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Methotrexate Alters the Fatty Acid Composition of NC Adenocarcinoma Cells in Culture

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The effects of methotrexate and indomethacin alone and in combination have been examined on the fatty acid (FA) composition of total cellular lipids in cultured NC adenocarcinoma cells. These studies show that methotrexate can alter the lipid content of cancer cells. Methotrexate 16 ng/ml incubated with NC cells for 2 days increased the content of various FAs. When used alone, indomethacin 1 μg/ml or methotrexate 8 ng/ml had no significant effect, but in combination caused FA increases, usually to about the same extent as with the higher concentration of methotrexate alone. No FA changes were seen up to 3 h with these drug concentrations or with methotrexate up to 10 μg/ml alone or with INDO 1 μg/ml. These effects may explain previous findings that indomethacin potentiates methotrexate, an interaction which may be important in cancer therapy.
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INTRODUCTION

OUR INVESTIGATIONS into methotrexate and indomethacin began several years ago because of our interest in prostaglandins and cancer, and the possibility of using prostaglandin synthesis inhibitors in treating malignancy. Indomethacin prolonged the survival of mice with NC cancer [1] and potentiated methotrexate *in vitro* and *in vivo* probably by enhancing its uptake and/or retention [2, 3]. This effect, which is potentially of importance for cancer therapy, was probably not due to methotrexate displacement from binding sites on the serum proteins, or to inhibition of either prostaglandin formation, cAMP phosphodiesterase, or calcium transport [3, 4].

Fatty acids (FAs) are incorporated into membrane phospholipids where they are important determinants of structure and activity, and they can also be used in cells as an energy source. Changing the FAs can alter membrane fluidity and permeability [5], transport mechanisms, receptor binding, and production of prostaglandins and other eicosanoids [6–8].

There is evidence that FAs influence cancer therapy, and *vice versa*. Dietary enrichment with polyunsaturates increased the accumulation of doxorubicin and methotrexate by tumour cells [9, 10]. Doxorubicin caused an overall rise in the unsaturated FA content of guinea-pig hepatoma cells [11], but little is known about other FA changes resulting from cytotoxic chemotherapy. The present study has, therefore, determined the changes in the FA composition of NC adenocarcinoma cells incubated with methotrexate, and has examined the possibility that methotrexate potentiation by indomethacin involves such changes. The increased tumour content of unsaturated FAs that we have found with methotrexate might alter the variables mentioned above.

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MATERIALS AND METHODS

Cells and cell culture

An NC tumour arose spontaneously in the mammary region of a WHT/Ht mouse [12] and has been passaged in the same strain since then. Metastasis occurs, usually to the lungs and mediastinum. NC cells obtained from ascites fluid grow in culture as a suspension, with a doubling time of 36 h.

A primary culture of peritoneal NC carcinoma cells was obtained from the ascites of a WHT/Ht mouse 10–14 days after an intraperitoneal injection of NC tumour. To ensure a similar age of all cells, the primary cultures were grown as a suspension for 6 days, centrifuged (200 *g*, 10 min), disaggregated in 1 ml trypsin/EDTA solution (0.05:0.02%) per 100 ml of original culture, and stored in liquid nitrogen for up to 4 weeks. After thawing, the cells were cultured for 7 days (75 cm^2 flasks; modified Eagle's medium containing 10% newborn bovine serum, 50 U/ml each of penicillin and streptomycin, 300 mg/l L-glutamine, and 1% non-essential aminoacids from Flow Laboratories). Cells were disaggregated again by trypsin treatment and subcultured in fresh medium.

