Recombinant human tumour necrosis factor-α suppresses synthesis, activity and secretion of lipoprotein lipase in cultures of a human osteosarcoma cell line

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The effect of recombinant human tumour necrosis factor-α (TNF-α) on synthesis, activity and secretion of lipoprotein lipase (LPL) was examined using a human osteosarcoma cell line, osteosarcoma Takase (OST). Treatment of OST cells with TNF-α decreased LPL synthesis, resulting in a decrease in expression of activity and secretion of LPL. When OST cells were incubated with glycerol tri[1-^{14}C]palmitate, TNF-α decreased dose- and time-dependently the production of $^{14}CO_2$ and the amounts of radioactivity incorporated into cellular triacylglycerol and phospholipid. The similar reduction of synthesis and activity of LPL as suppression of CO_2 production and cellular lipid synthesis indicated that the suppression of $^{14}CO_2$ production and ^{14}C-labelled lipid synthesis was secondary. TNF-α also suppressed expression of proliferating cell nuclear antigen, indicating that it had an anti-proliferative activity on OST cells. The findings suggest that one cause of the anti-proliferative activity of TNF-α is the suppression of the LPL-mediated supply of non-esterified fatty acids as an energy source for growth.

INTRODUCTION

Tumour necrosis factor is a polypeptide cytokine produced primarily by monocytes and macrophages in response to endotoxin, inflammatory agents and neoplastic growth. It has cytotoxic activity against various transformed cells, but has little effect on normal cells [1–5]. Tumour necrosis factor causes haemorrhagic necrosis, resulting in vascular collapse, tumour anorexia and subsequent tumour death. It also activates neutrophils *in vitro* and tumour necrosis factor-stimulated neutrophils exhibit an anti-tumour effect in culture [6–8].

In vitro [9–11] and *in vivo* [12] experiments have shown that tumour cells can use fatty acids (FA) as an energy source for growth. These FA are supplied either as non-esterified fatty acids (FFA) bound to albumin or as triacylglycerols in circulating chylomicrons and very-low-density lipoproteins. FFA are directly taken up by the tissues, whereas triacylglycerol is hydrolysed to FFA and monoacylglycerol by lipoprotein lipase (LPL) before uptake by the tissues.

Although there are many reports that tumour necrosis factor suppresses adipose tissue LPL activity *in vitro* [13–16] and *in vivo* [17,18], there has been no report on the effect of tumour necrosis factor on LPL activity and lipid metabolism in tumour cells. Herein, we describe the effect of recombinant human tumour necrosis factor-α (TNF-α) on synthesis, activity and secretion of LPL, and on the oxidation of and the cellular lipid synthesis from triacylglycerol-derived FA in a human osteosarcoma cell line, osteosarcoma Takase (OST) [19]. We also report on the effect of TNF-α on expression of proliferating cell nuclear antigen (PCNA), which is a non-histone nuclear protein ($M_r = 36000$) expressed in proliferating cells [20–22].

MATERIALS AND METHODS

Cell culture

A human osteosarcoma cell line, osteosarcoma Takase (OST), was a kind gift from Dr. Katsuro Tomita, Department of Orthopaedic Surgery, School of Medicine, Kanazawa University, Japan. OST cells were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B in 25-cm^2 flasks or 60-mm-diam. plates, and the medium was changed every 2 days. Cells were grown to about 80% confluence for each experiment.

Assay of LPL activity

The plates were replenished with the medium containing TNF-α at the indicated concentrations and cells were incubated for 22 h at 37 °C. Then the medium was replaced with fresh medium containing the appropriate additive and cells were incubated for 2 h at 37 °C. The medium was separated from cells, filtered through a 0.2-μm-pore-size filter, and used for prompt assay of LPL. Cellular LPL activity was measured in the acetone/ether powder extract of cells. Cells were harvested into 1.2 ml of 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 2% (w/v) BSA and 20 μg/ml of heparin, and sonicated briefly at 0 °C. An aliquot of the homogenate was used for preparing an acetone/ether powder [23]. The extract was made by adding the powder to ice-cold 50 mM NH_4Cl/NH_4OH buffer (pH 8.2) containing 20 μg/ml of heparin, letting the mixture stand at 0 °C for 1 h, sonicating briefly at 0 °C, centrifuging for 10 min at 4 °C and 1200 ***g***, and decanting the supernatant for assay.

