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REPORTS

EFFECTS OF METHOTREXATE ON PROLIFERATION OF HUMAN KERATINOCYTES IN VITRO

B. ALLEN FLAXMAN, M.D., ROBERT A. HARPER, PH.D., STEPHEN CHIARELLO, M.D., AND ANDREA M. FELDMAN, B. A.

Department of Medicine (Subsection of Dermatology), Brown University and the Miriam Hospital, Providence, Rhode Island, and The Skin and Cancer Hospital (RAH), Temple University Health Sciences Center, Philadelphia, Pennsylvania, U. S. A.

Normal human keratinocytes, propagated as epithelial outgrowths in vitro, were exposed to different concentrations of methotrexate (MTX) for different periods of time. After a 1-hr exposure, DNA synthesis was inhibited in a reversible manner. No change in the mitotic index was observed. After a 6-hr exposure, both DNA synthesis and mitosis were inhibited, again in a reversible fashion. Prolonged exposure (24 hr) resulted in irreversible mitotic inhibition even when followed by recovery periods of 168 hr. The effective concentrations of MTX in vitro were similar to those described previously in vivo.

The mitotic-inhibiting properties of methotrexate (MTX) have been exploited for many years in the treatment of psoriasis [1,2]. Much of our knowledge of the pharmacology of MTX comes from in vitro studies using *nonepidermal* cells. While the effect of MTX on human epidermis has been partially analyzed in vivo [3–5], there has been little analysis along similar lines using an in vitro system. Normal human epidermal keratinocytes can be propagated on artificial surfaces in vitro where, in the absence of underlying connective tissue, they produce a stratified, squamous epithelium showing basal cell proliferation and many of the morphologic and chemical changes characteristic of keratinization [6–8]. Proliferative events in this system can be studied with ease and the effects of drugs and pharmacologic agents evaluated [9]. The present investigation describes some responses of cultured human keratinocytes to MTX.

MATERIALS AND METHODS

The techniques for growing epithelial cell sheets of keratinocytes from explants of normal human skin have been described elsewhere [6–8]. For studies of the effect of MTX on mitosis and labeling index, 3 to 4 explants were placed on a single coverslip (multiple coverslips/experiment). For direct determination of incorporation of [^{3}H]deoxyuridine and [^{3}H]thymidine into DNA, the technique of multiple (20) primary explants was used [10]. All studies were carried out on cells after 10 to 12 days in vitro. Different experiments were performed on skin from different donors. Thus, while each control and its experimental counterpart was carried out on skin from the same donor, skin from multiple donors was needed to provide sufficient material to evaluate all the factors described under *Results*. As pointed out elsewhere [11], baseline rates of proliferation seem to vary in vitro according to the donor; but any effects of drugs on this baseline appear to be consistent from donor to donor.

MTX (Lederle), diluted with culture fluid, was added in a variety of final concentrations. Details of length of exposure, recovery period, etc., are described under *Results*. The mitotic index (M.I.) is the ratio of number of mitotic figures to interphase nuclei and was determined by a method described elsewhere [12]. To determine the labeling index (L.I.), i.e., the percentage of cells in DNA synthesis, [^{3}H]thymidine (sp act 6.7 Ci/mM) or [^{3}H]deoxyuridine (sp act 10 Ci/mM) was added at a level of 2 μCi/ml to cultures for 1 hr followed by fixation and autoradiography [13]. All exposures following dipping in emulsion (Kodak NTB-2) were at 4°C for 2 weeks. The ratio of labeled to unlabeled interphase nuclei was determined for a total of 1000 cells per outgrowth. Quantitative measurement of incorporation of [^{3}H]thymidine or [^{3}H]deoxyuridine into DNA by scintillation counting has been described elsewhere [10].

RESULTS

Experiments in which keratinocytes were exposed for 24 hr to different concentrations of MTX showed that 0.1 or 1.0 μg/ml was always effective in causing nearly total mitotic inhibition. There was no effect using 0.01 μg/ml. The 1.0 μg/ml level was chosen for most subsequent experiments.

