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Metabolic Modification by Insulin Enhances Methotrexate Cytotoxicity in MCF-7 Human Breast Cancer Cells

OLIVER ALABASTER,*† BARBARA K. VONDERHAAR‡ and SAMIR M. SHAFIE‡

*Cancer Research Laboratory, George Washington University, Washington, D.C., U.S.A. and ‡Laboratory of Pathophysiology, National Cancer Institute, Bethesda, Maryland, U.S.A.

Abstract—*Insulin, which activates and modifies metabolic pathways in MCF-7 human breast cancer cells, is shown to increase the cytotoxic effect of methotrexate up to ten thousand-fold* in vitro. *This enhanced cytotoxicity is not due to an increased bound intracellular drug level, an increased growth rate or an increase in S phase cells, but may involve the modification or activation of biochemical pathways associated with cell growth, even in cells not undergoing DNA synthesis. This observation supports the hypothesis that tumor cell sensitivity to chemotherapy could be increased by using agents that can activate the biochemical or metabolic pathways that determine the cytotoxic process.*

INTRODUCTION

IT IS well established that rapidly growing, high growth fraction tumors are more sensitive to chemotherapy than slow growing, low growth fraction tumors [1]. This reflects significant metabolic or biochemical differences between rapidly cycling, slow cycling and non-cycling cells. It would therefore be desirable to modify the metabolic characteristics of slow cycling and non-cycling cells in such a way that they develop drug sensitivity comparable to that of fast cycling cells.

Although pharmacological studies of chemotherapeutic agents have identified biochemical and metabolic pathways that are related to the cytotoxic process, the precise biochemical lesions that actually cause the irreversible process of cell death have not been clearly defined. Nevertheless, physiological agents such as hormones are known to produce many metabolic changes in target cells. The possibility thus arises that changes, for example, in the hormonal milieu of tumor cells, could be created for the express purpose of inducing changes in the biochemical profile of these cells, thereby enhancing their drug sensitivity. Put most simply, if a drug's cytotoxicity depends upon the disruption of one biochemical pathway which is dormant, then the drug should have no lethal effect. If this pathway is active, then the consequences of its disruption should be lethal to the cell.

Insulin (INS) has been shown to activate metabolic pathways in the human breast cancer cell line MCF-7 [2] that can lead to increased DNA, RNA and protein synthesis [3], and can also increase their growth under certain experimental conditions[4]. In view of these metabolic effects, and because there is so much clinical experience in the manipulation of INS, it was selected as the metabolic modifier in experiments designed to test this hypothesis. Methotrexate (MTX) was selected because it is a cell cycle-specific antifolate drug that is much more effective against fast cycling *S* phase cells than against resting *S* phase cells [5]. It has been widely used in the treatment of human tumors and is currently included in many breast cancer treatment regimens.

This study was therefore designed to examine the effect of INS on MTX cytotoxicity to MCF-7 cells and thereby establish the principle that appropriate metabolic manipulation can increase tumor cell sensitivity to cytotoxic drugs.

MATERIALS AND METHODS

Cell culture

The MCF-7 cells were cultured in T_{25} Falcon flasks using minimal essential medium with Hank's balanced salt solution (MEM) supplemented with 2mM L-glutamine, 100 units penicillin–100 μg streptomycin per ml, 10%

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†Mailing address: Dr. Oliver Alabaster, George Washington University, Dept. of Medicine, Suite 625, 2150 Pennsylvania Avenue, N.W., Washington, D.C. 20037, U.S.A.

fetal calf serum (Flow Laboratories, Maclean, VA) and 10 μg bovine INS (Sigma Chemical Co., St. Louis MO) per ml.

For drug experiments, actively growing (log phase) cells were subcultured at a density of 10^6 cells/T_{25} flask without INS. Twenty four hours after plating, the flasks were divided into two groups. The first group continued to be cultured with the same medium containing MTX (Lederle, Pearl River, NY) at concentrations of 10^{-10}–10^{-4} M. The second group was cultured with the same MTX concentrations in an identical way, except that the medium contained INS. Control cultures in each group did not contain MTX, but those in the second group received INS. Media were replaced every two days and the drug effect was determined by measuring the total DNA mass on days 1, 4 and 7 of cell culture. At each time point cells from triplicate flasks were collected by scraping and pelleted with cold 10% TCA. DNA mass was then determined on each individual pelleted sample according to the method of Kissane and Robbins, using salmon sperm DNA as the standard [6]. In previous experiments, changes in DNA mass correlated consistently with changes in cell number as measured in a hemocytometer.

