Original 和漢医薬学会誌4, 116－123, 1987

Inhibition by ginsenoside Rb_1 of morphological change of rat adipocytes induced by tumor necrosis factor

Keizo SEKIYA,*[a] Shigeru ARICHI[b] and Hiromichi OKUDA[a]

[a] *Department of Medical Biochemistry, School of Medicine, Ehime University*
[b] *The Research Institute of Oriental Medicine, Kinki University*

(Received May 20, 1987. Accepted August 5, 1987.)

Abstract

Recombinant human tumor necrosis factor (TNF) was found to enhance the morphological change of cultured rat epididymal adipocytes to fibroblast-like cells associated with loss of cellular lipid. TNF caused distinct increase in the percentage of fibroblast-like cells at 10^{-10} M, and its effect was dose-dependent. This TNF-induced morphological change was effectively prevented by ginsenoside Rb_1, which is a component of ginseng. Of the ginsenosides tested, only those containing 20(S)-protopanaxadiol genin prevented the TNF-induced morphological change. Since TNF is known to inhibit lipoprotein lipase (LPL) biosynthesis, we examined its effect on LPL activity in cultured adipose tissue, which we measured directly and indirectly. After culture of the tissue for 4 days, no LPL activity could be detected by direct assay in either control or TNF-treated tissue, and ginsenoside Rb_1 did not increase the LPL activity. The LPL activity was also assayed indirectly by measuring the rate of reesterification of labeled fatty acids released from exogenous triacylglycerol in the tissue. TNF(10^{-9} M) caused 82% inhibition of the reesterification and ginsenoside Rb_1 (30 μg/ml) almost completely prevented this inhibition by TNF. Ginsenoside Rb_1 did not affect the inhibitory action of TNF on L929 cell growth. Ginsenosides are components of ginseng, which is a herbal preparation of *Panax ginseng* C. A. MEYER. The present data suggest that ginsenosides may be useful clinically in counteracting the antilipogenic effect of TNF.

Key words ginsenoside, tumor necrosis factor, adipocyte, cachexia, dedifferentiation
Abbreviations LPL, lipoprotein lipase ; TNF, tumor necrosis factor

Introduction

Tumor necrosis factor (TNF) a product of activated macrophages, can be expressed by recombinant DNA methods in *Escherichia coli*,[1,2] and is known to be specifically toxic to many tumor cells *in vivo* and *in vitro*.[3-6]

Cachectin, which is produced by endotoxin-stimulated macrophages, suppresses key lipogenic enzymes *in vivo* and *in vitro*.[7-10]

These two monokines were recently shown to have similar N-terminal amino acid sequences and similar bioactivities.[11,12] Beutler and Cerami reported that cachectin/TNF at subnanomoler concentrations caused nearly complete suppression of LPL in 3T3-L1 cells.[10] Since lipoprotein lipase (LPL) is known to be present in adipocytes, TNF may exert an inhibitory action on adipose differentiation of 3T3-L1 cells, and a stimulatory action on dedifferentiation of mature adipocytes by suppressing LPL activity.

In the presnt investigation, TNF was found to induce morphological change of cultured mature adipocytes. Furthermore, some ginsenosides, which are components of *Ranax ginseng* C. A. MEYER, were shown to prevent the morphological change induced by TNF.

*〒791-02 愛媛県温泉郡重信町志津川
愛媛大学医学部生化学第二教室 関谷敬三
Shigenobu, Onsen-gun, Ehime 791-02, Japan

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **4**, 116－123, 1987

Materials and Methods

Chemicals : Recombinant human TNF was kindly supplied by Fujisawa Pharmaceutical Co., Osaka, Japan. Ginsenosides were supplied in part by Japan Korea Red Ginseng Co., Ltd., Kobe, Japan. Dulbecco's modified Eagle's medium was purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Fetal bovine serum was from M. A. Bioproucts, MD, USA. Culture flasks and dishes were from Falcon. [Carboxyl-^{14}C] triolein (sp. act. 111.8 mCi/mmol) and [methyl-^{3}H] thymidine (sp. act. 87 Ci/mmol) were from NEN Research Products. Other chemicals were reagent grade products from Wako Pure Chemical Co., Osaka, Japan.