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Reprint requests to: Dr. B. A. Flaxman, Department of Medicine (Subsection of Dermatology), Brown University and the Miriam Hospital, 164 Summit Avenue, Providence, Rhode Island 02906.

Abbreviations:
DHFA: dihydrofolic acid
L.I.: labeling index
M.I.: mitotic index
MTX: methotrexate

TABLE I. *Effect of different durations of exposure to MTX (1 μg/ml) on M.I. and L.I.*

Controls (no MTX) had culture fluid changed at same time as experimentals when MTX was washed out in recovery experiments. Horizontal lines dividing Table serve to group experiments performed on cultures from skin of the same donor. The entire series was repeated two times on skin from other donors and the same results were obtained.

Duration of exposure to MTX (*hr*)	Recovery period (*hr*)	M.I. ± SD	L.I. (%) [^{3}H]deoxyuridine	L.I. (%) [^{3}H]thymidine
0	0	26 ± 1	16.2	
1	0	29 ± .5	1.0	
0	24	16 ± .5	16.0	
1	24	18 ± 0	15.0	
0	0	31.9 ± 4	19.8	
6	0	13.5 ± 7	0	
0	24	28.7 ± 3	17.9	
6	24	39.8 ± 5	21.3	
0	0	4.4 ± 6	19.2	20.0
24	0	4 ± 2	.1	42.0
0	24	37 ± 2	7.0	8.5
24	24	0	0.5	12.1
0	48	38 ± 3	6.6	4.9
24	48	2 ± 0	0.14	10.8
0	168	15 ± 4	5.9	5.9
24	168	0	1.4	0.8

Table I shows the results of several experiments using MTX at a concentration of 1 μg/ml. A 1-hr exposure to the drug caused no change in the M.I., but did decrease the L.I. using [^{3}H]deoxyuridine by greater than 90%. [^{3}H]deoxyuridine can be used as a marker for de novo DNA synthesis [4]. The absence of an effect on the M.I. results from the continued movement of cells from G_2 into M, the former having a value of about 7 hr in this system [13]. Thus, the effect on DNA synthesis is not yet reflected in the M.I. after a 1-hr exposure to MTX. If MTX was washed out and the cells allowed to recover for 24 hr, the L.I. ([^{3}H]deoxyuridine) returned to control values. Thus, the drug had a rapid, reversible effect on suppression of DNA synthesis.

A 6-hr exposure to MTX (Tab. I) resulted in a 58% decrease in M.I. During this exposure, many of the cells in G_2 have passed into and through M but no new cells have moved through S into G_2 due to the block by MTX. Those mitoses present probably derive from cells that were in early G_2 at the time MTX was added. The L.I. ([^{3}H]deoxyuridine) was again decreased to zero. If MTX was washed out and a 24-hr recovery in normal culture fluid allowed, both the M.I. and L.I. ([^{3}H]deoxyuridine) returned to values slightly above those of the controls. Thus, the effects of a 6-hr exposure to 1 μg/ml of MTX also appeared to be reversible.

Twenty-four-hour exposure to MTX (Tab. I) resulted in almost total suppression of mitotic activity. While the L.I. ([^{3}H]deoxyuridine) was also nearly zero, the L.I. ([^{3}H]thymidine) more than doubled. [^{3}H]Thymidine, which is also a marker for DNA synthesis, can be incorporated into DNA in the presence of MTX via an alternate salvage pathway, thereby avoiding the enzymatic block caused by the drug. The autoradiographic results were confirmed by direct determination of the amount of radioactive precursor incorporated into DNA using both [^{3}H]thymidine and [^{3}H]-deoxyuridine (Tab. II). It should be noted that autoradiography provides a relatively crude measure of labeled precursor incorporation. For example, a L.I. of nearly zero with [^{3}H]deoxyuridine after 24 hr with 1 μg/ml of MTX (Tab. I) correlated with a decreased but still significant incorporation of [^{3}H]deoxyuridine into DNA as measured by scintillation counting after 2.5 μg/ml of MTX (Tab. II). In other experiments, MTX was washed out after 24 hr and different groups of treated cells were allowed to recover for 24, 48, or 168 hr (Tab. I). In all cases the M.I. remained at zero or only slightly above even though some incorporation of [^{3}H]thymidine and [^{3}H]deoxyuridine continued.

