport studies were carried out as described in Materials and methods using an extracellular MTX concentration of 1 μM. K141 and K161 are normal epidermal cell lines

Table 2. Comparison of exogenous thymidine on MTX inhibition of cell growth and DNA synthesis in normal and malignant human epidermal cells

Cell line	Cell growth: ID_{50} (μM)[a]		Thymidine incorporation: ID_{50} (μM)[a]	
	Tdr (3 μM)	No TdR	TdR (3 μM)	No TdR
SCC-25	0.05	0.03	0.03	0.06
SCC-15	0.03	0.03	0.08	0.03
NM-110	0.02	0.03	0.002	0.002
NEC-1	>1,000	0.03	>1,000	0.2
NEC-2	>1,000	0.04	>1,000	0.03

[a] The inhibitor of growth and DNA synthesis was determined using DFBS (SCC-25, SCC-15, and NM-110) and specially prepared KGM that lacked thymidine (NEC). The results were taken from a representative experiment

These results indicate that differences in MTX transport are not sufficient to account for the observed difference in sensitivity.

The effect of exogenous Tdr on the differential sensitivity to MTX in terms of the growth and DNA synthesis of normal and malignant keratinocytes is shown in Table 2. For the malignant cell lines and NM-110 cells, this was accomplished by using 10% DFBS in DMEM with or without 3 μM TdR. For the NEC cell lines, KGM was especially prepared without Tdr since conventional KGM is routinely supplemented with 3 μM TdR. The results show that for the malignant SCC-25 and SCC-15 cell lines, the presence or absence of 3 μM TdR had no effect on the response of the cells to MTX. In contrast, the NEC lines displayed a differential sensitivity of 4 orders of magnitude that correlated with the presence of 3 μM thymidine. In the absence of 3 μM TdR, the MTX-induced cytotoxicity obtained in normal keratinocytes approached that achieved in the malignant cell lines, suggesting that the differential sensitivity to MTX was related to differences in Tdr metabolism. The nonmalignant immortal cell line NM-110 demonstrated a pattern of response similar to that of the malignant cells, since 3 μM TdR could not abolish its sensitivity to MTX.

Discussion

There have been few studies comparing the effects of chemotherapeutic agents on tumor cells and their respective normal counterparts. Tsuji et al. [13] have shown differential sensitivities to 5-FU in normal melanocytes and malignant melanomas. More recently, Firestone et al. [4] have shown a differential sensitivity to MTX and FUDR in normal human epidermal cells and SCC cells. Specifically, the latter authors found that normal cells were 3–4 orders of magnitude less sensitive to MTX and FUDR, since testing with additional chemotherapeutic drugs failed to reveal differences in drug sensitivity (Table 1).

There are a number of possible mechanisms of resistance to MTX, among which are increased DHFR activity, alteration of DHFR properties, decreased MTX uptake, inactivation of MTX, increased cellular pools of reduced folate and Tdr, and Tdr salvage [6, 7, 12]. In their preliminary investigation into the mechanism of differential sensitivity to MTX in normal and malignant human epidermal cells, Firestone et al. [4] ruled out increased DHFR activity. Alternatively, MTX might inhibit purine biosynthesis more extensively in tumor cells and thereby resist rescue by TdR.

In defining the mechanism of differential sensitivity, we undertook studies of MTX transport as well as Tdr salvage. MTX transport was not shown to be a factor in the differential sensitivity of cells to MTX. As shown in Fig. 1, at times ranging from 10 to 60 min after drug administration, the differences observed in intracellular MTX levels among cell lines were negligible.

Our results suggest that normal cells can use exogenous Tdr at physiologic concentrations to decrease their sensitivity to MTX [Table 2], suggesting that Tdr salvage is a major determinant of decreased MTX sensitivity in normal human epidermal cells in citro. This observation is consistent with the findings of DeLapp et al., who showed that newborn mouse skin preferentially salvaged deoxypyrimidine nucleotide over de novo synthesis [3]. More recently, Schwartz et al. [11] have demonstrated that human keratinocytes have an effective Tdr salvage mechanism. In marked contrast, the SCC lines tested in the present study were incapable of exploiting exogenous TdR (at physiologic levels) to preclude the cytotoxic effects of MTX.

Interestingly, the NM-110 cell line, a keratinocytic line shown to be immortal but nontumorigenic, could not use exogenous TdR to overcome MTX cytotoxicity. This result might suggest that the ability to salvage TdR is lost early in the process of cellular transformation. The relative ability of normal keratinocytes to salvage Tdr might prove to be a useful marker of cell immortality.

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