Similar experiments were also conducted to ascertain the range of MTX concentrations that could be influenced by INS. MCF-7 cells were established in T_{25} flasks as in the first experiment and were exposed to MTX 10^{-12}–10^{-6} M ± INS (10 μg/ml) 24 hr after plating. At this time, cells from triplicate control flasks (− INS) were harvested and pooled for total DNA mass estimation. Media were replaced every 2 days, and on day 7 cells from triplicate flasks in each drug group (± INS) were also pooled for total DNA mass estimation. Corresponding controls ± INS were also obtained.

A subsidiary set of experiments were also carried out to determine the influence of INS concentration. MCF-7 cells were established in T_{25} flasks as described above and, 24 hr after plating, 10^{-10} M MTX, with INS at concentrations of 0.1, 1.0 and 10 μg/ml was added to groups of triplicate flasks. Corresponding control groups containing these INS concentrations were also established. On day 7 cells were harvested from respective drug-treated and control flasks, as in previous experiments.

Cytokinetic analysis

Relative DNA content distributions for cell cycle analysis were derived from aliquots of MCF-7 cells on the seventh day of culture in the first series of experiments. These cells had been exposed to various concentrations of MTX for six days, with and without supplementary INS. Cells were harvested from T_{25} flasks mechanically using a syringe and cannula, washed in phosphate-buffered 0.85% saline and fixed in 70% ethanol. Fixed cells were subsequently analyzed in a Los Alamos Flow Cytometer and Cell Sorter, using the method previously described [7]. Duplicate samples were analyzed.

[^{3}H]-*Methotrexate uptake*

To assess the influence of INS on long term MTX uptake by MCF-7 cells, six T_{25} flasks containing MCF-7 cells in exponential growth were combined to produce a cell suspension. These cells were then divided into 4 large centrifuge tubes and the medium removed. Cells were resuspended in two tubes, in medium containing 5×10^{-8} M labeled MTX (15 mCi S.A., Amersham, Illinois) in one tube and in medium containing 5×10^{-8} M labeled MTX + INS (10 μg/ml) in the other tube. Two control tubes contained cells and the respective media with unlabeled MTX. These tubes were then maintained in a shaking water bath at 37°C and equal aliquots of cells were removed from each tube in duplicate at 30, 60, 120 and 240 min respectively. Each cell aliquot was washed three times in 0.85% NaCl and the final cell pellets were resuspended in 1 ml 1 M NaOH. Radioactivity in each dissolved cell pellet was then determined in a liquid scintillation spectrometer. Radioactivity in the supernatants from the last wash was undetectable in each sample, indicating effect removal of all free [^{3}H]-MTX.

RESULTS

The cytotoxic effect is defined as either reversible or irreversible cell damage. Figure 1 illustrates the effect of 10^{-10} M–10^{-4} M MTX on the growth of MCF-7 cells, with and without supplementary INS. Note that cells were exposed to the drug ± INS for six days. INS itself did not increase the growth rate or the final cell number in the controls. Evidently, 10^{-10} M and 10^{-8} M MTX had no cytotoxic effect in the absence of additional INS. However, these concentrations were cytotoxic in the presence of INS, so that 10^{-10} M *with* INS was equivalent to 10^{-6} M *without* INS. Cells remaining in flasks in which there was cytotoxicity were grossly deformed morphologically; nonetheless they excluded Trypan blue. Attempts to grow these remaining cells in drug-free medium were unsuccessful in subsequent experiments.

