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Fatty Acid Composition of Normal and Malignant Cells and Cytotoxicity of Stearic, Oleic and Sterculic Acids *in vitro*

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The aim of this study was to investigate the hypothesis that saturated fatty acids are differentially cytotoxic to cancer cells. Three studies were undertaken to: (1) measure the toxicities of stearic and oleic acids to normal and malignant cells *in vitro*, (2) assess if there is any relationship between toxicity and relative fatty acid composition and (3) determine whether the relative fatty acid composition of a cancer cell line could be modified by sterculic acid, an inhibitor of delta-9-desaturase. Stearic (18:0) and oleic (18:1) acids inhibited the colony-forming abilities of five human cancer cell lines and two non-neoplastic cell lines in a dose-dependent fashion. The concentration of oleic acid required to reduce colony formation ability by 50% was 2.5–6.0-fold greater than that of stearic acid. Addition of sterculic acid to a cancer cell line resulted in steady-state levels of stearic acid and increasing percentage of oleic acid.
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INTRODUCTION

CANCER CELLS appear to have an altered balance of saturated to monounsaturated fatty acids. For example, the ratio of stearic to oleic acid is lower in hepatocellular carcinoma than normal liver [1]. Similarly, increased proportions of oleic acid are found in experimental tumours [2–4], hepatoma cell lines [5] and virally transformed cell lines [6, 7]. These observations led to the concept that tumour cell growth might be modulated by addition of exogenous saturated fatty acids such as stearic acid [8].

The aim of this study was to investigate the possibility that fatty acids possess anticancer activity. Three studies were undertaken. These were: (1) to compare the *in vitro* toxicities of saturated stearic acid with monounsaturated oleic acid to normal and neoplastic cells, (2) to determine whether there is any relationship between percentage fatty acid compositions and toxicity, and (3) to determine if percentage fatty acid composition of one malignant bladder cell line (RT112) is influenced by sterculic acid. This compound inhibits delta-9-desaturase, the enzyme which desaturates stearic to oleic acid [3].

Previous studies *in vitro* have assessed the differential cytotoxicity of stearic and oleic acids to one or two cell lines [9–16]. This is the first study to assess toxicity using a panel of human neoplastic and non-neoplastic cells *in vitro*. Human cancer cell lines were chosen from tumour types with a wide range of sensitivity to chemotherapy, including curable testicular cancer, moderately sensitive bladder cancer and relatively resistant colon cancer. The sensitivities of these cell lines to chemotherapeutic drugs reflects the clinical response of their tumours of origin [17]. The sensitivities of the cancer cell lines were compared with those of a continuous cell line derived from normal urothelium, HU609, and an untransformed normal fibroblast cell line derived from fetal lung, HFL.

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MATERIALS AND METHODS

Cell lines

Five human cancer cell lines were used. Two were derived from transitional cell carcinomas of the bladder, RT112 and RT4 [18], 2 from non-seminomatous testicular germ-cell tumours, SuSa and 833K [19, 20], and one from a colon carcinoma, HT29 [21]. Two cell lines derived from normal tissue were used. HU609 was derived from human ureteric epithelium [22] and HFL from human fetal lung fibroblasts. All cell lines were maintained under identical conditions as monolayers in RPMI 1640 medium (Gibco) supplemented with 5% heat-inactivated fetal calf serum (Sigma) and 2 mmol L-glutamine (Gibco). Each cell line was used over a restricted range of 10 *in vitro* passages to minimise any changes that might occur during long-term culture. One batch of fetal calf serum was used for these studies.

