STUDIES ON CELL GROWTH STIMULATING SUBSTANCES OF LOW MOLECULAR WEIGHT

PART 2. EXFOLIAZONE AND LAVANDUCYANIN, POTENT GROWTH PROMOTING SUBSTANCES OF RAT LIVER CELL LINE, RLN-8, PRODUCED BY *Streptomyces exfoliatus* AND *Streptomyces aeriouvifer*[a]

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Exfoliazone and lavanducyanin isolated from *Streptomyces exfoliatus* BT-38 and *Streptomyces aeriouvifer* CL-190, respectively, showed strong growth promoting activities to liver cell RLN-8 established from normal Donryu rat. When RLN-8 cells were cultured in EAGLE's minimal essential medium containing 1% fetal bovine serum, exfoliazone significantly stimulated the growth of RLN-8 cells. However, no effect was observed under serum-free conditions. Effective dose of exfoliazone was at the range of 0.004~0.1 μg/ml. Cell proliferation was confirmed by MTT assay and by the increases of cell number and DNA synthesis.

Lavanducyanin also stimulated the growth of RLN-8 cells in the same medium. It showed growth promoting activity at lower concentrations than exfoliazone and the effective dose was at the range of 0.0001~0.06 μg/ml. Analogous compounds of exfoliazone and lavanducyanin also promoted the growth of RLN-8 cells. In addition, exfoliazone and lavanducyanin enhanced the growth of NIH 3T3 and T601 cells. These results indicate that exfoliazone, lavanducyanin and their related compounds seem to be a new type of growth promoting substances with low molecular weight produced by microorganisms, and that they can partially substitute for functions of serum. Since 12-*O*-tetradecanoylphorbol-13-acetate (TPA) did not show the growth promoting activities under the same conditions, the action mechanism(s) of exfoliazone and lavanducyanin are different from that of TPA.

Many kinds of animal cell lines are being utilized for commercial production of biologically active proteinaceous substances such as monoclonal antibodies and growth factors. Most cell cultures require the addition of serum to synthetic media for their maintenance and growth, and proteins contained in the serum make difficult the purification of the desired proteinous products.

It is generally necessary to supplement the growth promoting factors to culture cell lines maintained in serum-free media or in media with low serum concentration. Many growth promoting factors have been found; most of them are, however, of high molecular weight, unstable and expensive.

Recently, some growth promoting substances with low molecular weight were found from foods[2], blue-green alga[3] and protein hydrolysates[4]. However, few growth promoting substances have been isolated from microorganisms. Therefore, we attempted to screen substances with low molecular weight of microbial

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origin which proliferate animal cells without serum or at low serum conditions.

In the course of our screening using a medium with low serum concentration, we have found that lavanducyanin[5] and exfoliazone[6] previously isolated as an antitumor substance and an antifungal substance, respectively, by our group displayed strong growth promoting activity. In this paper, we report the growth promoting action of these compounds.

Materials and Methods

Materials

Lavanducyanin[5] and exfoliazone[6] were isolated from *Streptomyces aeriouvifer* CL-190 and *Streptomyces exfoliatus* BT-38, respectively, as reported previously. Pyocyanin was isolated from *Pseudomonas aeruginosa* (IAM1514) by the methods of ROBERT *et al.*[7]. Questiomycin A was synthesized according to OSMAN and BASSIOUNI[8]. *N*-Acetylquestiomycin A was prepared by acetylation of questiomycin A[9].

Cell Culture

RLN-8[10] established from normal Donryu rat was used to investigate the biological activities of lavanducyanin and exfoliazone. The cells were routinely grown in a humidified incubator (95% air and 5% CO_2) at 37°C with EAGLE's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS).