Ceiling culture of adipocytes : Adipocytes were isolated from the epididymal fat pads of Wistar rats by the method of Rodbell,[13] washed gently 6 times by culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin) and inoculated into medium filled culture flasks (Falcon #3012). The flasks were capped and cultured at 37℃.[14] For addition to culture, TNF was dissolved in 0.9% NaCl containing 0.5% bovine serum albumin, while ginsenosides were dissolved at 10 mg/ml in 20% ethanol (in 0.9% NaCl), and diluted appropriately with 0.9% NaCl. Control cultures received the respective vehicles only. After culture for 3 days, microphotographs of adipocytes in 10 different fields were taken and cell counts were made on at least 2,000 cells. Adipocyte which likes a non-sphere shape and had one more plastic attached processes and one more lipid globes was counted among the fibroblast-like cells. The each fiblobrast-like cell was present separately and cluster of fiblobrast-like cells was not found in the culture. And growth rate of fiblobrasts in preparation of adipocytes were not potentiated by TNF. These points indicate that the fiblobrast-like cell was dedifferentiated form from adipocyte and not from fiblobrast.

Assay of LPL activity : Epididymal adipose tissue was removed from 6-week-old rats and cut up with scissors. Then the tissue was washed 6 times with culture medium with centrifugation at $100\times g$ for a few minutes and a 50 μl aliquots of the packed sliced tissue were cultured in 24 well plates (Falcon #3047) in 1 ml of the same medium. After one and three days, the medium was changed and test materials were added. On day 4, the tissue was sonicated in 1 ml of 50 mM NH_4Cl buffer (pH 8.0) containing 10 units/ml heparin, which release LPL from bound from, the homogenate was centrifuged for 10 min at $10,000\times g$, and the supernatant was used for LPL assay.

LPL activity was measured by the method of Nilsson-Ehle and Schotz.[8] Briefly, a stock emulsion of ^{14}C-triolein and phosphatidylcholine in glycerol was prepared. Before assay, 1 volume of the stock emulsion, 4 volumes of 3% bovine serum albumin (fraction V) in 200 mM Tris-HCl buffer (pH 8.1) and 1 volume of serum from starved rats (heat inactivated at 60℃ for 10 min) were mixed and incubated at 37℃ for 15 min (activated substrate). For assay, 50 μl of this activated substrate mixture was added to 50 μl of the tissue supernatant and the mixture was incubated for 1 hr at 37℃.

For assay of reesterified fatty acids, triolein emulsion (20 μl of "activated substrate" for LPL assay) was added to the culture on day 4 and culture was continued for one more day. The tissue was then washed 4 times with ice-cold 0.9% NaCl, which reduced the count of extracellular radioactivity to the normal background level. Then, intracellular lipids were extracted in Dole's extraction mixture[19] with sonication for 1 min. The mixture was shaken and 1.5 ml heptane and 1 ml water were added, and the mixture was shaken and centrifuged at 2,000 rpm for 10 min with table top centrifuge. Then heptane layer was washed with same volume of 0.05 N NaOH in 50% ethanol to remove free fatty acids. The radioactivity of the upper heptane layer was counted.

Evaluation of growth of L929 cells : L929 cells, whose growth is known to be inhibited by TNF, were cultured in 35 mm culture dishes (Falcon #3001) in the medium described above. On day 1 TNF and ginsenoside Rb_1 were added, on day two 0.1 μCi of ^{3}H-thymidine (2 μCi/ml) was

added and culture was then continued for 24 hr. The cells were washed three times with ice-cold 5% TCA and the radioactivity of the TCA-insoluble fraction was counted. Control L929 cells were in the logarithmic growth phase.

