REPORTS

EFFECTS OF METHOTREXATE ON PROLIFERATION OF HUMAN KERATINOCYTES IN VITRO

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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 [3H]deoxyuridine and [3H]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, [3H]thymidine (sp act 6.7 Ci/mM) or [3H]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 [3H]thymidine or [3H]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.
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.

<table>
<thead>
<tr>
<th>Duration of exposure to MTX (hr)</th>
<th>Recovery period (hr)</th>
<th>M.I. ± SD</th>
<th>L.I. (%)[3H]deoxyuridine</th>
<th>L.I. (%)[3H]thymidine</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>26 ± 1</td>
<td>16.2</td>
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<td>0</td>
<td>29 ± .5</td>
<td>16.0</td>
<td>15.0</td>
</tr>
<tr>
<td>0</td>
<td>24</td>
<td>16 ± .5</td>
<td>19.8</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>18 ± 0</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>31.9 ± 4</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24</td>
<td>28.7 ± 3</td>
<td>21.3</td>
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<td>0</td>
<td>12.1</td>
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<tr>
<td>6</td>
<td>24</td>
<td>39.8 ± 5</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>4.4 ± 6</td>
<td>19.2</td>
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</tr>
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<td>4 ± 2</td>
<td>1</td>
<td>42.0</td>
</tr>
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<td>37 ± 2</td>
<td>7.0</td>
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<td>12.1</td>
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<tr>
<td>24</td>
<td>48</td>
<td>28 ± 3</td>
<td>6.6</td>
<td>4.9</td>
</tr>
<tr>
<td>24</td>
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<tr>
<td>24</td>
<td>168</td>
<td>15 ± 4</td>
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<tr>
<td>24</td>
<td>168</td>
<td>0</td>
<td>1.4</td>
<td>0.8</td>
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</table>

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 [3H]deoxyuridine by greater than 90%. [3H]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 G2 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. ([3H]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 G2 have passed into and through M but no new cells have moved through S into G1 due to the block by MTX. Those mitoses present probably derive from cells that were in early G2 at the time MTX was added. The L.I. ([3H]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. ([3H]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. ([3H]deoxyuridine) was also nearly zero, the L.I. ([3H]thymidine) more than doubled. [3H]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 [3H]thymidine and [3H]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 [3H]deoxyuridine after 24 hr with 1 µg/ml of MTX (Tab. I) correlated with a decreased but still significant incorporation of [3H]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 [3H]thymidine and [3H]deoxyuridine continued.

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 [3H]thymidine. One hour prior to adding MTX, [3H]thymidine was added and then

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Deoxyuridine</td>
<td>2610</td>
<td>1547</td>
</tr>
<tr>
<td>[3H]Thymidine</td>
<td>6213</td>
<td>22,865</td>
</tr>
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</table>

Each value represents the average for two dishes of cells. The entire experiment was repeated and the results were confirmed.
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 complemented 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 \( \mu \text{g/ml} \). 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 G1, which were not affected by the drug and then entered S phase, G2, 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 \( \mu \text{g/ml} \) 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 \( \mu \text{g/gm} \) 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 \( \mu \text{g/ml} \) in Eagle's minimal essential medium plus additional folic acid in serum). There was an increased L.I. \((^{3}H\text{thymidine})\) 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. \((^{3}H\text{thymidine})\) even persisted after the 24-hr and 48-hr recovery period, eventually dropping off drastically by 168 hr. A similar increase in L.I. \((^{3}H\text{thymidine})\) 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 mitototic 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

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