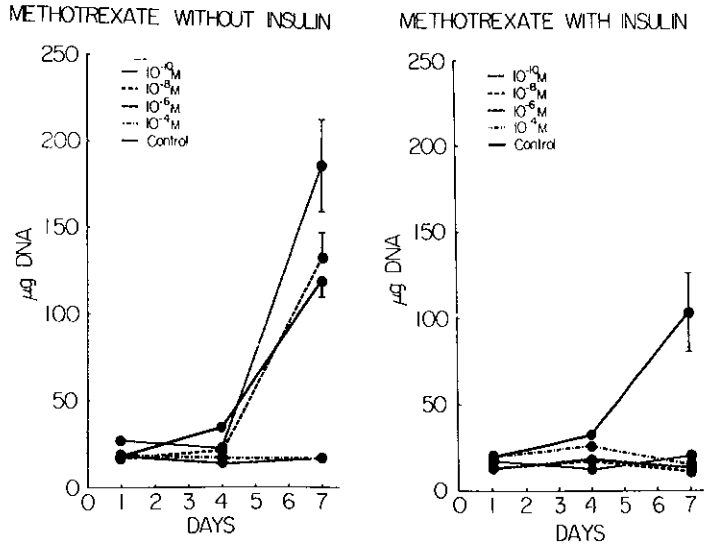

Fig. 1. Effect of MTX with and without INS on the growth of the human breast cancer cell line MCF-7. MTX was administered on day 1 at 10^{-10} M–10^{-4} M. The effect on cell growth was measured by calculating the total DNA mass which had been previously shown to correlate directly with cell number. The addition of supplementary INS made a dose of 10^{-10} M MTX as cytotoxic as 10^{-6} M MTX without INS. Results are expressed as the mean ± S.D.

Figure 2 illustrates the relative DNA content of MCF-7 cells on day 7, following six days exposure to MTX with and without added INS. The first peak represents the frequency of cells with a G_0/G_1 DNA content and the second peak represents the frequency of cells with a G_2 (or mitotic) DNA content. The S phase cells are located between these peaks. It is evident that the proportion of cells in S phase was considerably increased in all cell cultures in which growth was inhibited by MTX. In contrast, the proportion of cells in S phase was not increased in those cultures in which growth was unaffected, although there was a transient increase in S phase cells in these cultures on day 4 (data not shown). This apparent increase in S phase cells could theoretically be produced either by selective loss of G_0/G_1 cells by cell death, or more probably, by progression of G_1 cells into S phase that are subsequently unable to complete DNA synthesis.

Figure 3 illustrates the range of MTX concentrations that can be influenced by INS. These results are expressed as per cent relative growth. This was derived by taking the ratio of DNA mass on day 7 to that on day 1 for the controls (− INS) as normal growth (100%). The effect of INS alone, MTX alone and MTX + INS were then expressed as a percentage of normal growth. It is evident that INS alone had no significant effect on growth, as in the previous experiments. MTX alone again produced growth stimulation in the range of 10^{-11} M–10^{-8} M. Only at 10^{-7} M did MTX alone begin to produce growth inhibition. However, MTX at 10^{-11} M with INS produced significant growth inhibition which was roughly equivalent to that produced by 10^{-7} M MTX without INS; a 10,000-fold difference in MTX concentration.

In the experiments in which MTX at 10^{-10} M was examined with a range of INS concentrations, there was no significant difference in growth inhibition, nor was there any effect on corresponding control cultures.

The mean results of duplicate experiments on [^{3}H]-MTX uptake are presented in Fig. 4. After an initial acceleration of drug uptake at 30 min and 60 min by INS, the bound intracellular drug level reached a steady state at 120 min and was then unaffected by INS.

DISCUSSION

These data demonstrate that INS can increase the cytotoxic effect of MTX up to 10,000-fold in MCF-7 cells *in vitro*. The data also indicate that MCF-7 cells are relatively resistant to MTX since prolonged exposure to MTX alone, at lower concentrations, paradoxically produced growth stimulation unless combined with INS. Growth enhancement by MTX has been reported previously as an incidental finding in EL-4 lymphoma cells [8]. In MTX-treated cell cultures that showed increased