Assay Method

RLN-8 cells were grown to semiconfluence. The cells were collected by centrifugation (130 $g \times 5$ minutes) and washed with serum-free MEM. The washed cells were suspended in MEM containing 1% FBS 1×10^4 cells/ml) and seeded into 96-well plates (100 μl/well). Microbial broth sterilized by passing through membrane filter was diluted 10-fold with serum-free MEM and this solution was put into wells (10 μl/well). After incubating for 4 days, the growth promoting activity was examined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) assay[11,12]. After removal of the medium, formazan deposits formed were dissolved in DMSO and the absorbance at 577 nm was measured with a microplate reader.

Cell Number and Assay of DNA Synthesis

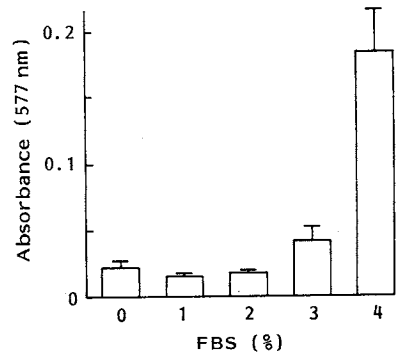
RLN-8 cells (2×10^4 cells/ml) were seeded into 6-well plates (2 ml/well) with different concentrations of serum and cultured for 4 days. The cell number was counted with a Coulter Counter after detaching the cells using 0.2% trypsin containing 0.02% EDTA. DNA synthesis was determined by [^{3}H]thymidine incorporation. To each culture dish was added 0.2 μCi of [^{3}H]thymidine, and incubated for 2 hours. The cells were hydrolyzed in NaOH for 5 minutes, acidified with TCA and passed through Whatman GF/C filters and then the filters were dried. The radioactivities of the filters were measured in toluene scintillation cocktail using a liquid scintillation counter.

Results

Assay Method and Results of Screening

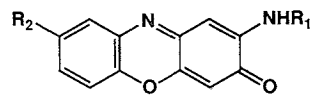
The effect of serum concentration of RLN-8 cell growth was measured by MTT assay. As shown in Fig. 1, the cell growth was distinctively suppressed at serum condition lower than 3%. Based on this result we selected MEM supplemented with 1%

Fig. 1. Effect of serum concentration on growth of RLN-8 cells.

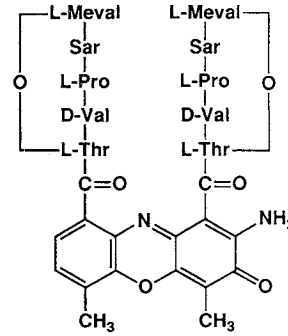


After 4 days cultivation, cell growth was measured by MTT assay.

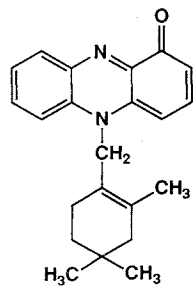
Fig. 2. Structures of exfoliazone, lavanducyanin and their related compounds.



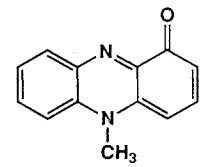
	R_1	R_2
Exfoliazone	$COCH_3$	CH_2OH
Questiomycin A	H	H
N-acetylquestiomycin A	$COCH_3$	H



Actinomycin D



Lavanducyanin



Pyocyanin

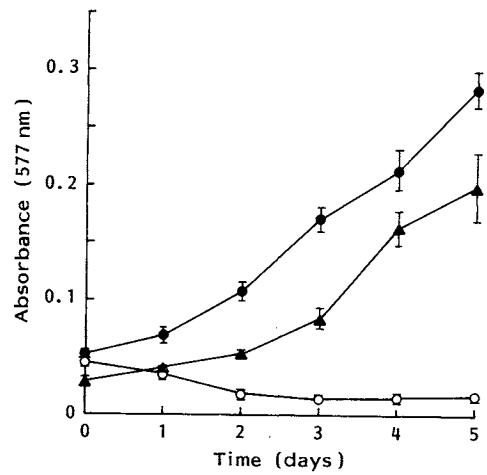
serum as the assay condition to find substances which can substitute completely or partially for serum. Broth filtrates of about 1,000 microorganisms were tested by this screening system. As a result, *S. exfoliatus* BT-38 and *S. aeriouvifer* CL-190 were found to produced very strong growth stimulating substances, exfoliazone[6] and lavanducyanin[5], which had been isolated as an antitumor substance and an antifungal substance, respectively, by our group. The structures of these metabolites and their related compounds are shown in Fig. 2.