Drugs or vehicle were added as follows: (a) control (indomethacin and vehicles); (b) indomethacin 1 μg/ml and methotrexate vehicle; (c) methotrexate 8 ng/ml and indomethacin vehicle; (d) methotrexate 16 ng/ml and indomethacin vehicle; (e) methotrexate 8 ng/ml and indomethacin 1 μg/ml. After incubation for 1 h, 3 h or 2 days (37°C, 5% CO_2 in humidified air), the cells were harvested by centrifugation (10 min, 200 *g*) and suspended in 25 ml 154 mmol/l NaCl; 1 ml was removed for total cell counts (Coulter Counter or haemocytometer), viable cell counts (trypan blue exclusion/haemocytometer), and for measuring the size of cells incubated for 2 days with methotrexate 8 and 16 ng/ml with or without indomethacin (microscope and calibrated graticule). For each sample, 200–600 cells were counted to determine viability and total numbers, and 50–60 cells were measured. After a second centrifugation, the cells were resuspended in fresh 154 mmol/l NaCl (4×10^6 cells/ml) prior to FA analysis. Further experiments were performed with aliquots removed from the cells incubated with methotrexate 10, 100, 1000 and 10 000 ng/ml with or without indomethacin 1 μg/ml for 1 and 3 h.

Table 1. Effects of indomethacin and methotrexate on the total fatty acid composition of murine NC adenocarcinoma cells after 2 days in culture

FA	Untreated	Indomethacin (1 μg/ml)	Methotrexate (8 ng/ml)	Methotrexate (16 ng/ml)	Indomethacin (1 μg/ml) plus methotrexate (8 ng/ml)
14:0	5.4 (1.0)	5.1 (1.2)	4.9 (0.9)	6.3 (1.3)	5.3 (1.0)
16:0	21.0 (3.4)	21.4 (5.3)	22.1 (3.1)	31.4 (5.1)†	30.2 (4.3)*
16:1	11.4 (2.4)	13.4 (2.9)	11.4 (1.8)	16.0 (2.5)	14.6 (1.9)
18:0	13.2 (1.6)	13.6 (2.6)	14.8 (1.6)	19.4 (2.4)*	20.8 (3.0)†
18:1	21.1 (2.0)	22.8 (6.2)	26.3 (3.1)	39.9 (5.0)†	40.4 (6.8)†
18:2	1.4 (0.3)	1.6 (0.4)	1.3 (0.1)	1.6 (0.1)	1.8 (0.5)
18:4	1.2 (0.2)	1.2 (0.2)	1.5 (0.4)	1.4 (0.3)	1.4 (0.2)
20:2	7.3 (0.8)	8.4 (1.4)	7.8 (1.1)	9.4 (1.4)	9.2 (1.5)
20:3	1.4 (0.3)	1.3 (0.3)	1.4 (0.5)	1.2 (0.2)	0.9 (0.2)†
20:4	16.0 (1.9)	16.7 (4.4)	20.1 (2.9)	31.1 (4.7)†	31.8 (5.8)†
20:5	16.0 (2.2)	17.2 (4.2)	20.5 (3.3)	29.2 (4.5)*	25.1 (4.7)*
22:6	2.4 (0.5)	2.7 (0.8)	3.0 (0.7)	4.4 (0.8)	4.5 (1.1)
n-3	19.5 (2.8)	21.0 (4.9)	25.0 (4.1)	35.0 (5.5)*	30.9 (5.7)*
n-6	26.0 (2.8)	28.0 (6.3)	30.6 (3.8)	43.3 (5.7)†	43.9 (7.2)†
S	39.6 (5.6)	40.1 (8.9)	41.9 (5.4)	57.0 (8.6)*	56.3 (8.1)
U	78.0 (8.5)	85.2 (19.2)	93.3 (11.5)	134.1 (16.7)†	129.8 (21.0)*
T	117.6 (13.9)	125.3 (27.8)	135.2 (16.1)	191.2 (24.6)†	186.1 (28.7)*
16:0/16:1	1.90 (0.09)	1.68 (0.26)	2.01 (0.14)	1.95 (0.08)	2.05 (0.11)
18:0/18:1	0.62 (0.02)	0.65 (0.03)	0.57 (0.03)	0.49 (0.01)†	0.54 (0.05)
18:2/20:3	1.36 (0.43)	0.10 (0.01)	2.07 (0.70)	1.52 (0.21)	2.65 (0.52)*
20:3/20:4	0.09 (0.02)	0.10 (0.03)	0.08 (0.03)	0.05 (0.02)	0.04 (0.02)†
18:2/20:4	0.09 (0.01)	1.47 (0.26)	0.07 (0.01)	0.06 (0.01)	0.07 (0.01)
n-6/*n*-3	1.41 (0.17)	1.45 (0.21)	1.30 (0.13)	1.31 (0.14)	1.52 (0.17)*
S/U	0.50 (0.03)	0.45 (0.03)	0.45 (0.04)	0.42 (0.03)	0.44 (0.03)