A stock emulsion containing 5 mCi of tri[9,10(*n*)-^{3}H]oleoylglycerol, 1.13 mmol of trioleoylglycerol, 60 mg of phosphatidylcholine and 9 ml of glycerol was prepared [23]. One vol. of the stock emulsion, 19 vol. of 3% (w/v) BSA in 0.2 M Tris/HCl buffer (pH 8.2) and 5 vol. of heat-inactivated (56 °C, 10 min) serum from starved rats were mixed and incubated at 37 °C for 15–30 min. For assay, 100 μl of this activated substrate mixture, containing 2.0 μCi of tri[9,10(*n*)-^{3}H]oleoylglycerol, 450 nmol of trioleoylglycerol and 34 nmol of BSA, was added to 100 μl of the diluted tissue extract and medium, and incubated at

Abbreviations used: LPL, lipoprotein lipase; TNF-α, recombinant human tumour necrosis factor-α; PCNA, proliferating cell nuclear antigen; FA, fatty acids; FFA, non-esterified fatty acids.

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37 °C for 60 min. One m-unit of lipolytic activity represents release of 1 nmol of FA/min at 37 °C.

Incorporation of [^{35}S]methionine into total protein, LPL and PCNA

OST cells were incubated for 21.5 h at 37 °C in 3 ml of the medium containing TNF-α at the indicated concentrations in 60-mm-diam. plates. Cells were then washed once with Dulbecco's PBS and incubated in 1.5 ml of methionine-deficient Dulbecco's modified Eagle's medium containing the appropriate additive. After 30 min, 160 μCi of [^{35}S]methionine was added to each plate and the plates were incubated for 2 h at 37 °C. Cells were washed once with PBS, harvested in 0.5 ml of lysis buffer (pH 7.5) containing 0.2 M Tris, 3% Triton X-100, 1% *N*-lauroylsarcosine, 0.15 M NaCl and 1 mM PMSF, and sonicated briefly at 0 °C, and centrifuged for 20 min at 4 °C and 15000 ***g***. Aliquots of the supernatants were used for measurement of the amount of radioactivity incorporated into total protein of cells by 10% trichloroacetic acid precipitation [23].

^{35}S-labelled LPL was immunoprecipitated from an aliquot of the supernatant with chicken antiserum to bovine LPL, which was kindly given by Dr. Thomas Olivecrona, University of Umeå, Umeå, Sweden, and resolved by SDS/PAGE [23].

To precipitate ^{35}S-labelled PCNA, an aliquot of the supernatant was mixed with 20 μg of mouse monoclonal antibody to PCNA (PC10, DAKO Japan Co., Ltd.) in 1 ml of 0.1 M sodium borate buffer (pH 8.0) containing 0.5 M NaCl, 1% Triton X-100, 5 mM EDTA, 10 μg/ml of leupeptin, 1 μg/ml of pepstatin, 3.5 μg/ml of aprotinin and 0.1 mM PMSF. After 48 h, 200 μg of rabbit anti-(mouse IgG) was added and incubation was continued for 16 h at 4 °C. The immunoprecipitate was collected, washed and resolved by SDS/PAGE as described previously [23].

Autoradiographs were obtained by exposure of Kodak X-Omat film to gels at −80 °C for 14 days. The radioactive band corresponding to LPL or PCNA was cut out from the gel and the excised gel was dissolved in 1 ml of 30% hydrogen peroxide at 65 °C overnight. Radioactivity was determined in a liquid scintillation counter.

$^{14}CO_2$ production and ^{14}C-labelled lipid synthesis in OST cells

A stock emulsion containing 0.5 mCi of glycerol tri[1-^{14}C]palmitate, 113 μmol of tripalmitoylglycerol, 6 mg of phosphatidylcholine, and 0.9 ml of glycerol was prepared. One vol. of the stock emulsion, 19 vol. of 3% (w/v) BSA in 0.2 M Tris/HCl buffer (pH 8.2), and 5 vol. of heat-inactivated serum from starved rats were mixed and incubated at 37 °C for 15–30 min. This mixture was used as the activated substrate mixture. In another series of experiments, we used a mixture of 100 μCi of [1-^{14}C]palmitic acid and 169 μmol of palmitic acid in 1 ml of ethanol.