Effects of Exfoliazone and Related Compounds on the Growth of RLN-8 Cells

The effect of exfoliazone on the growth of RLN-8 cells in the presence of 1% serum (MEM + 1% FBS) is shown in Fig. 3. It promoted the growth of RLN-8 cells after cultivating for 2 days and the growth rate was greater than that in MEM supplemented with 10% FBS. Without exfoliazone, however, no growth was observed in the presence of 1% serum.

Fig. 3. Growth curves of RLN-8 cells with or without exfoliazone.

● MEM + 1% FBS + exfoliazone (0.02 μg/ml), ○ MEM + 1% FBS, ▲ MEM + 10% FBS.

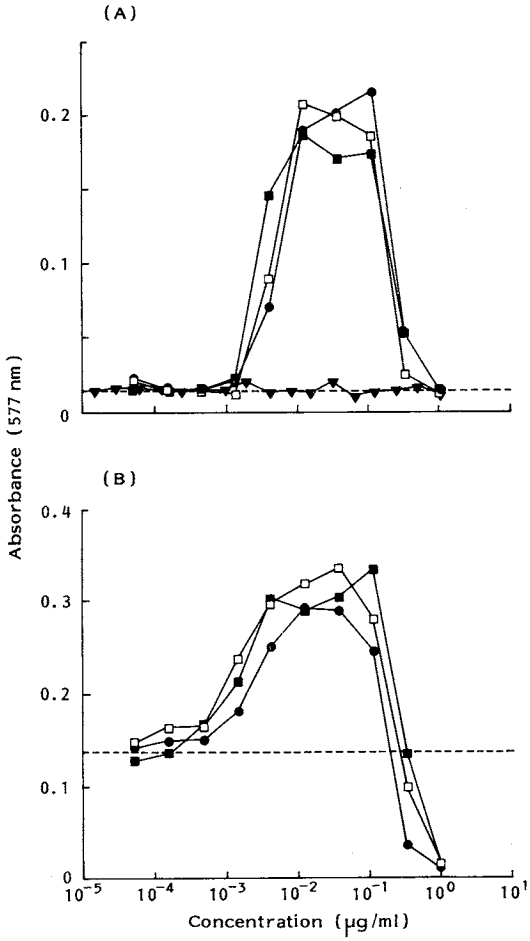


Cell growth was measured by MTT assay.

The effects of exfoliazone and related compounds on the growth of RLN-8 cells in MEM supplemented

Fig. 4. Effects of exfoliazone and related compounds on RLN-8 cells.

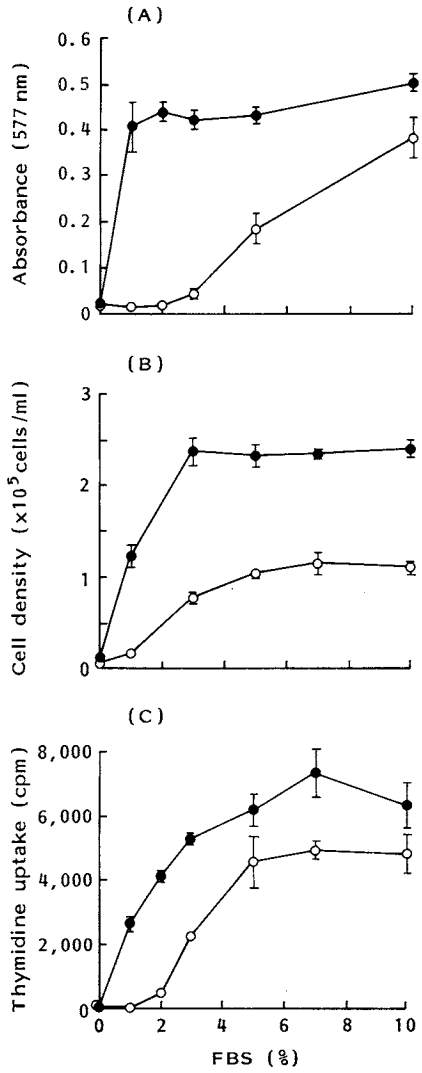
(A) MEM + 1% FBS, (B) MEM + 10% FBS, ● exfoliazone, □ questiomycin A, ■ *N*-acetylqusetiomycin A, ▼ actinomycin D, ----- control level.