Clonogenic assays

A single cell suspension of exponentially growing cells was plated at a density of 200–2000 cells (depending on the plating efficiency of the cell line) in 5 ml medium in 5 cm Petri dishes (Nunc). Cells were incubated at 36.5°C in 5% CO_2 for 48 h, after which the medium was replaced with medium containing a range of concentrations of fatty acids in the experimental dishes or 0.5% alcohol in the controls. Three replicate dishes were used for each fatty acid concentration and five for the controls. Stearic, oleic (Sigma) and sterculic acid (Lord Zuckerman Research Centre, Reading) were dissolved in ethyl alcohol (BDH) and further diluted in culture medium so that the final alcohol concentration did not exceed 0.5%. All fatty acids were made up fresh for each assay. After 12 days culture, colonies were fixed in methanol and stained with 10% Giemsa (BDH). A colony was classified as a group of 50 or more cells. Each assay was repeated at least three times. The mean number of colonies at each fatty acid concentration was expressed as a percentage of the mean number of colonies in the control dishes, and percentage survival plotted on a logarithmic scale against fatty acid concentration on a linear scale. Points which lay on the straight part of the curve were assessed for linear regression analysis (using "Oxstat" software) for each experiment. The IC_{50} value (the concentration necessary to inhibit 50% of colony formation) for each experiment was calculated and the mean determined from a minimum of three experiments for each fatty acid and each cell line. Comparisons of IC_{50} values between assays were tested for statistical significance using the Student's *t*-test ("Oxstat" software).

Table 1. *Concentration of stearic and oleic acid reducing colony forming ability by* 50% (IC_{50})

Cell line origin		Stearic* (μmol/l)	Oleic* (μmol/l)	Ratio of IC_{50} values oleic/stearic
Malignant				
Testis	SuSa	9.7 (4.8)	58.4 (8.1)	6.0
Testis	833K	17.6 (0.6)	94.0 (8.8)	5.3
Bladder	RT112	15.3 (2.7)	79.3 (10.6)	5.2
Bladder	RT4	26.8 (1.5)	88.4 (2.6)	3.3
Colon	HT29	26.9 (0.5)	121.4 (2.8)	4.5
Normal				
Urothelium	HU609	25.1 (1.9)	89.6 (4.2)	3.6
Fetal lung fibroblasts	HFL	30.5 (0.4)	77.0 (2.4)	2.5

* Mean (S.E.M.).

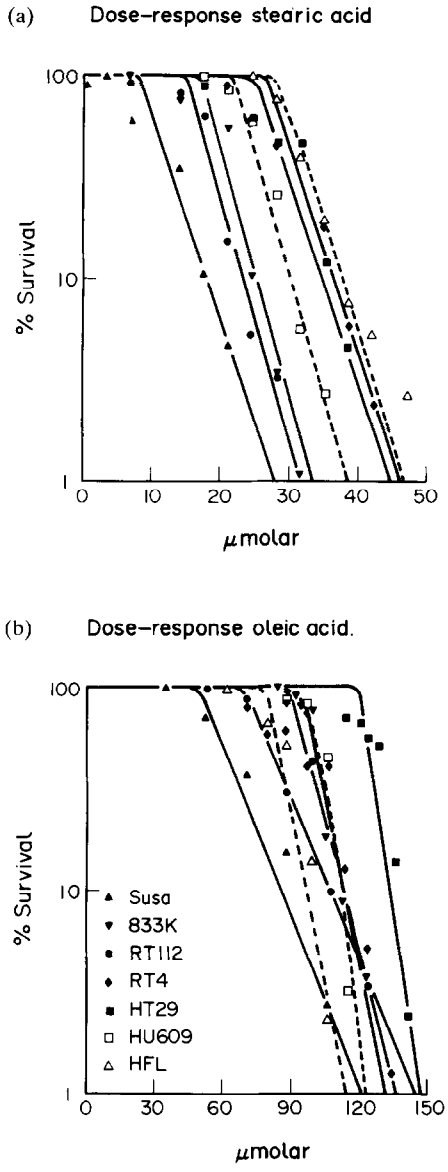

Fig. 1. Dose–response curves of (a) stearic and (b) oleic acids to five malignant (Susa, 833K, RT112, RT4, HT29) and two normal (HU609, HFL) cell lines.