The $^{14}CO_2$ production was measured by the method of Ichiyama et al. [24] with modification. About 80% confluent cells cultured in 25-cm^2 flasks were incubated for 24 h at 37 °C in 2 ml of RPMI-1640 medium containing TNF-α at the indicated concentrations. The medium was changed for 2 ml of fresh medium containing the appropriate additive. After 2 h, either 0.25 ml of the activated substrate mixture or 20 μl of the palmitic acid mixture was added. Then the flask was sealed with a culture tube (16 mm × 100 mm) containing a piece of 20% β-phenethylamine-soaked filter paper (Whatman 3MM), on to which the $^{14}CO_2$ produced is adsorbed. Then cells were incubated for 4–22 h at 37 °C. The reaction was terminated by addition of 0.1 ml of 0.5 M H_2SO_4 and the flask was kept for 10 min on ice. The radioactivity adsorbed on to the filter paper was measured in a liquid scintillation counter. The DNA content in the cells cultured for 24 h in the flask sealed with a culture tube was identical to that in the cells cultured in an atmosphere of 5% CO_2 in air (results not shown). In both cases, the cells attached to the bottom of the flask excluded Trypan Blue. These findings indicate that the cells cultured in a sealed flask were growing normally during the experimental period.

Cells were washed twice with ice-cold PBS, harvested in PBS, and collected by centrifugation for 10 min at 4 °C and 1200 ***g***. Cellular lipids were extracted with a mixture of chloroform and methanol (2:1, v/v) and separated by TLC on silica gel plates using a solvent system of hexane/ether/acetic acid (60:40:2, by vol.). Each extract contained oleic acid, mono-oleoylglycerol, dioleoylglycerol and trioleoylglycerol as inner standards. Lipids were visualized by exposure to iodine vapour. Silica gel corresponding to each lipid was cut out, and radioactivity was measured in a liquid scintillation counter.

DNA measurement

DNA was measured fluorometrically by the method of Hinegardner [25] using calf thymus DNA as standard.

RESULTS

Effect of TNF-α on LPL activity

OST cells were incubated for 24 h in the absence or presence of TNF-α, and LPL activity in the extract of the acetone/ether

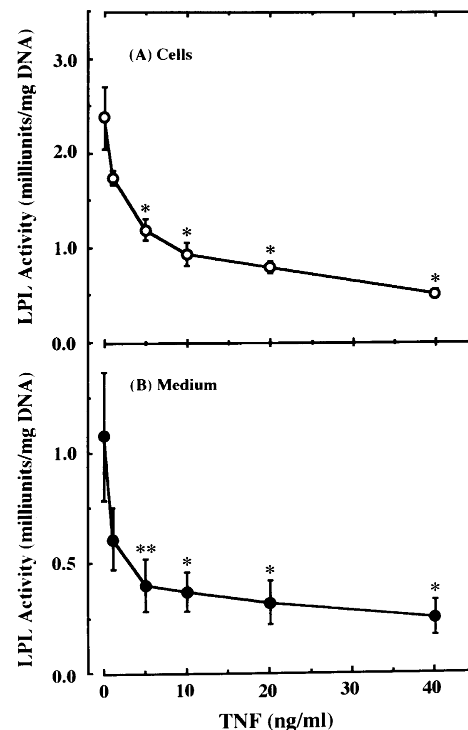

Figure 1 Dose-dependency of reduction of LPL activity by TNF-α

OST cells were incubated for 22 h in the medium containing TNF-α at the indicated concentrations. Then the medium was replaced with fresh medium containing the appropriate additive and cells were incubated for 2 h at 37 °C. The medium was separated and filtered through a 0.2-μm filter for prompt assay of LPL activity. Cells were harvested, sonicated, and used to make the acetone/ether powder for assay of cellular LPL activity. (**A**) LPL activity in the extract of acetone/ether powder of cells. (**B**) LPL activity released to the medium during the last 2 h of the 24-h treatment. Values given are means ± S.D. of three plates. $^{*}P < 0.01$, $^{**}P < 0.05$ (compared with the activity measured in the absence of TNF-α).

