MACROPHAGE-SPECIFIC EFFECT ON LIPID METABOLISM BY AN ANTIBIOTIC, ASCOFURANONE

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Ascofuranone (AF) enhanced glucose consumption of splenocytes and macrophages, while it enhanced incorporation of [^{14}C]acetate into macrophages but not into splenocytes. When using tumor cell lines, it inhibited the incorporation of [^{14}C]acetate into lymphoma cell lines, YAC-1 and P388, and a thymoma, L5178Y, while it stimulated that into P388D1, which is derived from P388 and has macrophage-like characteristics. Incorporation of [^{14}C]acetate into a mammary carcinoma FM3A was also stimulated by AF. In contrast, AF stimulated uptake of methylglucose in all cell lines tested. The effect of AF was further studied using mouse myeloid leukemia, M1 cells. AF slightly stimulated the incorporation of [^{14}C]acetate into undifferentiated M1 cells, and strongly stimulated that of hydrocortisone-differentiated M1 cells. In contrast, AF suppressed the incorporation of [^{14}C]acetate into retinoic acid-differentiated M1 cells. Glucose consumption of these three types of M1 cells was all stimulated. From these results, we conclude that AF specifically stimulates the incorporation of [^{14}C]acetate into macrophages while it generally stimulates glucose uptake of the cells.

Ascofuranone (AF) is an isoprenoid antibiotic produced by a phytopathogenic fungus *Ascochyta viciae*.[1] We have reported that the antibiotic is active against experimental tumors including L1210, Ehrlich carcinoma and sarcoma 180; in all the cases studied, AF is effective when administered prior to the tumor implantation.[2] This suggests that the antitumor activity of the antibiotic is host-mediated. In fact, it significantly activates splenic endogenous cytotoxic activity following ip administration.[3] *In vitro* study of the effect of AF on immune mediators shows the cytospecific stimulation of macrophages:[4] AF stimulates glucose consumption, interleukin 1 (IL-1) production and tumoricidal activity of macrophages while it inhibits the mitogenic response, interleukin 2 (IL-2) production, and formation of interleukin 2 receptors of T cells in response to concanavalin A. Natural killer activity of splenocytes is also inhibited.[4] Since AF modulates lipid metabolism *in vivo*[5,6] and *in vitro*,[7] these cytospecific effects on the immune response are assumed to be associated with the modulation of lipid metabolism.

In this report, we examined the effects of AF on the lipid metabolism of splenocytes and macrophages to study the correlation of its effect on lipid metabolism and on immune system.

Materials and Methods

Chemicals

Ascofuranone of more than 99% purity was supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo). AF was dissolved in methanol at 10 mg/ml and diluted to appropriate concentrations with medium. Less than 0.5% methanol had no effect on the cells. Lipopolysaccharide (LPS, lipopolysaccharide B from *Escherichia coli*) was purchased from Difco Laboratories (Detroit, Michigan, U.S.A.).

Preparation of Cells

A single cell suspension of splenocytes, the erythrocytes in which had been ruptured by a treat-

ment with ammonium chloride, was prepared as described previously.[2] Inflammatory macrophages were induced in a peritoneal cavity by ip injection of 0.5 ml of 10% thioglycolate broth (Difco) or 2 ml of 1.2% sodium caseinate (Wako Pure Chemical Industries Ltd., Osaka) 4 days prior to the collection. Experiments were performed after removing plastic non-adherent cells as described previously.

Cell Culture

Cells except M1 were cultured in RPMI 1640 medium (Nissui Seiyaku, Tokyo) supplemented with 50 μg/ml kanamycin and 10% fetal bovine serum (Flow Laboratories, North Ryde, N.S.W., Australia). M1 cells were cultured in EAGLE's minimum essential medium (Nissui Seiyaku) containing 10% donor horse serum (Flow Laboratories), twice the amount of original vitamins and amino acids and 50 μg/ml kanamycin. M1 cells were differentiated by culturing with 0.1 μM hydrocortisone (Sigma Chem. Co., St. Louis, MO., U.S.A.) or 10 μM retinoic acid (Sigma Chem. Co.) for 48 hours at 37°C.