Fatty acid content of cell lines

To measure percentage fatty acid composition, cells were plated in 25 cm^2 flasks in 5 ml of culture medium and incubated for 4 days. The medium was replaced and 24 h later exponentially growing cells detached using 0.05% trypsin in 0.016% versene (ethylenediamine-tetra-acetic acid disodium salt). The cells were washed four times with phosphate buffered saline (PBS) in 10 ml neutral capped glass tubes with Teflon-lined lids (FBG Trident Ltd, Bristol). Total lipids were extracted by the Slayback method [23]. The lipids were saponified with 15% methanolic potassium hydroxide (BDH) and the free fatty acids liberated from their potassium salts with hydrochloric acid. The free fatty acids were extracted further with benzene (BDH) and methylated with 10% boron trichloride in methanol (Sigma) [24]. After purification with the addition of water, the benzene

phase containing fatty acids was dried down under nitrogen and redissolved in trimethylpentane (BDH). Extractions of the fatty acid methyl esters were analysed by temperature-programmed gasl liquid chromatography [1]. The methodology used in this study assesses total lipid content of the cells. The fatty acid composition of each cell line was measured on a minimum of three samples.

The fatty acids detectable using this system were: 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 18:3 (linolenic acid), 20:0 (arachidic), 20:1 (11-eicosenoic acid) and 20:4 (arachidonic acid). These fatty acids were identified according to the retention times of standard samples (Sigma). A solvent blank and two control samples were also run. The values of the individual fatty acids were expressed as a percentage of the total. Minor peaks were also present, but these could not be reliably quantitated.

Influence of sterculic acid on relative fatty acid content of RT112 cells

5000 RT112 cells were plated in 5 cm Petri dishes in 5 ml of culture medium. The cells were incubated at 36.5°C in 5% CO_2 for 48 h and fresh medium containing 18 μg/ml sterculic acid or 0.02% alcohol added, this being the highest concentration of alcohol to which the cells were exposed. After further 24, 48, 72 and 96 h culture periods the cells were removed using trypsin from triplicate dishes. The cells were then treated as described in the previous section and all samples were analysed within 1 week. The experiment was repeated three times. The effect of sterculic acid on the relative fatty acid content of the other cell lines was not studied.

RESULTS

Cytotoxicity

The sensitivities of the cell lines to fatty acids are shown in Table 1 and Fig. 1. Stearic acid sensitivities were similar in the two normal cell lines (HU609 and HFL) and two cancer cell lines RT4 and HT29, while the bladder cancer line RT112 and the testis cancer lines SuSa and 833K were more sensitive. Oleic acid sensitivities were similar amongst the cell lines, with the exception of SuSa, which was more sensitive, and HT29, which was more resistant. The cell lines were between 2.5 and 6.0 times more sensitive to stearic than oleic acid comparing molar concentrations of the two fatty acids. Sterculic acid sensitivities of two cancer and one normal cell line (833K, RT112 and HU609) were similar (57.0–65.9 μmol/l), but the colon cancer line HT29 was not killed by concentrations up to 170 μm.

Fatty acid composition

The mean percentage fatty acid composition of the five cancer and the two normal cell lines are shown in Table 2. Two-way analysis of variance showed significant differences in fatty acid composition between the five malignant cell lines and also between the two normal cell lines. The greatest differences in the fatty acid composition between the 5 malignant cell lines were seen in the palmitoleic (16:1), stearic (18:0) and oleic (18:1) acid composition. When the fatty acid compositions of the two normal cell lines were compared, the normal urothelial cells (HU609) contained a greater proportion of oleic (18:1) and a lower percentage of linoleic (18:2) and arachidic (20:0) acids than the normal fibroblasts (HFL).