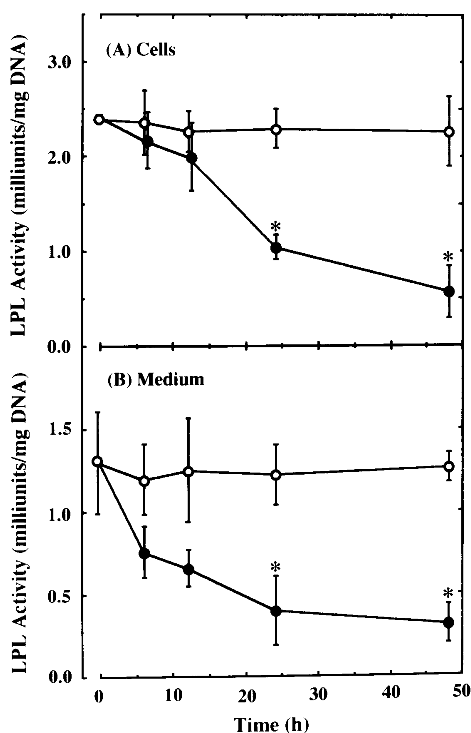

Figure 2 Time course of reduction of LPL activity by TNF-α

OST cells were incubated for 4–48 h in the absence (○) or presence (●) of 10 ng/ml of TNF-α. Then the medium was replaced with fresh medium containing the appropriate additive and cells were incubated for 2 h at 37 °C. The medium was separated from cells and filtered through a 0.2-μm filter for prompt assay of LPL activity. Cells were harvested, sonicated, and used to make the acetone/ether powder for assay of cellular LPL activity. (**A**) LPL activity in the extract of acetone/ether powder of cells. (**B**) LPL activity released to the medium during the last 2 h of treatment. Values given are means ± S.D. of three plates. $^*P < 0.01$ (compared with the activity measured in the absence of TNF-α).

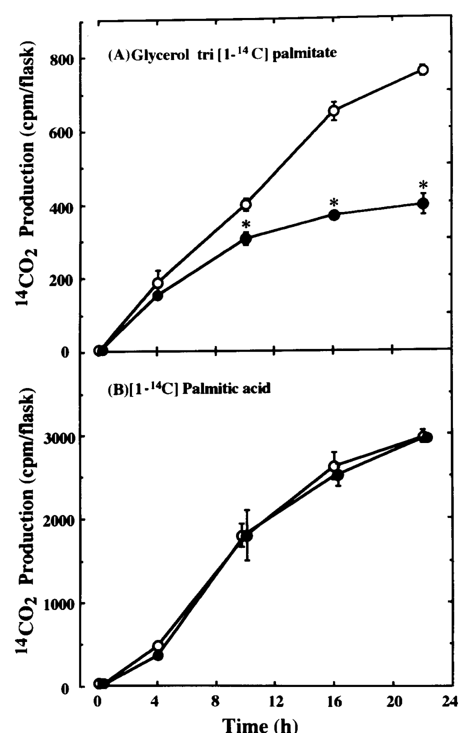

Figure 3 Effect of TNF-α on $^{14}CO_2$ production by OST cells

OST cells were incubated for 24 h at 37 °C in the absence (○) or presence (●) of 10 ng/ml of TNF-α. The flasks were replenished with fresh medium containing the appropriate additive. After 2 h, either glycerol tri[1-^{14}C]palmitate (**A**) or [1-^{14}C]palmitic acid (**B**) was added and cells were incubated as described in the Materials and methods section. The amount of $^{14}CO_2$ was determined by measuring radioactivity adsorbed into β-phenethylamine-soaked filter paper. Values given are means ± S.D. of three flasks. $^*P < 0.01$ (compared with control cells).

powder of cells and that released into the medium during the last 2 h of the 24-h-long treatment were measured. TNF-α decreased LPL activity in a dose-dependent manner. TNF-α at 10 and 40 ng/ml decreased cellular LPL activity by 60 % and 78 % respectively (Figure 1A), and release of activity to the medium by 66 % and 77 % respectively (Figure 1B).