S and U, saturated and unsaturated FA, respectively. Results are microgrammes per 8×10^6 cells (S.E.), $n = 6$. P values: * < 0.05, † < 0.02.

Total lipid extraction

Extraction of the total lipids (phospholipids, neutral lipids and free FAs) was carried out according to the method of Folch *et al.* [13]. Briefly, 1 ml sample, 2 ml methanol, 100 μl internal standard (heptadecanoic acid in chloroform, 10–100 μg, depending on the amount and characteristics of the sample), and 3.9 ml chloroform were mixed on a vortex for 1 min and then centrifuged (2000 *g*, 10 min, 4°C). After evaporating the chloroform phase to dryness at 37°C under a stream of N_2, the total lipid extract was dissolved in di-isopropyl ether/1-butanol (6:4, 2 ml), and 1 ml 50 mmol/l aqueous NaCl was added. After mixing on a vortex and centrifugation, the upper organic phase containing the neutral lipids, phospholipids and free FAs was evaporated at 37°C under nitrogen.

Saponification and methylation of lipids

The lipid extract was dissolved in methanol/KOH (98:2 v/w, 800 μl), saponified by heating in a boiling water bath for 5 min, and then cooled for 2–3 min. BF_3-methanol reagent (14%, 1 ml) was added to methylate the FAs which were then extracted with hexane and dried under nitrogen.

Gas chromatography

FA methyl esters were analysed by capillary gas chromatography [Packard Model 436 GC; splitter type injector (250°C); flame ionisation detector (250°C); Alltech Superox polyethylene glycol column (30 × 0.32 mm bonded FS07); Shimadzu integrator]. The carrier gas was N_2 (20 ml/min), and the oven temperature was programmed from 150 to 230°C at 2°C/min.

Statistics

The results were analysed using the Student's *t*-test for paired data (two-tailed).

RESULTS

The FA composition of the total lipids extracted from untreated NC adenocarcinoma cells grown in culture for 2 days is shown in Table 1. The major FAs were 16:0, 16:1, 18:0, 18:1, 20:2, 20:4 and 20:5.

Cells treated with either indomethacin 1 μg/ml or methotrexate 8 ng/ml alone for 2 days showed no significant changes in FA composition or content compared with untreated cells (Table 1; all P values > 0.1). However, in cells treated for 2 days with methotrexate at 16 ng/ml, the following changes occurred: there was more 16:0, 18:0, 18:1, 20:4 and 20:5 compared with the controls (increases of 50, 47, 89, 94 and 83%, respectively, $P < 0.05$–0.02); the total FA content was 63% higher, with more *n*-3 (18:4, 20:3, 20:5, 22:6) and *n*-6 (18:2, 20:2, 20:4) FAs (44 to 80% increase, $P < 0.05$ to 0.02); the ratio of 18:0/18:1 was 21% less ($P < 0.02$), indicating increased delta 9-desaturase activity.

Similarly, with indomethacin 1 μg/ml plus methotrexate at the lower concentration of 8 ng/ml (amounts which on their own had no significant effect), the cells had more 16:0, 18:0, 18:1,

Table 2. Effects of treatments on cell numbers and sizes

	Cell number (% change)	P	Cell size (μm)	P
Untreated	152.2 (28.8)	0.003	2.2 (0.05)	
Indomethacin 1 μg/ml	97.2 (41.9)	0.07	2.1 (0.03)	
Methotrexate 8 ng/ml	69.5 (30.8)	0.07	2.5 (0.13)	< 0.1
Methotrexate 16 ng/ml	−14.3 (17.0)		2.4 (0.09)	< 0.1
Indomethacin + MTX 8	−19.2 (18.9)		2.6 (0.10)	< 0.1

The cells were incubated for 2 days (six experiments for cell numbers, four experiments for size). Changes in numbers are compared with starting counts.
Mean (S.E.).