TABLE II. *Effects of 24-hr exposure to 2.5 μg/ml MTX on incorporation of [^{3}H]deoxyuridine and [^{3}H]thymidine into DNA (cpm/μg DNA)*

Each value represents the average for two dishes of cells. The entire experiment was repeated and the results were confirmed.

	Control	Experimental
[^{3}H]Deoxyuridine	2610	1547
[^{3}H]Thymidine	6213	22,865

Since a 6-hr exposure to MTX resulted in reversible depression of mitosis and DNA synthesis, which is similar to in vivo effects in psoriasis [4], it was of interest to determine the source of the dividing cells seen after the recovery period. The fate of the MTX-inhibited cells was studied by prelabeling with [^{3}H]thymidine. One hour prior to adding MTX, [^{3}H]thymidine was added and then

washed out thoroughly with three rinses of "cold" culture fluid. MTX was added for 6 hr, washed out for 24 hr, and the cultures fixed. Many labeled mitotic figures were found, indicating that although cells in S phase had been blocked temporarily in DNA synthesis, they were able to recover completely and proceed on to mitosis. Occasional labeled pyknotic nuclei were found which probably derived from cells that did not recover or else from recovered postmitotic cells that had keratinized.

DISCUSSION

Studies of MTX in the treatment of cancer have been carried out first in vivo and then complimented and greatly extended by in vitro observations. The use of MTX in treatment of a benign disease such as psoriasis has been accompanied by a series of studies in vivo that have provided much valuable information recording effects of the drug on keratinocyte proliferation [3–5]. There has, however, been very little work attempted in vitro to corroborate and extend the in vivo findings. Indeed, Karasek [14] reported that, compared with other water-soluble antimetabolites in vitro, MTX was quite ineffective in blocking growth of human keratinocytes, even at a concentration of 1000 μg/ml. Quite clearly our own study shows this not to be the case and, in fact, normal human keratinocytes in vitro seem to be susceptible to the same range of concentrations of MTX as are effective in vivo [4,15,16] and for other types of cells in vitro [17–19]. A possible explanation for the difference between our findings and those of Karasek may lie in the method of analysis. Karasek measured gross changes in size of keratinocyte outgrowths whereas we looked specifically for changes in cytologic and biochemical parameters.

The characteristics of reversible inhibition and recovery from short exposure to MTX in vitro for normal keratinocytes compare closely with the recovery characteristics described in psoriasis in vivo [4]. It has been reported that concentrations of MTX which inhibit DNA synthesis or are lethal for psoriatic keratinocytes in vivo [5] and in organ culture in vitro [20] have less inhibition and no serious damaging effect on normal keratinocytes. Our preliminary studies of psoriatic keratinocytes in outgrowth cultures show responses to MTX identical to those of normal keratinocytes. Thus, the so-called selective effect of MTX may reflect only as-yet unidentified in vivo environmental factors not present in vitro. This correlates well with other data that suggest there is no inherent difference between normal and psoriatic keratinocytes [9].

While many mitoses found 24 hr after a 6-hr exposure and washout of MTX may have represented cells previously in G_1 which were not affected by the drug and then entered S phase, G_2, and M during the recovery period, some represented cells previously blocked in S phase which then recovered. This was shown by prelabeling with [^{3}H]thymidine. Although a few labeled pyknotic nuclei were found, most postrecovery label was present in interphase nuclei or over readily identified metaphase chromosomes. Similar experiments in vivo have stressed the presence of labeled pyknotic nuclei in MTX-treated psoriasis lesions [5], indicating that a proportion of blocked cells do not recover. Since the present in vitro studies show that keratinocyte recovery is possible, the effect of MTX in psoriasis probably is due only partially to its cell-killing ability, the remainder being related to a reversible prolongation of the cell cycle. It should be noted that a 6-hr exposure to 1 μg/ml of MTX in vitro probably approximates the in vivo exposure following parenteral MTX inasmuch as tissue levels of the drug have been measured at 0.46 to 0.85 μg/gm 5 hr after injection [16].