Figure 2 shows the time course of reduction of LPL activity by TNF-α. TNF-α decreased LPL activity time-dependently. Up to 12 h TNF-α had no effect on LPL activity, but incubation for 24 h and 48 h decreased cellular LPL activity by 55 % and 75 % respectively (Figure 2A), and release of activity to the medium by 67 % and 75 % respectively (Figure 2B).

Effect of TNF-α on synthesis of LPL and total protein

OST cells incorporated [^{35}S]methionine into total protein at a linear rate up to 2 h, and the amount of radioactivity in total protein of TNF-α-treated cells [$(2.84 \pm 0.33) \times 10^8$ c.p.m./mg of DNA, $n = 3$] was identical to that of control cells [$(2.86 \pm 0.10) \times 10^8$ c.p.m./mg of DNA, $n = 3$], indicating that TNF-α-treated cells synthesized proteins normally.

The ^{35}S-labelled immunoprecipitated LPL was resolved by SDS/PAGE, and the amount of radioactivity in LPL was measured. ^{35}S-labelled LPL of TNF-α-treated cells migrated on SDS/PAGE with the same mobility ($M_r = 57000$) as that of control cells (results not shown). Treatment of cells with TNF-α inhibited LPL synthesis in parallel with the activity. The amount of radioactivity in LPL of TNF-α-treated cells (1022 ± 84 c.p.m./mg of DNA, $n = 3$) was 38 % of that of control cells (2718 ± 457 c.p.m./mg of DNA, $n = 3$). The percentage of radioactivity in LPL to that in total protein was 0.00095 % in control cells and 0.00036 % in TNF-α-treated cells.

Effect of TNF-α on $^{14}CO_2$ production in OST cells

The effect of TNF-α on the oxidation of FA to CO_2 in OST cells was examined by incubation with glycerol tri[1-^{14}C]palmitate. The amount of $^{14}CO_2$ increased linearly during the 22-h incubation period, indicating that the amount of radioactivity added to the medium was sufficient to be oxidized and used for synthesis of cellular lipids. Treatment of cells with TNF-α suppressed the rate of $^{14}CO_2$ production (Figure 3A). TNF-α at 10 and 40 ng/ml decreased the amount of $^{14}CO_2$ by 56 % and 69 %, respectively, after 22 h (Table 1).

When cells were incubated with [1-^{14}C]palmitic acid, the amount of $^{14}CO_2$ increased with no difference between control and TNF-α-treated cells during the 22-h incubation period (Figure 3B), indicating that TNF-α had no effect on the uptake and oxidation of FA.

Effect of TNF-α on ^{14}C-labelled lipid synthesis in OST cells

The effect of TNF-α on ^{14}C-labelled lipid synthesis in OST cells was examined by incubation with glycerol tri[1-^{14}C]palmitate.

Table 1 Effect of TNF-α on CO_2 production and lipid synthesis in OST cells

OST cells were incubated for 24 h in complete medium with 0, 10 and 40 ng/ml of TNF-α. The plates were replenished with fresh complete medium containing the appropriate additive. After 2 h, glycerol tri[1-^{14}C]palmitate was added and the cells were incubated for 22 h. The amount of $^{14}CO_2$ was determined by measuring the radioactivity adsorbed into β-phenethylamine-soaked filter paper. The radioactivity incorporated into each lipid was measured by TLC. Values given are means ± S.D. of three flasks. $^{*}P < 0.05$, $^{**}P < 0.01$ (compared with control cells). Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; PL, phospholipid.