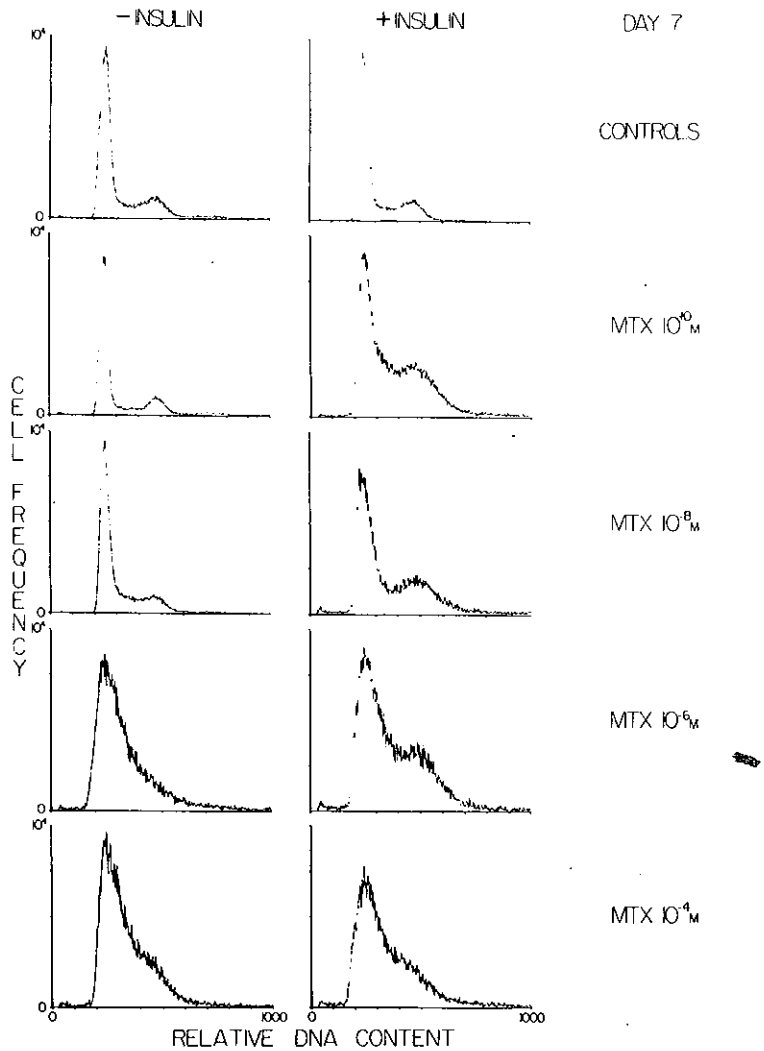

Fig. 2. Relative DNA content distributions obtained on day 7 of the experiment. The control histograms are unaffected by INS and there is little change in those in which MTX was ineffective (10^{-10} M and 10^{-8} M without INS). The remaining histograms were derived from cell cultures in which MTX was cytotoxic, and revealed a relative increase in S phase cells. Histograms derived from duplicate cell cultures were identical.

growth, there was no increase in S phase in the corresponding DNA histograms. This implies that there was a shortening of the cell cycle time which affected all cell cycle phases proportionately. The addition of INS, however, prevented the growth-stimulating effect of MTX. Nevertheless, growth enhancement represents a potentially serious side effect of MTX exposure.

The mechanism by which INS enhances MTX cytotoxicity is not clear. The growth curves of the controls (± INS) do not differ significantly, therefore INS itself does not increase the growth rate, nor is it directly cytotoxic. Thus, an increased growth rate is not the cause of enhanced MTX cytotoxicity. This is also supported by the DNA histograms where the controls are essentially identical, excluding the possibility that INS had increased the proportion of S phase cells that are known to be preferentially sensitive to MTX. However, MTX cytotoxicity is associated with a relative increase in S phase cells, whether or not this cytotoxicity is associated with the addition of INS.

Another possible effect of INS is on MTX transport. However, since the experiments that demonstrated the effect of INS were prolonged, it seems likely that changes in initial free-drug uptake would be less significant than the bound intracellular drug level. Studies of the bound MTX level thus provides indirect information on drug uptake as well as an estimate of the level of dihydrofolate reductase.