Determination of Glucose Consumption

Glucose concentration in the medium was determined by a blood analyzing system (Chugai Pharmaceutical Co., Ltd.) using an enzymatic method. Glucose consumption was computed using the following formula: Glucose consumption (%) = 100 × (1 − glucose at the termination of the culture/glucose in fresh medium.)

Incorporation of [^{14}C]Acetate

Cells were cultured with 2 μCi [^{14}C]acetate (New England Nuclear, Boston, MA., U.S.A.) for 4 hours. At the termination of culture, splenocytes and cultured cell lines except M1 were harvested by automatic cell harvester. Incorporation of radioactivity into TCA insoluble fraction of M1 cells was determined as described previously.[7] Incorporation into macrophages was determined by counting radioactivity of cellular lipid extracted by BLIGH-DYER method.[8] Almost the same level of radioactivity was detected by these three methods, suggesting that most of the acetate was incorporated into lipid fraction.

For analyzing the synthetic profile of simple lipids, cellular lipids extracted by BLIGH-DYER method were developed on a silica gel plate with the solvent system *n*-hexane - ether - acetate, 90: 10: 1. Radioactivity in each spot was detected by autoradiography.

Methylglucose Uptake

One million cells in 200 μl volume were incubated for 5 minutes in the presence of AF and 2 μCi [^{3}H]methylglucose (New England Nuclear). At the termination of culture, 2 ml of ice-cold phosphate buffered saline (PBS, 0.8% NaCl, pH 7.6) were added. Cells were washed three times with ice-cold PBS before the determination of radioactivity.

Results

Effect of AF on Glucose and Lipid Metabolism of Splenocytes and Macrophages

As shown in Table 1, AF stimulated both resident and caseinate-stimulated inflammatory macrophages to consume more glucose in the medium, like LPS, a known activator of macrophages. Maximum stimulation was shown at 10 μg/ml AF. AF also stimulated glucose consumption of splenocytes although AF has no mitogenic effect on lymphocytes but rather inhibits their functions.[4] One of the representative experiments is dipicted in Table 2. G-10 passed splenocytes, which were almost free from macrophages, were also stimulated to consume glucose to the same level as untreated splenocytes, indicating that lymphocytes but not macrophages in splenocytes consumed the glucose. A comparable low dose of AF, 5 μg/ml, most strikingly stimulated the glucose consumption of both non-treated and G-10 passed splenocytes. The difference in the culture period, 20 hours for macrophages and 8 days for splenocytes, would explain the difference in concentrations required for maximum

Table 1. Stimulation of glucose consumption of macrophages by ascofuranone.

Addition (μg/ml)	Glucose consumption (%)	
	Resident	Caseinate-induced
Control	14.3	16.5
AF 5	22.4 (157)*	21.4 (130)
10	26.4 (185)	28.3 (172)
20	25.8 (180)	33.5 (203)
40	19.6 (137)	23.9 (145)
LPS 1	22.4 (157)	22.0 (133)

Plastic adherent cells of 2×10^6 resident or caseinate-induced peritoneal cells were cultured for 20 hours, and glucose consumption was determined at the termination of the culture.

* Values in parentheses represent percent of control value.

Table 2. Stimulation of glucose consumption of splenocytes by ascofuranone.

Addition (μg/ml)	Glucose consumption (%)	
	Unfractionated	G-10 passed
Control	59.3	51.5
AF 2.5	75.0 (126)*	62.6 (122)
5	80.1 (135)	66.6 (129)
10	60.1 (101)	33.3 (65)

Unfractionated or G-10 passed splenocytes (10^7 cells/ml) were cultured for 8 days in the presence of AF, and glucose consumption was determined at the termination of the culture.

* Values in parentheses represent percent of control value.

stimulation of glucose consumption of macrophages and splenocytes.

In contrast to glucose consumption, incorporation of [^{14}C]acetate into the splenocytes was inhibited in a dose-dependent manner, whereas that into the macrophages was slightly increased (Fig. 1). Increase was observed at 0.4 and 2 μg/ml, while 10 μg/ml AF was inhibitory. Thus, AF affects the lipid metabolism of splenocytes and macrophages differently.

Fig. 1. Comparison of incorporation of [^{14}C]acetate into macrophages and splenocytes.