More prolonged exposure to MTX was followed by irreversible mitotic inhibition, even though "recovery" periods of up to 1 week were allowed. This was consistent with observations in other systems that "toxicity depends on duration of exposure to specific inhibitory levels of drug" [21]. Recovery took place in culture fluid containing folic acid (1 μg/ml in Eagle's minimal essential medium plus additional folic acid in serum). There was an increased L.I. ([^{3}H]thymidine) and incorporation of [^{3}H]thymidine into DNA after 24 hr with MTX which clearly bore no relation to the actual state of health of the cells since mitotic recovery never took place. The higher L.I. ([^{3}H]thymidine) even persisted after the 24-hr and 48-hr recovery period, eventually dropping off drastically by 168 hr. A similar increase in L.I. ([^{3}H]thymidine) has been noted in psoriasis after MTX [4] and interpreted as a sign that the cells were not dying. Clearly, that interpretation is not supported by the present in vitro studies.

The increased incorporation of [^{3}H]thymidine directly into DNA in the face of a block by MTX apparently takes place via the "salvage" pathway [22] and reflects compensation for inhibition at the level of the "de novo" pathway. The importance of the de novo pathway in human skin has been stressed [23], although the salvage pathways to DNA synthesis may be more important in the skin of other species [24]. Failure of cells to recover following a 24-hr exposure to MTX may not be related only to a prolonged block in DNA synthesis but also to inhibition of other pathways dependent on 1-carbon units which leads to overall cell decay [17,25,26]. Many studies have stressed the primary role of MTX as an inhibitor of dihydrofolic acid reductase (DHFA reductase), thereby limiting the availability of reduced folate for synthesis of thymidine monophosphate (TMP) from deoxyuridine monophosphate (DUMP) in the de novo synthesis of DNA [20,27,28]. A portion of MTX is tightly, but reversibly, bound to DHFA reductase [29]. The most important inhibitory effect of the drug, however, may be related to the level of excess free MTX which interacts with a "low-affinity" receptor site [30]. Effects on other aspects of

metabolism have also been demonstrated and include direct inhibition of thymidylate synthetase [17], and inhibition of synthesis of purines [25], RNA [26], and proteins [31]. The relative contribution of each of these effects to mitotic inhibition or cell killing has been discussed [18]. Since the cell cycle in the keratinocyte in vitro system is about 59 hr [13], after 24-hr exposure to MTX a sizeable cell population would still exist outside of S phase, and the DNA synthesis of these cells would not be affected by the drug. This suggests that the other aspects of cell metabolism are affected to the point of irreversible recovery.

The present experiments point out that human keratinocytes in vitro show a sensitivity and behavior to MTX quite similar to that of keratinocytes from patients with psoriasis in vivo. It seems possible. therefore, that this in vitro system may be of real use in the screening and evaluation of other drugs of potential value in treatment of skin disease.