Cell growth was measured by MTT assay.

Fig. 5. Effect of FBS concentration on growth promoting activity of exfoliazone.

Growth promoting activity was measured by MTT assay (A), Cell number (B) and [^{3}H]thymidine incorporation (C), ● with exfoliazone (0.02 μg/ml), ○ without exfoliazone.



with 1% FBS are shown in Fig. 4A. Exfoliazone showed the growth promoting effect at the concentration from 0.004 to 0.1 μg/ml. Questiomycin A and *N*-acetylquestiomycin A, analogues of exfoliazone, also promoted the growth of RLN-8 cells at the same dose. On the other hand, actinomycin D with a phenoxazine unit in the structure did not proliferate RLN-8 cells. Exfoliazone and its analogous compounds also exhibited the same activities in MEM supplemented with 10% FBS (Fig. 4B). The effective doses of these growth promoting substances were almost same as those in 1% serum condition. At the concentrations higher than the optimal dose, however, the growth was suppressed due to the cell toxicity of these compounds. At the concentrations lower than the optimal dose, the growth levels were almost equal to the control level. These results suggest that

exfoliazone and its related compounds possess both the growth stimulatory and inhibitory effects on RLN-8 cells.

Effect of Serum Concentration on the Growth Promoting Activity of Exfoliazone

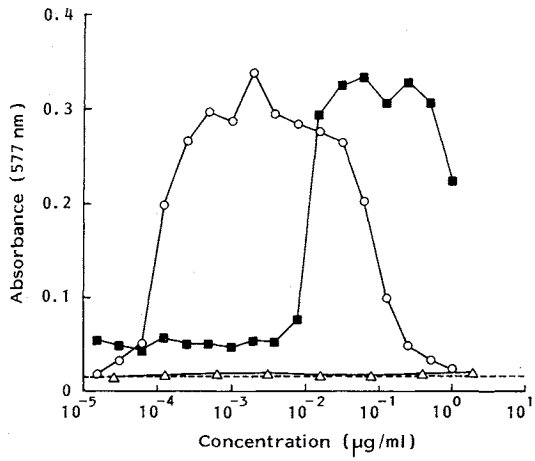
The growth promoting effect of exfoliazone on RLN-8 cells was determined at varying concentrations of serum (Fig. 5). At concentrations between 1% to 10%, exfoliazone clearly promoted the growth of the cells. However, no significant growth promotion was observed at serum-free condition. These results suggest that exfoliazone can partially substitute for some functions of serum but can not substitute completely. The growth promoting activity of exfoliazone was also confirmed by the increase of cell number and [^{3}H]thymidine incorporation (Fig. 5).

Effects of Lavanducyanin and Related Compound on RLN-8 Cells Growth

Lavanducyanin and its analogous substance, pyocyanin, also promoted the growth of RLN-8 cells. The effective dose of lavanducyanin was 0.0001 ~ 0.06 μg/ml under low serum conditions. It is to be emphasized that lavanducyanin stimulated the growth of RLN-8 cells more potent than any other compounds tested so far (Fig. 6). The tumor-

Fig. 6. Effects of lavanducyanin and related compounds on growth of RLN-8 cells.