2×10^5 plastic adherent peritoneal cells induced by thioglycolate broth (○), or 2×10^5 splenocytes (●) were cultured for 4 hours in the presence of [^{14}C]acetate and AF. Radioactivity incorporated into the cells was determined. Each points represent average percent to control incorporation of triplicate cultures.

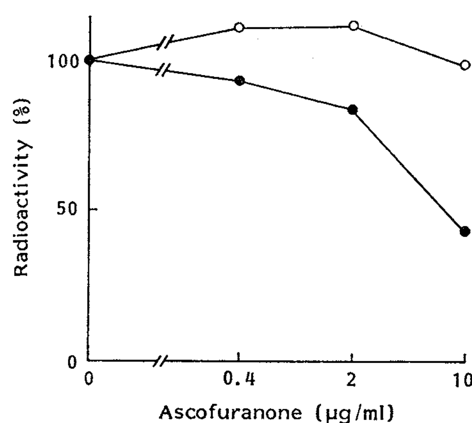
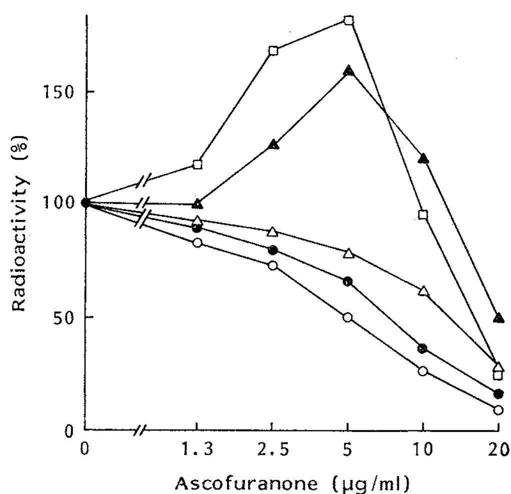


Fig. 2. Comparison of incorporation of [^{14}C]acetate into various cultured cell line.

2×10^5 cells (△: L5178Y, ○: P388, ▲: P388D1, ●: YAC-1, □: FM3A) were cultured for 4 hours in the presence of [^{14}C]acetate and AF, and radioactivity incorporated into the cells was determined. Each points represent average percent to control value of triplicate cultures.



Effect of AF on Glucose and Lipid Metabolism of Cultured Cell Line

Since the splenocytes and macrophages are both constituted from heterogeneous populations, the experiments using homogeneous cells are needed. Cultured tumor cell lines maintained *in vitro*

Fig. 3. Comparison of synthetic profiles of simple lipid from [^{14}C]acetate in P388 and P388D1.

P388 and P388D1 were cultured with [^{15}C]acetate and AF for 4 hours. Lipid extracted from the cells were developed on TLC and the radioactivity was detected by autoradiography.

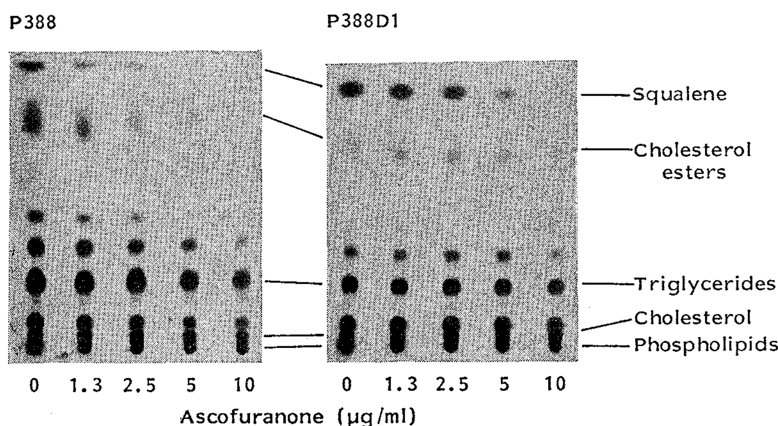


Table 3. Comparison of methylglucose uptake in various cell lines.

Cell lines	Radioactivity incorporated (cpm)			Statistics*
	Control	AF (5 μg/ml)	T/C (%)	
L5178Y	397±34**	187±50	123	NS
P388D1	701±29	1,103±78	154	$P<0.002$
P388	614±59	856±231	139	NS
YAC-1	908±114	1,136±187	125	NS
FM3A	526±67	848±112	161	$P<0.02$

Cells were incubated for 5 minutes with AF and [^{3}H]methylglucose, and the radioactivity taken up into the cells were determined.