Results

Isolated rat epididymal adipocytes contain a single lipid droplet and float in medium. We cultured adipocytes in sealed culture flasks completely filled with medium. After a few days, a few percent of the adipocytes came in contact with the upper surface of the flasks and became fibroblast-like with loss of their intracellular lipid (Fig. 1)

We found that addition of TNF to the culture increased the number of morphologically changed adipocytes (Fig. 2). TNF at 10^{-10} M increased the percentage of fibroblast-like cells distinctly, and

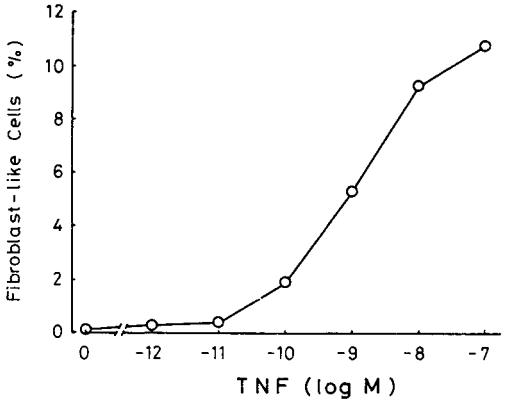

Fig. 2 TNF-induced morphological change of cultured rat adipocytes.
Adipocytes were prepared from 5-week-old rats. Data are for one of three reproducible experiments.

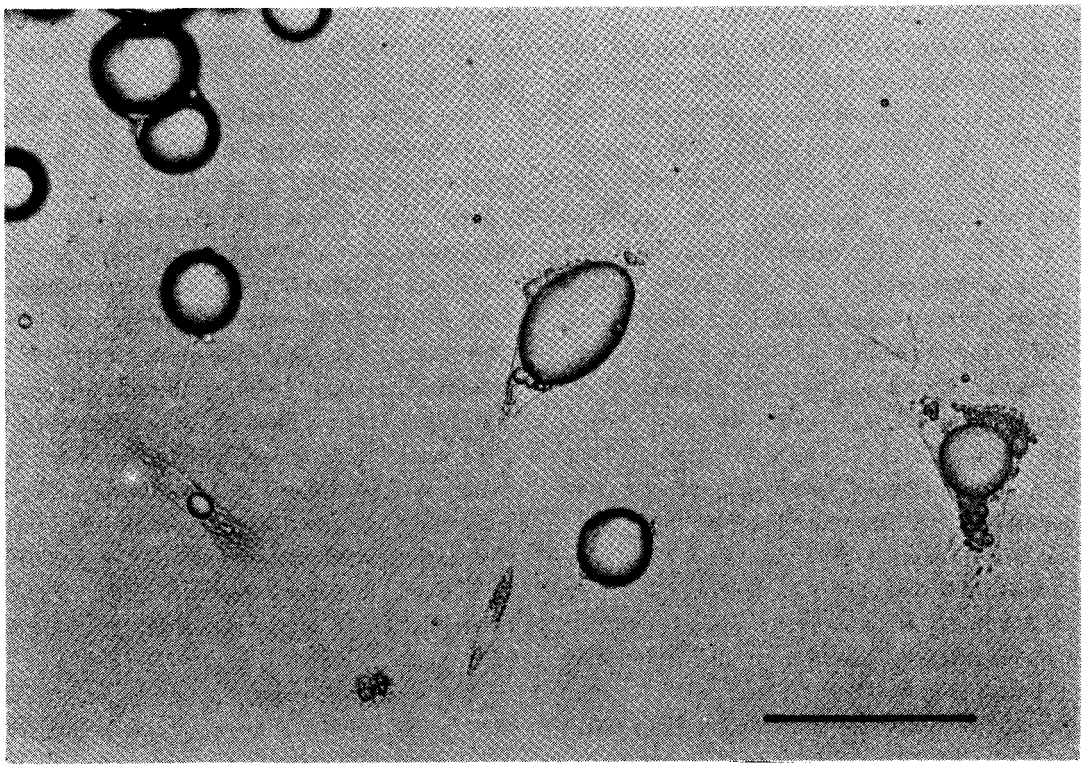

Fig. 1 Morphology of isolated adipocytes on day 3.
Adipocytes were prepared from 3-week-old rats. During culture, some adipocytes lost intracellular lipid and became fibroblast-like. The typical appearance of cells in a culture with 10^{-9} M TNF is shown (bar=100 μm, ×400).