20:4 and 20:5 (44, 58, 92, 99 and 57%, $P < 0.05$–0.02), but in contrast there was 36% less 20:3 ($P < 0.02$). Contents of *n*-3 and *n*-6 FAs were 59–69% higher ($P < 0.05$–0.02). The 18:2/20:3 ratio (dependent on delta-6-desaturase activity) increased by 95%, whereas the 20:3/20:4 ratio (dependent on delta-5-desaturase activity) was 56% less.

Incubation of NC cells for 1 or 3 h with methotrexate 8, 16 or 10–10 000 ng/ml alone, indomethacin 1 μg/ml alone, and both drugs together had little or no effect on the cell numbers or their FA composition, total FA amounts or the FA ratios.

The mean cell numbers increased over the 2 day incubation in the groups given vehicle, indomethacin 1 μg/ml or methotrexate 8 ng/ml, but not with methotrexate 16 ng/ml or MTX 8 ng/ml + indomethacin (Table 2).

The viability of the cells, harvested for FA analysis following 1 h of culture, then centrifuged twice (200 *g*) and washings in 154 mmol/l NaCl, was mainly similar regardless of the drug treatments [mean (S.E.) 75 (5%)–81 (2%)]. After 2 days of culture the viability ranged from 52 (6%) to 63 (4%) (all experimental groups), and the cell sizes tended to be larger in the methotrexate groups (Table 2); no giant cells were seen.

DISCUSSION

Our studies show that methotrexate can alter the lipid content of cancer cells, which may have important therapeutic implications. The composition and degree of FA unsaturation influence cell membrane functions and characteristics including fluidity, permeability and thickness. Drugs that increased the sensitivity of guinea-pig hepatoma cells to humoral immune killing also reduced the amount of FA saturation [5], and lipid changes can affect the entry of antineoplastic drugs into tumour cells [9, 10]. However, it is not feasible to predict the changes from our measurements of FAs, since although the amount of unsaturated FAs increased with methotrexate 16 ng/ml and with indomethacin plus methotrexate 8 ng/ml, the mean amount of saturated FAs was also higher, and the tendency for a lower (12–16%) mean saturated/unsaturated FA ratio was not statistically significant. Furthermore, our measurements include all cell lipids, although this may not affect our interpretation since the FA changes in whole cells are thought to reflect those in the plasma membrane [11].

Methotrexate potentiation by indomethacin was shown by the increased amounts of the major FAs with MTX 8 ng/ml plus indomethacin 1 μg/ml, but not by either given alone. Perhaps this increase of the FA changes, to extents generally similar to those with methotrexate 16 ng/ml, might help explain the indomethacin-induced potentiation of methotrexate cytotoxicity to NC and other tumour cells in culture for 2 days or longer (Refs 3, 4; and in the present study).

The cause of the changes is not clear. Methotrexate arrests cells in the S-phase, and this has metabolic consequences. We, therefore, do not know the extent to which the FA changes reflect a general mechanism or are specific for methotrexate. Nor is it clear to what extent the FA changes may either cause or result from the decrease in NC cell numbers in culture. Cell death and/or inhibition of replication themselves might not cause the FA changes, since there tended to be fewer cells after 2 days with the lower methotrexate concentration, but there was little or no change in FAs. Furthermore, although there were fewer cells with the higher concentration of methotrexate alone or with the lower concentration plus indomethacin, these remaining cells were washed twice and spun down gently, and any debris from disintegrated cells was presumably left behind. The per cent viability of the remaining cells was similar in the test and control groups, as indicated by trypan blue exclusion, and the same number of cells was used for the FA analyses.