In order to assess differences in the relative fatty acid composition between normal and malignant cell lines, each malignant cell line was compared separately with each normal cell line. The proportion of stearic acid was significantly lower in all five malignant cell lines and the proportion of oleic and palmitoleic acids was significantly greater in four out of five of the malignant cell lines compared with the normal fibroblasts. Compared with normal urothelium, four out of five of the malignant cell lines contained a significantly greater proportion of oleic acid. Both malignant bladder cell lines contained a significantly greater proportion of palmitoleic acid than the normal urothelium but there were no other consistent differences in relative fatty acid composition between normal and malignant bladder.

The IC_{50} (concentration necessary to inhibit 50% of colony formation) of stearic and oleic acid for each cell line (Table 1) was plotted against the relative percentage of each of the fatty acids. The correlation coefficient (r) and its significance were calculated (Table 3). There was no correlation in percentage fatty acid composition and the IC_{50} of stearic acid. The IC_{50} of

Table 2. Relative percentage fatty acid content of five malignant and two normal cell lines

		Malignant cell lines					Normal cell lines	
		Testis		Bladder				
Fatty acid		SuSa	833K	RT112	RT4	Colon HT29	Urothelium HU609	Fibroblasts HFL
Palmitic	(16:0)	16.91 (1.11)	22.30 (1.74)	21.13 (0.57)	21.72 (0.91)	23.48 (1.12)*	19.46 (1.55)	19.53 (0.93)
Palmitoleic	(16:1)	4.65 (2.11)	5.74 (0.24)†	13.59 (2.24)‡‖	18.31 (1.51)‡¶	11.03 (1.08)‡¶	4.19 (0.65)	3.26 (0.71)
Stearic	(18:0)	16.83 (1.24)*	15.83 (0.27)†	14.94 (2.02)*	5.02 (0.45)‡¶	14.34 (0.90)‡§	18.02 (1.13)	21.13 (1.19)
Oleic	(18:1)	38.47 (2.37)*	41.00 (1.85)‡	35.59 (2.74)	44.04 (0.51)‡¶	40.71 (0.90)‡	38.55 (0.66)‡	30.99 (1.26)
Linoleic	(18:2)	3.48 (0.90)	1.16 (0.70)†	2.55 (0.08)	1.05 (1.05)*	2.87 (0.39)*	2.58 (0.57)*	4.96 (0.73)
Linolenic	(18:3)	1.30 (0.64)	< 0.05	< 0.05	1.30 (1.14)	< 0.05	2.44 (0.63)	0.98 (0.78)
Arachidic	(20:0)	8.42 (1.96)	9.37 (2.33)	4.92 (2.49)*	3.06 (1.62)†§	3.51 (0.66)†‖	7.75 (0.77)*	10.73 (1.14)
Eicosenoic	(20:1)	4.29 (1.02)	0.97 (0.56)	4.26 (3.92)	2.21 (2.09)	< 0.05	1.18 (0.30)	2.10 (0.44)
Arachidonic	(20:4)	2.18 (0.38)	3.41 (1.55)	2.82 (0.16)	1.22 (0.14)	2.98 (0.26)	2.56 (0.77)	3.35 (0.57)
Minor peaks		3.49 (0.84)	0.99 (0.57)	0.20 (0.20)*	2.07 (0.76)	0.87 (0.29)*§	3.28 (0.92)	2.97 (0.68)
No. of samples		8	4	3	3	7	6	6

Values represent mean (S.E.M.).
Significant difference: compared with HFL: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$; compared with HU609: § $P < 0.05$, ‖ $P < 0.01$, ¶ $P < 0.001$.

Table 3. Correlation coefficients between fatty acid composition and IC_{50} values of stearic and oleic acids

Fatty acid		Correlation coefficients: Stearic	Oleic
Palmitic	(16:0)	0.811	0.918*
Palmitoleic	(16:1)	0.676	0.267
Stearic	(18:0)	0.490	0.399
Oleic	(18:1)	0.490	0.399
Linoleic	(18:2)	−0.481	−0.257
Linolenic	(18:3)	−0.125	−0.593
Arachidic	(20:0)	−0.760	−0.478
Eicosenoic	(20:1)	−0.741	−0.910*
Arachidonic	(20:4)	−0.221	0.354

* Significant correlation ($P < 0.05$).

oleic acid was associated ($P < 0.05$) with the palmitic (16:0) acid and the eicosenoic (20:1) acid content of the cells.