Table I Effects of TNF and ginsenoside Rb_1 on cultured rat adipocytes from 3-week-old and 7-week-old rats.

Additions	Fibroblast-like cells (%) 3-week-old	7-week-old
Control	0.58±0.34[1]	0.06
Ginsenoside Rb_1 30 μg/ml	2.61	0
TNF 10^{-10} M	4.08	—
+Ginsenoside Rb_1 30 μg/ml	1.94	—
TNF 10^{-9} M	8.71±1.06	1.80
+Ginsenoside Rb_1 30 μg/ml	1.43±0.24	0.38

1 : Statistical analyses were carried out by Student's t-test (n : 3−4). The value (mean±S.E.) for TNF (10^{-9} M) was significantly increase ($p<0.01$) than the control value or that with TNF+ginsenoside Rb_1.

the effect of TNF was dose-dependent.

The percentage of fibroblast-like cells was higher in adipocytes from 3-week-old rats than in those from 7-week-old rats (Table I). In adipocytes from rats of both ages, increased ratio of fiblobrast-like cells induced by TNF was almost completely prevented by ginsenoside Rb_1, which is a component of ginseng (Table I). In adipocytes from 3-week-old rats, ginsenoside Rb_1 increased the ratio of fiblobrast-like cells, but not in those from 7-week-old rats. This difference may be due to batch variation in preparated adipocytes.

We also examined the effects of other ginsenosides on TNF-induced morphological change of adipocytes to study structure-activity relationship (Table II). Ginsenosides Rb_2, Rc and Rd were also found to possess same activity as ginsenoside Rb_1 have. But ginsenosides Re, Rg_1, Rh_2 and Ro did not. Ginsenosides are classified into three groups according to their genin structure (Fig. 3). The results show that all ginsenosides containing 20(S)-protopanaxadiol genin except ginsenoside Rh_2 had activity.

Next we examined the effects of TNF and ginsenoside Rb_1 on lipoprotein lipase (LPL) activity in rat adipose tissue cultured in 24-well plates. No LPL activity was detectable in control tissue, indicating that its activity decreased considerably during culture for 4 days (Table III). Therefore, we used another method for LPL assay ; namely measurement of reesterified fatty acids derived from extracellular triacylglycerol emulsion. TNF (10^{-9} M) caused 82% inhibition of the uptake of triacylglycerol possibly indicating reduction of LPL activity in the tissue. Ginsenoside Rb_1 (30 μg/ml) prevented this inhibition by TNF.

Ginsenoside Rb_1 had no effect on inhibition of growth of L929 cells by TNF (Fig. 4).

Table II Effects of various ginsenosides on TNF-induced morphological change of cultured rat adipocytes.

Additions	Fibroblast-like cells (%) Exp.1[1]	Exp.2[2]
Control	0.25	0.20
TNF 10^{-9} M	6.6	9.7
+G. Rb_1	1.9	1.3
+G. Rb_2	1.9	
+G. Rc	1.9	
+G. Rd	2.1	
+G. Re	4.4	8.7
+G. Rg_1	5.2	
+G. Rh_2	4.8	
+G. Ro		9.7

Concentrations of 30 μg/ml of ginsenosides (G.) Rb_1, Rb_2, Rc, Rd, Re and Rg_1, 20 μg/ml of G. Rh_2 were used.

1 : In experiment 1 (Exp.1), adipocytes were prepared from 5-week-old rats.

2 : In experiment 2 (Exp.2), adipocytes were prepared from 3-week-old rats.