REFERENCES

1. Van Scott, EJ, Auerbach R, Weinstein GD: Parenteral methotrexate in psoriasis. Arch Dermatol 89:550–556, 1964
2. Rees RB, Bennett JH, Maibach HI, Arnold HL: Methotrexate for psoriasis. Arch Dermatol 95:2–11, 1967
3. Weinstein GD, Frost P: Abnormal cell proliferation in psoriasis. J Invest Dermatol 50:254–259, 1968
4. Weinstein GD, Goldfaden G, Frost P: Methotrexate: mechanism of action on DNA synthesis in psoriasis. Arch Dermatol 104:236–243, 1971
5. Weinstein GD, Valasco J: Selective action of methotrexate on psoriatic epidermal cells. J Invest Dermatol 59:121–127, 1972
6. Flaxman BA, Lutzner MA, Van Scott EJ: Cell maturation and tissue organization in epithelial outgrowths from skin and buccal mucosa in vitro. J Invest Dermatol 49:322–332, 1967
7. Flaxman BA: Replication and differentiation in vitro of epidermal cells from normal human skin and from benign (psoriasis) and malignant (basal cell carcinoma) hyperplasia. In Vitro 8:237–256, 1972
8. Flaxman BA: Cell identification in primary cell cultures from skin. In Vitro 10:112–118, 1974
9. Flaxman BA, Harper RA: In vitro analysis of the control of keratinocyte proliferation in human epidermis by physiologic and pharmacologic agents. J Invest Dermatol 65:52–59, 1975
10. Flaxman BA, Harper RA: Primary cell culture for biochemical studies of human keratinocytes. Br J Dermatol 92:305–309, 1975
11. Harper RA, Flaxman BA: Effect of pharmacologic agents on human keratinocyte mitosis in vitro. III. Inhibition by histamine and methylated analogs. J Invest Dermatol 65:400–403, 1975
12. Chopra DP, Yu RJ, Flaxman BA: Demonstration of tissue specific inhibitor of mitosis of human epidermal cells in vitro. J Invest Dermatol 59:207–210, 1972
13. Chopra DP, Flaxman BA: Human epidermal cell cycle in vitro. Br J Dermatol 87:13–17, 1972
14. Karasek MA: Effects of water-soluble antimetabolites on epithelial cell growth in vitro, Psoriasis, Proceedings of the International Symposium. Edited by EM Farber, AJ Cox. Stanford, Calif, Stanford University Press, 1971, pp 271–276
15. Zurek WZ, Ojima Y, Anderson LL, Collins GS, Oberfield RA, Sullivan RD: Pharmacologic studies of methotrexate in man. Surg Gynecol Obstet 126:331–338, 1968
16. Liguori VR, Giglio JJ, Miller E, Sullivan RD: Effects of different dose schedules of amethopterin on serum and tissue concentrations and urinary excretion patterns. Clin Pharmacol Ther 3:34–40, 1962
17. Borsa J, Whitmore GF: Studies relating to the mode of action of methotrexate. III. Inhibition of thymidylate synthetase in tissue culture cells and in cell-free systems. Mol Pharmacol 5:318–332, 1969
18. Borsa J, Whitmore GF: Cell killing studies on the mode of action of methotrexate on L-cells in vitro. Cancer Res 29:737–744, 1969
19. Hryniuk W, Fischer GA, Bertino JR: S-phase cells of rapidly growing and resting populations: differences in response to methotrexate. Mol Pharmacol 5:557–567, 1969
20. Weinstein GD, McCullough JL: Effects of methotrexate esters on normal and psoriatic skin. Arch Dermatol 111:471–475, 1975
21. Chabner BA, Myers CE, Coleman CN, Johns GD: The clinical pharmacology of antineoplastic agents. N Engl J Med 292:1107–1113, 1975
22. Kuebbing D, Werner R: A model for compartmentation of de novo and salvage thymidine nucleotide pools in mammalian cells. Proc Natl Acad Sci USA 72:3333–3336, 1975
23. Weinstein GD, Friedland A, McCullough J: Biologic and biochemical actions of methotrexate in psoriasis (abstr). J Invest Dermatol 66:277, 1976
24. DeLapp NW, Karasek MA: Importance of pyrimidine nucleotide salvage pathways for DNA synthesis in skin. J Invest Dermatol 66:306–312, 1976
25. Hryniuk WM: Purineless death as a link between growth rate and cytotoxicity by methotrexate. Cancer Res 32:1506–1511, 1972
26. Friedland A: Effect of methotrexate on deoxyribonucleotide pools and DNA synthesis in human lymphocytic cells. Cancer Res 34:1883–1888, 1974
27. O'Brien JS: The role of the folate coenzymes in cellular division. A review. Cancer Res 22:267–281, 1962
28. Bertino JR: The mechanism of action of the folate antagonists in man. Cancer Res 23:1286–1306, 1963
29. Bertino JR: Folate antagonists, antineoplastic and immunosuppressive agents, Handbook of Experimental Pharmacology, vol 38, part 2. Edited by AC Sartorelli, DG Johns. New York, Springer-Verlag, 1974, pp 468–483
30. Goldman ID: The mechanism of action of methotrexate. I. Interaction with a low-affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts. Mol Pharmacol 10:257–274
31. Holland JF: Symposium on the experimental pharmacology and clinical use of antimetabolites. VII. Folic acid antagonists. Clin Pharmacol Ther 2:274–409, 1961