Effects of sterculic acid on the relative fatty acid composition of RT112 cells

Under normal culture conditions the relative percentage of palmitic and stearic acids decreased and those of oleic acid increased in the human bladder cancer cell line, RT112. Sterculic acid reversed the changes in the 18-carbon fatty acids, resulting in steady-state levels of stearic acid and a fall in the proportion of oleic acid. Sterculic acid caused a relative increase in the palmitic acid composition (Fig. 2).

DISCUSSION

The aim of this study was to investigate the hypothesis that saturated fatty acids might show differential toxicity to cancer cells. Stearic acid was more cytotoxic than oleic acid, in agreement with five previous *in vitro* studies [9–13]. It has also been shown that the cytotoxic effect of stearic acid can be abrogated by simultaneous addition of oleic acid [9, 11, 14]. However, two studies disagree and found oleic acid to be more toxic than stearic acid [15, 16]. The discrepancies are not due to the fatty acid content of serum, since stearic acid has been shown to be more toxic than oleic acid using serum-free medium [9], delipidized serum [11] and in this study 5% fetal calf serum.

We do not know why fatty acids are cytotoxic. Non-specific cytotoxicity may occur as a result of lipid droplets or lipid inclusions in the cytoplasm of the cell (steatosis), leading to irreversible cell degeneration [25]. This is likely to be a general phenomenon following exposure of cells to fatty acids. However, individual fatty acids may have specific effects on cell membrane function [26]. In order to determine whether the cytotoxicity of stearic acid is due to a membrane-specific effect, studies need to be undertaken to determine if exogenous free fatty acids are incorporated into the cell membrane. This is likely as other studies have shown a change in total percentage fatty acid content reflects a change in membrane fatty acid content [27, 28].

The two normal cell lines have different fatty acid compositions. HFL is a low-passage culture of embryonic fibroblasts and is neither transformed nor immortal. HU609 is a continuous cell line derived from human urothelium and is immortal but is not tumorigenic in nude mice [18]. The HU609 cell line has a fatty acid profile closer to that of the malignant cell lines. Therefore the differences in relative fatty acid composition in this *in vitro* study may reflect changes occurring as a result of immortalisation, rather than differences between malignant and normal cells. In support of this hypothesis, increased oleic acid composition was seen in rat fibroblasts transformed by herpes simplex virus and simian virus 40 (SV40), transformed W1–38 cells [29], and Rous sarcoma virus (RSV) transformed chick embryo fibroblasts [6] compared with non-transformed fibroblasts. Similarly more oleic acid was seen in proliferative neu-