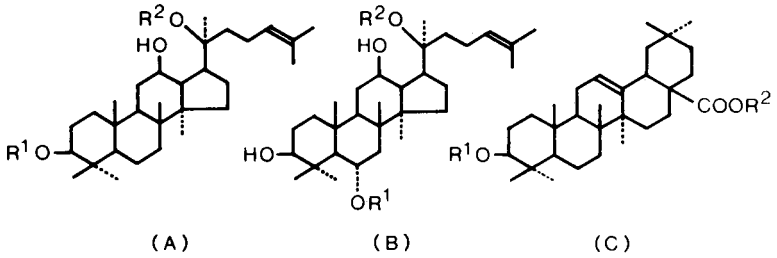

Fig. 3 Structures of ginsenosides.
(A) 20(S)-Protopanaxadiol : $R^1=R^2=H$
Ginsenoside Rb_1 : R^1=glucose 2-1 glucose, R^2=glucose 6-1 glucose ; ginsenoside Rb_2 : R^1=glucose 2-1 glucose, R^2=glucose 6-1 arabinose (pyr) ; ginsenoside Rc : R^1=glucose 2-1 glucose, R^2=glucose 6-1 arabinose (fur) ; ginsenoside Rd : R^1=glucose 2-1 glucose, R^2=glucose ; ginsenoside Rh_2 : R^1=glucose, $R^2=H$.
(B) 20(S)-Protopanaxatriol : $R^1=R^2=H$
Ginsenoside Re : R^1=glucose 2-1 rhamnose, R^2=glucose ; ginsenoside Rg_1 : R^1=glucose, R^2=glucose.
(C) Oleanolic acid : $R^1=R^2=H$
Ginsenoside Ro : R^1=glucuronic acid 2-1 glucose, R^2=glucose.

Table III Effects of TNF and ginsenoside Rb_1 on extracellular triacylglycerol uptake and lipoprotein lipase activity of cultured rat adipose tissue.

Additions	Reesterified fatty acid (μ moles/g tissue)	LPL activity
Control	1.12±0.18[1]	ND
TNF 10^{-9} M	0.20±0.06	ND
+G. Rb_1	0.97+0.21	ND

1 : Statistical analyses were carried out by Student's t-test (n=3). The value (mean±S.E.) for reesterified fatty acid with TNF only was significantly less ($p<0.01$) than the control value or that with TNF+ginsenoside Rb_1 (G. Rb_1 30 μg/ml). ND means not detectable.

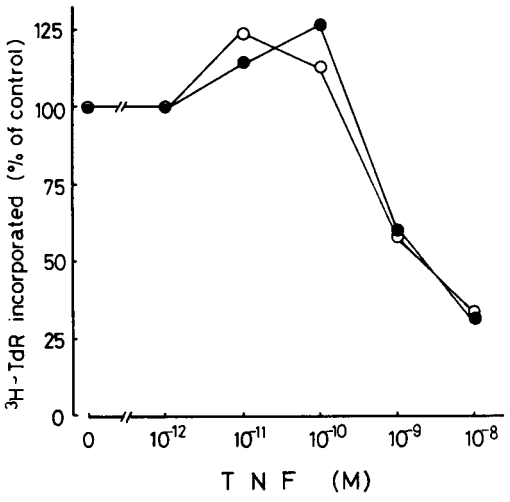

Fig. 4 Effect of ginsenoside Rb_1 on TNF-induced cell growth inhibition of L929 cells.
Incorporation of radioactivity was measured in the absence (○) and presence (●) of ginsenoside Rb_1 (30 μg/ml). Data were means of triplicate dishes.

Discussion

In this work we found that recombinant human tumor necrosis factor enhanced the morphological change of mature rat adipocytes *in vitro* associated with loss of cellular lipid.

TNF was first detected in the sera of mice treated with bacteria and endotoxin.[3] It is produced by macrophages, and causes hemorrhagic necrosis of Meth A sarcoma *in vivo* and inhibitions of growth of many murine and human tumor cells *in vitro*,[3-6] and it has recently been suggested to be closely related, or possibly identical to another macrophage-derived protein, cachectin.[11,12]

Cachectin is known to inhibit lipoprotein lipase and other lipogenic enzyme activities, and its accumulation in animals during bacterial infection is thought to cause some toxic symptoms and cachexia.[7-10]

The morphological change of adipocytes induced by TNF that we observed in this work may also be involved in the conditions known as cachexia induced by malignancies and certain infections, in which the animal loses weight continuously. This morphological change is thought to represent an intermediate state in dedifferentiation of adipocytes to fibroblast-like cells.