* Student's t-test.

** Mean±SD of triplicate cultures.

NS: Not significant ($P>0.05$).

are suitable for this purpose. As shown in Fig. 2, AF inhibited the incorporation of [^{14}C]acetate into lymphomas, YAC-1 and P388, and a thymoma, L5178Y. However, it markedly enhanced the incorporation into P388D1, a sub-cell line which is derived from P388 but has morphological and functional characteristics of macrophages.[9,10] AF also stimulated the incorporation into a mammary carcinoma, FM3A. Maximum stimulation was observed at 5 μg/ml AF, where enhancement was 60% with P388D1 and 82% with FM3A (Fig. 2). The minimum inhibitory concentration of AF against the growth of these cell lines was 20 μg/ml.

When the profile of the synthesis of simple lipid from acetate was compared for P388 and P388D1, marked differences were noted for the spots of squalene and cholesterol esters. With P388D1, more radioactivity was incorporated into squalene and less into cholesterol esters, as compared with P388. The effect of AF were also evident from these spots. The radioactivity of squalene was reduced in both cell lines, whereas that of cholesterol esters was enhanced in P388D1 and was reduced in P388 (Fig. 3).

Glucose consumption during 20 hours was also studied using those cultured cell lines. There was no obvious difference in the effect, probably because of the growth inhibitory effect of AF. As

Table 4. Comparison of glycolysis of differentiated and undifferentiated M1 cells.

Addition (μg/ml)	Glucose consumption of M1 cells (%)		
	None	Hydrocortisone	Retinoic acid
Control	28.7	39.6	35.7
AF 5	41.9 (146)*	60.4 (153)	26.3 (74)
10	59.0 (206)	65.0 (164)	32.6 (91)
20	64.8 (226)	71.1 (180)	45.5 (127)
40	47.3 (165)	53.6 (135)	34.0 (95)
LPS 1	31.3 (109)	58.3 (147)	25.4 (71)

2×10^5 M1 cells, untreated or treated with 0.1 μM hydrocortisone or 10 μM retinoic acid, were incubated with AF. Glucose consumption was determined 16 hours later.

* Values in parentheses represent percent of control value.

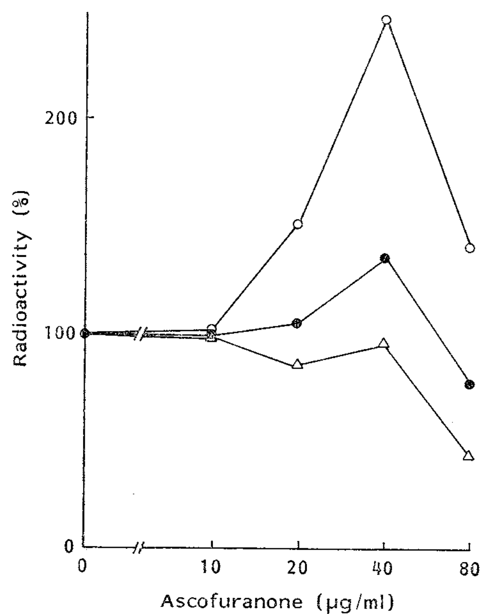
the stimulation of [^{14}C]acetate incorporation was shown within 4 hours, we examined uptake of glucose for shorter period using [^{3}H]methylglucose. As shown in Table 3, AF stimulated methylglucose uptake of all cells used. Stimulation was more than 50% with P388D1 and FM3A, and was statistically significant. On the other hand, stimulation in L5178Y, P388 and YAC-1 was less than 50%.