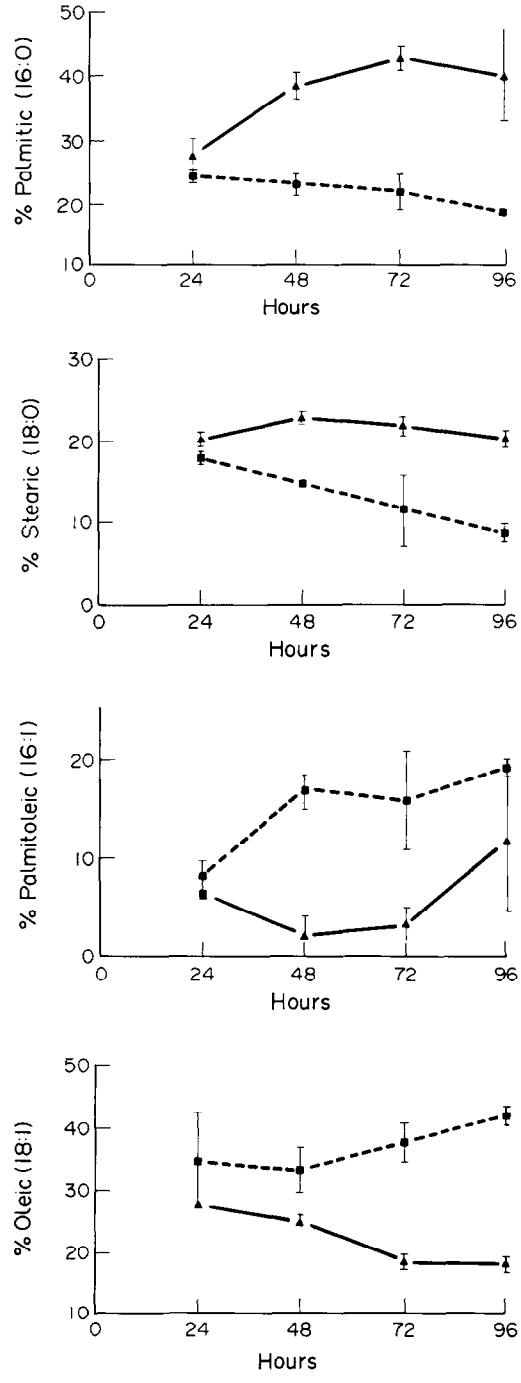

Fig. 2. Palmitic, palmitoleic, stearic and oleic acid composition of RT112 cells after addition of 18 μg/ml sterculic acid to the culture medium for 96 h. – –■– – control, —▲— sterculic.

ronal or glial cells than transformed cells [29]. However, in contrast to these findings, increases in the percentage of palmitic and stearic and decreases in the percentage of palmitoleic and oleic acid in total cellular lipid were seen in SV40 transformed hamster fibroblasts compared with normal fibroblasts [30].

One limitation of this study is the use of serum-supplemented medium, as it is possible that the cytotoxicity of stearic and oleic acid are influenced by the fatty acid content of the culture medium and supplements. Extracellular fatty acid is taken into the cell in the form of free fatty acids]26]. The free fatty acid content of one batch of fetal calf serum at 5% was 3.6 μmol/l stearic acid and 9.9 μmol/l oleic acid [9], compared with IC_{50} values of stearic acid of 9.7 to 30.5 μmol/l (2.8 to 8.7 μg/ml) and IC_{50} values of oleic acid of 58.4 to 121.4 μmol/l (16.5 to 34.2 μg/ml). Significantly higher concentrations of stearic and oleic acids are needed to kill cells. The concentrations of these fatty acids found in 5% serum are non-toxic.

The sensitivity of the malignant cell lines to stearic acid was not associated with fatty acid composition. However, there was a correlation between the sensitivity of the malignant cells to oleic acid and the palmitic (16:0) and eicosenoic (20:1) composition. Studies on further cell lines would be needed to confirm these findings.

Sterculic acid inhibits the formation of oleic acid [3] by *de novo* synthesis within the cell and by desaturation of exogenous stearic acid taken into the cell from the culture medium [31]. It caused a 2.1-fold increase in the percentage of stearic acid and a 2.4-fold decrease in the percentage of oleic acid of RT112 human bladder cancer. This was the only cell line on which the effects of sterculic acid were studied. Sterculic acid induced similar changes in the stearic and oleic composition of Morris hepatoma cells *in vitro* [31].

This study shows that all cancer cell lines studied contain a lower proportion of stearic acid than normal fibroblasts but not normal urothelial cells, and that by inhibiting delta-9-desaturase, the percentage stearic acid content of the malignant bladder cancer cell line (RT112) studied was increased. However, we failed to show any relationship between relative fatty acid compositions and the toxicity of stearic acid, and stearic acid did not show greater toxicity to all five cancer cell lines compared with the non-neoplastic cell lines tested.

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