Fibroblast-like cells in culture are reported to proliferate and accumulate intracellular lipid droplets when they become confluent.[14,20]

We examined the lipoprotein lipase activity of adipocytes associated with their morphological change during culture *in vitro*. But no LPL activity could be measured directly in the adipocytes because the cells were severely damaged during their isolation from the sealed culture flasks. Therefore, we used cultures of sliced adipose tissue instead of isolated adipocytes for estimation of lipoprotein lipase activity. The enzyme activity was found to decrease considerably during culture and not to be detectable in the medium (data not shown) and in the cells after culture for 4 days. Heparin-releasable LPL was also not detected (data not shown). However, LPL-mediated reesterification of fatty acids derived from exogenously added triacylglycerol could be detected in the culture of sliced adipose tissue. The difference between LPL activity and reesterification may depend on incubation time (LPL assay : 1 hr ; assay of reesterification : 24 hr). TNF was found to inhibit reesterification of fatty acids in the tissue.

It is interesting that some ginsenosides prevented the morphological change of adipocytes induced by TNF. Ginsenosides are classified into three groups according to their genin structure : 20(S)-protopanaxadiol, 20(S)-protopanaxatriol and oleanolic acid groups.[15-17] Of these ginsenosides, all those of the 20(S)-protopanaxadiol group except ginsenoside Rh_2 which had no sugar moiety at position R^2, inhibited induction of morphological change of rat adipocytes by TNF. Ginsenosides of the 20(S)-protopanaxatriol and oleanolic acid groups did not counteract the effect of TNF. Furthermore, ginsenoside Rb_1, which belongs to the 20(S)-protopanaxadiol group, also prevented the inhibition by TNF of LPL-mediated reesterification of fatty acids derived from exogenously added triacylglycerol in slices of rat adipose tissue.

Ginseng, which is a traditional herbal preparation of *Panax ginseng* C. A. MEYER, has been used as a remedy for general weakness.[21,22] And ginsenoside, a component of ginseng, is reported to have physiological and pharmacological effects[23] and increases lipogenesis and decreases blood lipids and glucose in rats.[24,25]

It is interesting that ginsenoside Rb_1 did not affect the inhibition of growth of L929 cells by TNF.

The present results suggest that ginsenosides of the 20(S)-protopanaxadiol group may possess a protective effect against TNF-induced cachexia in patients with cancer and infection.

Acknowledgement

This study was supported in part by a Grant from Ginseng Research Foundation.

和文抄録

腫瘍壊死因子（TNF）は癌細胞を殺す作用を持ち臨床応用に期待が持たれているが，他方，抗脂肪合成作用も持っていることが明らかになっている。そこで，我々はジンセノサイドのTNFに対する影響を検討した。TNFは培養ラット副睾丸脂肪細胞の線維芽様細胞への変化を増強させた。このTNFの作用は10^{-10} Mで明らかとなり濃度依存的であった。このTNFの作用は薬用人参の成分であるジンセノサイドRb_1によって阻止された。他のジンセノサイドについても検討した所，20(S)-protopanaxadiol系のものがTNFに拮抗することが分かった（例外，ジンセノサイドRh_2）。一方，TNFはリポプロテインリパーゼ（LPL）の生合成を阻害することが知られているので，この酵素についても検討した。脂肪組織を培養するとLPL活性が検出されず，ジンセノサイドRb_1を加えても変化はなかった。そこで，直接LPL活性を測定するのではなくLPLが関与している細胞外中性脂肪からの脂肪酸の再エステル化を測定した。TNF（10^{-9} M）は再エステル化を82%阻害したが，ジンセノサイドRb_1（30 μg/ml）によってその阻害が打ち消された。また，TNFは腫瘍細胞の成長を阻害することが知られているので，TNFに対して感受性を持つL929細胞の成長に対するジンセノサイドRb_1の影響を検討した。ジンセノサイドRb_1はTNFのL929の細胞成長阻止作用に対しては影響を及ぼさなかった。以上の結果より，ジオール系のジンセノサイドはTNFの臨床応用において，その抗脂肪合成作用を阻止することにより有用であると考えられる。

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