	Radioactivity (c.p.m./mg of DNA)					
	CO_2	TG	DG	MG	FFA	PL
Control cells	2711 ± 61	60 854 ± 11 086	1336 ± 193	75 ± 18	136 ± 29	4982 ± 739
TNF-α-treated cells						
10 ng/ml	1206 ± 131**	19 894 ± 5069*	159 ± 113**	38 ± 28	56 ± 19*	2594 ± 363*
40 ng/ml	841 ± 59**	11 744 ± 2453**	263 ± 66**	47 ± 56	9 ± 6**	1519 ± 278**

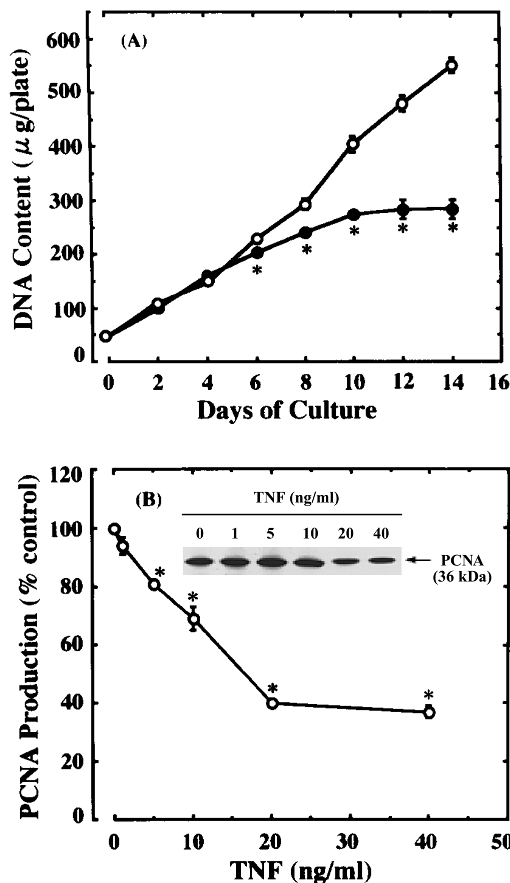

Figure 4 Effect of TNF-α on proliferation of OST cells

(**A**) OST cells were cultured in the absence (○) or presence (●) of 10 ng/ml of TNF-α. At the indicated intervals, cells were harvested and sonicated briefly at 0 °C, and DNA in the homogenate was measured. Values given are means ± S.D. of three plates. $^{*}P < 0.01$ (compared with DNA content of cells cultured in the absence of TNF-α). (**B**) ^{35}S-labelled PCNA was immunoprecipitated with monoclonal antibody to PCNA from the extract of cells incubated with [^{35}S]methionine, and resolved by SDS/PAGE. The radioactive band corresponding to PCNA was cut out and dissolved in 30% hydrogen peroxide and radioactivity was determined in a liquid scintillation counter. Radioactivity incorporated into PCNA in the cells incubated in the absence of TNF-α was 2552 ± 72 c.p.m./plate. Values given are means ± S.D. of three plates. $^{*}P < 0.01$ (compared with radioactivity in PCNA in cells incubated in the absence of TNF-α).

Cells incorporated much more radioactivity into triacylglycerol than into other lipids (Table 1). The amount of radioactivity in triacylglycerol was 12-times higher than in phospholipid. Treatment of cells with TNF-α decreased the amount of radioactivity incorporated into cellular lipids in a dose-dependent manner. TNF-α at 10 and 40 ng/ml decreased the amount of radioactivity incorporated into triacylglycerol by 67 % and 81 % respectively, and that incorporated into phospholipid by 48 % and 70 % respectively (Table 1). However, when cells were incubated with [1-^{14}C]palmitic acid, no difference in the amount of radioactivity incorporated into each lipid was observed between control and TNF-α-treated cells (results not shown).

Effect of TNF-α on proliferation of OST cells

To examine the effect of TNF-α on proliferation, OST cells were cultured in the absence or presence of 10 ng/ml of TNF-α, and DNA contents of the cultures were measured (Figure 4A). Cells cultured in the absence of TNF-α proliferated linearly during the 14-day culture period. The presence of TNF-α in the medium lowered the proliferative rate of cells. On Day 14, the DNA content of the plate of cells cultured in the presence of TNF-α was 52 % of that of cells cultured in its absence.

Next, cells were incubated with [^{35}S]methionine, and ^{35}S-labelled PCNA, a marker of cell proliferation, was immunoprecipitated from the cell extract and resolved by SDS/PAGE (Figure 4B). ^{35}S-labelled PCNA of TNF-α-treated cells migrated on SDS/PAGE with the same mobility ($M_r = 36000$) as that of control cells. TNF-α decreased the amount of radioactivity incorporated into PCNA in a dose-dependent manner. TNF-α at 10 and 40 ng/ml decreased PCNA production by 31 % and 67 % respectively.