Our previous work on the potentiation of methotrexate cytotoxicity by indomethacin found an increase of [^{3}H]methotrexate accumulation by NC cells within 1 h of incubation [3]. We thought that this may explain the potentiation of cytotoxicity, but recent evidence indicates that this may not be so. Because tritiated methotrexate was available only at a low specific activity (9.25 GBq/mmol), 2 μmol/l [^{3}H]methotrexate was previously used to obtain adequate tritium accumulation [3]. However, the resulting methotrexate concentration of approximately 960 ng/ml is roughly 100 times the amount needed to kill NC cells *in vitro*. Using [^{3}H]methotrexate at the much higher specific activity now available (370–740 MBq/mmol), but at the low concentrations used in previous cytotoxicity studies, tritium accumulation at 1 h was unchanged by indomethacin (DR Kuonen, EM Anderson, SJH, and A B). Nevertheless, indomethacin may still be useful for potentiating methotrexate clinically since therapeutic blood concentrations can be in the micromolar range.

Towards the end ofthe present study we incubated methotrexate with NC cells in high concentrations (up to 10 μg/ml) for 1 and 3 h, to determine if the early increased methotrexate accumulation might be associated with FA changes. The lack of early FA changes strongly argues against their participation in the mechanism by which indomethacin potentiates [^{3}H]-methotrexate accumulation by NC cells. Nevertheless, alteration of the cell lipids that occurs over 2 days with much lower methotrexate concentrations might account, at least in part, for the potentiation seen with longer term studies *in vitro* and *in vivo* [3]. Inhibition of cyclo-oxygenase does not seem to explain the methotrexate/indomethacin interaction [3], but we do not know if it contributes to changes in FA composition.

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Lack of Therapeutic Gain When Low Dose Rate Interstitial Radiotherapy is combined with Cisplatin in an Animal Tumour Model

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The interaction of cisplatin with low dose rate interstitial radiotherapy was studied in an animal tumour model with a range of dose rates commonly used in clinical low dose rate brachytherapy. Small pieces of R1-rhabdomyosarcoma were implanted subcutaneously in the flanks of female Wag/Rij rats. When the tumour had grown to the desired treatment volume, four afterloading catheters were inserted in the tumour in a square geometry, and a fixed spacing was attained by means of a template. Subsequently, four 2 cm Ir^{192} wires were inserted. A range of tumour doses of 20–120 Gy at a mean dose rate of 48 cGy/h was applied; 15 mins before the implant an intraperitoneal bolus injection of 3 mg/kg cisplatin was given. For growth delay and cure rate, no modification of the effects of low dose rate brachytherapy by the addition of cisplatin was observed. The observed effects of the combination of cisplatin with low dose rate interstitial radiation in relation to the animal tumour are discussed.
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INTRODUCTION

THE INTERACTION of cisplatin with high dose rate irradiation has been studied extensively on tumour and normal tissues, both *in vivo* and *in vitro*. A varying degree of enhancement of the radiation effect by cisplatin was observed *in vitro* and several mechanisms have been proposed to explain this, such as a true radiosensitising effect [1–3] and inhibition of sublethal and potential lethal damage repair [1,4,5]. These effects were sometimes more pronounced in the absence of oxygen [4,5] while occasionally just the opposite has been reported [5–7]. *In vivo*, different dose enhancement factors have been reported in tumours and normal tissues. Depending on dose, timing and sequence a maximum dose enhancement factor was observed when the highest dose of an intraperitoneal bolus injection of cisplatin was given just before the start of the irradiation or with fractionated drug and radiation treatment [8–11].

The inhibition of sublethal and potential lethal damage repair *in vitro*, the diminshed recovery in tumours in split dose experiments *in vivo* [12] and the supra-additive effects obtained *in vivo* after fractionated schedules of both the drug and radiation treatment in two animal tumour models [10,11,13,14] were of particular interest to the design of the present study. It was argued that, if the modification of the radiation effects by cisplatin is really based on inhibition of sublethal damage repair,

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