Difference in Effect of AF on Glucose and Lipid Metabolism in the Course of Macrophage Maturation

Cytospecific stimulation by AF of incorporation of [^{14}C]acetate into macrophages was further studied using mouse myeloid leukemia, M1 cells, which differentiate into mature macrophages or granulocytes when they were cultured with inducers.[11] When they were cultured with the typical chemical inducer, hydrocortisone, 70% of cells showed phagocytic activity, while only 5% of non-treated cells showed the activity. When the hydrocortisone-differentiated M1 cells were cultured with AF, 2.4-fold increase of [^{14}C]-acetate incorporation was observed. When untreated cells were cultured with AF, increase was also shown but it was only 40%. In both types of M1 cells, the optimal dose of AF for stimulation was relatively high, 40 μg/ml, and at this dose the cell growth was completely inhibited. Forty eight hour-treatment with retinoic acid also induced phagocytic activity in 40% of the cells. However, AF showed no stimulation of [^{14}C]acetate incorporation when cultured with them (Fig. 4).

Fig. 4. Comparison of incorporation of [^{14}C]acetate into differentiated and undifferentiated M1 cells.

2×10^5 M1 cells untreated (●), or treated with 0.1 μM hydrocortisone (○), or 10 μM retinoic acid (△), were cultured with AF and [^{14}C]acetate for 4 hours, and radioactivity incorporated into the TCA insoluble fraction were determined. Each points represent average percent to control value of triplicated culture.



A stimulating effect of AF on glucose consumption was observed with untreated, hydrocortisone-treated and retinoic acid-treated M1 cells, although the stimulation was less evident in retinoic acid-

treated M1 cells. The optimal dose for stimulation was 20 μg/ml. It should be noted that LPS stimulated glucose consumption of hydrocortisone-treated M1 cells but inhibited that of retinoic acid-treated M1 cells (Table 4).

Discussion

Present results show that AF stimulates the incorporation of [^{14}C]acetate into macrophages but inhibits that into splenocytes. This is further confirmed by the results using macrophage-like cell line, P388D1, as compared with the effect of AF on various lymphoid cell lines. It is interesting that mammary carcinoma, FM3A was affected by AF in a similar manner to P388D1, suggesting that P388D1 is metabolically similar to FM3A. Furthermore, incorporation of [^{14}C]acetate into a mouse myeloid leukemia M1 cells differentiated by hydrocortisone was evidently stimulated more than with undifferentiated M1 cells. Therefore, AF is likely to affect the cellular metabolism which is specifically developed in macrophages, in the course of their maturation. Since AF inhibits the functions of lymphocytes and activates that of macrophages, the cytospecific effect is to be associated with the immunological activity. In this respect, the fact that the incorporation of [^{14}C]acetate into retinoic acid-differentiated M1 cell was somewhat inhibited by AF is interesting because it implies that AF modulates the cellular metabolism differently depending on the subpopulations of macrophages. In fact, LPS inhibited glucose consumption of retinoic acid-differentiated M1 cells, while it enhanced that of hydrocortisone-differentiated M1 cells. Thus, hydrocortisone-differentiated M1 cells, a subpopulation which responds to LPS, could be specifically stimulated by AF. The relationships between other functions of macrophages, such as tumoricidal acitivity or IL-1 production, and susceptibility of lipid metabolism of the cells to AF need to be elucidated.

Although glucose consumption or methylglucose uptake was stimulated by AF in all types of the cells studied, the stimulation was most evident in the cells in which AF stimulated the incorporation of [^{14}C]acetate. Therefore, the effects on glucose metabolism and lipid metabolism seem to be coupled. The result that AF also stimulated glycolysis of splenocytes which is functionally inhibited by AF, suggests that AF directly functions on glycolysis. Therefore, it is likely that AF modulates lipid metabolism as a result of the modulation of glucose metabolism. However, the mechanism of stimulative incorporation of [^{14}C]acetate, remains to be elucidated. One possible explanation may be stimulation of lipogenesis, *i.e. de novo* synthesis of fatty acids. However, this is less plausible because incorporation of [^{3}H]glycerol into macrophages or P388D1 was rather suppressed by AF (unpublished data), as previously shown in L5178Y,[7] indicating that AF inhibited *de novo* synthesis of triglycerides. As an alternative explanation, of acetyl-CoA may be deprived and this could result in stimulation of incorporation of exogenously added acetate into acetyl-CoA. Deprivation of acetyl-CoA could result from suppression of the TCA cycle. If this is the case, glycolysis may could be stimulated to compensate for the suppressed production of ATP. More precise study to elucidate the effect of AF on lipid metabolism is now in progress.

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