DISCUSSION

LPL is synthesized and glycosylated in the endoplasmic reticulum, transported from the endoplasmic reticulum to the Golgi, and finally secreted from cells. Expression of LPL activity involves the LPL gene transcription, mRNA processing and translation, as well as post-translational modification [23,26–30]. The present study with [^{35}S]methionine showed that treatment of OST cells with TNF-α decreased the synthetic rate of LPL, but the ratio of activity and synthesis of LPL was identical in both groups of cells (control cells, 8.8×10^{-4} m-unit/c.p.m.; TNF-α-treated cells, 9.3×10^{-4} m-unit/c.p.m.). A study with endoglycosidase H, which cleaves the high-mannose-type oligosaccharide chain but not complex-type chain [31], showed that no difference of processing of oligosaccharide chains of LPL was observed between control and TNF-α-treated cells (results not shown). These findings indicate that TNF-α decreased LPL synthesis in OST cells, resulting in the decrease in expression of

LPL activity. Thus, as in adipose tissue, TNF-α may be a potent inhibitor of the LPL gene expression in OST cells.

Proliferating tumour cells require a large amount of energy for growth from conversion of glucose into lactate [32] and the oxidation of FFA to CO_2 [9–12]. In the present study, we found that OST cells use triacylglycerol-derived FA as substrate (Figure 3A). In general, triacylglycerol is hydrolysed into FFA and monoacylglycerol by LPL before uptake by the cells. Our data shows that $^{14}CO_2$ production from glycerol tri[1-^{14}C]palmitate was suppressed by TNF-α in parallel with LPL activity. Our previous study showed that in human sarcomas and carcinomas, the level of LPL activity was higher in the area containing a larger amount of PCNA-positive cells [33]. These findings indicate that LPL of tumour cells plays a role in providing FFA from triacylglycerol as an energy source for growth.

In OST cells incubated with triacylglycerol, TNF-α suppressed the rate of CO_2 production. It is likely that TNF-α suppressed the LPL-mediated production of FFA from triacylglycerol as the oxidative substrate, because treatment of cells with TNF-α caused a dose-dependent reduction of LPL activity (Figure 1), and the magnitude of its reduction was similar to that of CO_2 production. It is unlikely that TNF-α inhibited the uptake of FFA, because when OST cells were incubated with [1-^{14}C]palmitic acid, no difference in the rate of CO_2 production was observed between control and TNF-α-treated cells (Figure 3B). Thus, suppression of the oxidation of triacylglycerol FA to CO_2 is caused by the reduction of LPL activity by TNF-α.

When OST cells were incubated with glycerol tri[1-^{14}C]palmitate, most radioactivity was incorporated into triacylglycerol and phospholipid as Brenneman and Spector reported for Ehrlich ascites tumour cells [34]. However, they did not observe release of lipase activity from Ehrlich cells and suggested that some triacylglycerol was taken up intact. This is unlikely in our OST cells because the extent of suppression of incorporation of radioactivity into triacylglycerol was identical to that of reduction of LPL activity.

OST cells replicated at a lower proliferative rate when they were cultured in the presence of TNF-α (Figure 4A), suggesting that TNF-α had an anti-proliferative activity on OST cells. This was confirmed by the finding that treatment of OST cells with TNF-α caused a dose-dependent decrease in PCNA production (Figure 4B). Expression of PCNA is closely linked to the cell cycle. The level of PCNA in the nucleus increases during the late G1 phase immediately before the onset of DNA synthesis, becomes maximal during the S phase, and decreases again during the G2 and M phases [21,22]. PCNA is used as an immunocytochemical marker of proliferating cells [33,35,36].

In conclusion, LPL of tumour cells plays a role in supplying FFA from triacylglycerol FA as a source for energy and synthesis of cellular lipid components for growth. TNF-α decreased the synthetic rate of LPL in parallel with the activity, causing a decrease of supply of FFA from extracellular triacylglycerol. Thus, one of the causes of the anti-proliferative activity of TNF-α is the suppression of the LPL-mediated supply of FFA as an energy source for growth.

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