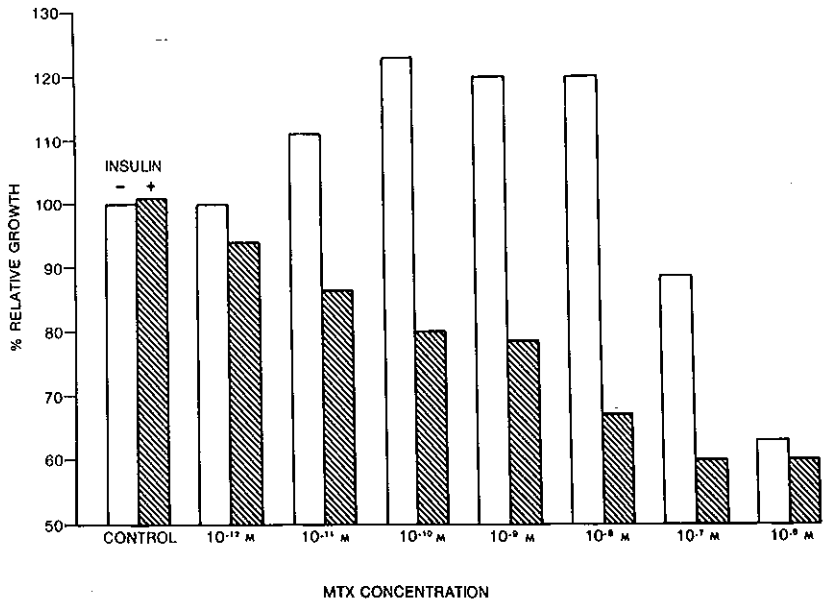

Fig. 3. Relative growth effect of MTX (10^{-12} M–10^{-6} M) ± INS on MCF-7 cells. DNA mass measurements were made on pooled cell samples from triplicate flasks on day 1 (24 hr after cell plating) and day 7 (six days after the addition of MTX). The ratio of total DNA mass of the controls (– INS) on day 7 to the total DNA mass of the controls on day 1 (– INS) represents normal growth (100%). Similar ratios are presented between the total DNA mass on day 7 for each MTX concentration ± INS and the control total DNA mass (– INS) on day 1. Each ratio is then expressed as a percentage of the normal growth ratio.

The similarity of the steady state levels of bound intracellular MTX presented in Fig. 4 suggests that although INS could have an effect on early MTX uptake, there is no effect on the steady-state level of bound MTX. Of course, this does not exclude an effect on the free intracellular MTX level or on the final level of bound intracellular MTX on day 7. Nevertheless, it would seem unlikely that differences in the *rate* of initial drug uptake during such a long period of drug exposure could account for the observed effects of INS.

One of the proposed mechanisms of cell death induced by MTX is that of unbalanced growth [9]. Under these conditions, RNA and protein synthesis continue in the presence of inhibition of DNA synthesis. The possibility thus exists that the anabolic effect of INS accentuates this effect. Moreover, since MTX is thought to act mainly upon cycling cells, the synergistic effect of INS presumably involves the activation of some metabolic or biochemical pathways associated with cell growth, without actually increasing the growth rate. The extent to which INS can induce these changes in G_0 or G_1 cells is not yet clear, though we have observed that MTX–INS-treated cells fail to grow after removing MTX, suggesting that the G_0/G_1 cells were irreversibly damaged. This observation is currently under further investigation.

In the dose–response experiments it was found that MTX at concentrations as low as 10^{-11} M were cytotoxic in the presence of INS and that the highest MTX concentration in which there was a demonstrable enhancement by INS was 10^{-7} M. On the other nand, although other investigators [2, 5] have used INS at a dose

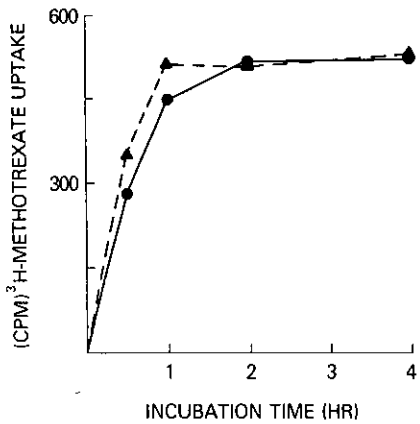

Fig. 4. MTX uptake by MCF-7 cells. Actively growing cells were incubated at 37°C with 5×10^{-8} M [^{3}H]-MTX ± INS for varying periods of time. At the end of incubation the cells were washed three times in 0.9% NaCl and the cell pellets were resuspended in 1 ml of 1 M NaOH, and the radioactivity determined. Results presented are the mean of 2 separate experiments, each done in triplicate.

of 10 μg/ml in the culture media for MCF-7 cells, we have found that growth is unaffected by various INS concentrations. Similarly, the cytotoxic effect of MTX at 10^{-10} M was equally enhanced by different INS concentrations.

For INS to usefully enhance MTX cytotoxicity *in vivo*, there should be a greater effect on tumor cells than normal cells. The prospect that malignant cells may be more reponsive to metabolic modification than normal cells is supported by the evidence that some rat mammary tumors grow only in the presence of INS [10, 11], whereas ductal growth and lobular–alveolar development occur in the normal gland even in diabetic rats [12, 13]. Experiments to assess MTX-INS interaction *in vivo* are currently underway.

It is premature to extrapolate these *in vitro* observations to a clinical trial, but it is possible that the 10,000-fold increase in MTX cytotoxicity produced by INS may establish not only a new way to increase the therapeutic effect of MTX, but also the principle that metabolic modifiers should be examined as a means to increase the tumoricidal effects of chemotherapeutic agents.

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