○ lavanducyanin, ■ pyocyanin, △ TPA, ------ control level.



Cell growth was measured by MTT assay.

Table 1. Growth stimulating spectra of exfoliazone and lavanducyanin.

Cell	FBS (%)	Growth		Origin
		EX[a]	LA[b]	
Hep G2	1	−	−	Human hepatocellular carcinoma
Chang liver	1	−	−	Human liver
Ac2F (DT)	1	+	NT	Rat liver
Hepatocytes[c]	0	−	−	Rat primary cultured
WI38	1	−	−	Human embryonic lung
NIH 3T3	5	++	++	Mouse embryo
T601	1	++	++	Mouse embryo
NIH 3T3/*ras*	1	−	−	Mouse embryo
CHL-IU	1	++	++	Chinese hamster lung
CHO	1	−	−	Chinese hamster ovary
TPH-1	1	−	−	Human myelomonocyte
BALL-1	1	−	−	Human B cell line
MG-63	1	−	−	Human osteogenic sarcoma
SK-N-SH	1	−	−	Human neuroblastoma
10H	1	−	−	Mouse hybridoma

[a] Exfoliazone (0.02 μg/ml). [b] Lavanducyanin (0.01 μg/ml). −; not significant, +; significant, difference of means between sample and control level at $P<0.05$, ++; significant, at $P<0.01$. NT; not tested. [c] Growth was measured by [^{3}H]thymidine incorporation. Others were measured by MTT assay.

promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that can activate protein kinase C did not show any growth promoting action in this assay system.

Growth Promoting Action of Exfoliazone and Lavanducyanin Against Other Cells

Table 1 shows *in vitro* activities of exfoliazone and lavanducyanin against 15 kinds of animal cell lines. Exfoliazone promoted the growth of Ac2F(DT), NIH 3T3, T601[13] and CHL-IU cells in addition to RLN-8 cells. No effect, however, was observed against rat primary cultured hepatocytes. The growth stimulating spectrum of lavanducyanin was almost identical with those of exfoliazone. Lavanducyanin showed no growth promoting action to T601 cells under serum-free conditions.

Discussion

Some phenoxazine and phenazine derivatives are well known to show antibiotic and antitumor activities. We have found the growth promoting action of these compounds for the first time. The antitumor agent adriamycin was reported to show a growth stimulating activity at low subtoxic concentrations[14]. This property is similar to those of exfoliazone and their analogous compounds. Unlike exfoliazone and lavanducyanin, however, adriamycin exhibited the growth stimulation activity under serum free condition suggesting the difference of their action mechanisms.

In agreement with the structural similarity between exfoliazone and lavanducyanin possessing a tricyclic ring system with heteroatoms in the middle ring, their growth promoting effects seem to be identical. For example, they do not stimulate cell growth under serum-free condition, but show very similar activity profiles to proliferate animal cells such as NIH 3T3, T601 and CHL-IU. Their growth promoting action was confirmed not only by MTT assay but also by the increase of cell number and DNA synthesis. Thus, exfoliazone, lavanducyanin and their analogous compounds have proven to be a new type of growth promoting substances with low molecular weight isolated from microorganisms.

It should be emphasized that the effective dose ranges of exfoliazone and lavanducyanin are wider than adriamycin by 2.5 times and 60 times, respectively. Therefore, they may be used as useful tools for investigations on cell proliferation and for cultivation of animal cells used for producing proteinaceous substances. The preliminary studies on the growth promoting mechanism of lavanducyanin have been reported[1].

MOTOHASHI[15] reported antitumor activities of phenothiazines and phenoxazines using a solid type of Ehrlich carcinoma *in vivo*. According to his results, questiomycin A and *N*-acetylquestiomycin A did not show antitumor activities and instead they promoted the weight gain of tumor cells than the control. We have not yet examined the growth promoting activities of exfoliazone and lavanducyanin *in vivo*. These compounds may also possess the growth promoting activity *in vivo*.

Acknowledgments

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