CYTOSTATIN, A NOVEL INHIBITOR OF CELL ADHESION TO COMPONENTS OF EXTRACELLULAR MATRIX PRODUCED BY *Streptomyces* sp. MJ654-NF4

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Cytostatin has been identified as a novel inhibitor of cell adhesion to components of extracellular matrix (ECM) in cultured broth of *Streptomyces* sp. MJ654-NF4. Though cytostatin did not inhibit EL-4 cell adhesion to ECM components such as laminin and fibronectin, it inhibited the adhesion of B16 melanoma cells to laminin and collagen type IV but not to fibronectin. It exhibited antimetastatic activity on B16 melanoma cells in mice. The cytotoxicity of cytostatin are also reported.

It is well known that tumor metastases and/or invasion and immune responses are mediated by adhesion to the extracellular matrices (ECM) such as laminin, fibronectin and collagen type IV[1~3].

To develop a new types of immunomodulator and inhibitors of tumor metastases in microbial products, we have attempted to find inhibitors of cell adhesion to ECM components. Earlier delaminomycins were found as a new class of immunosuppressant, although they did not inhibit tumor metastases[4]. During the screening program, we have succeeded in finding an inhibitor of tumor metastases, namely cytostatin having a new structure.

In this paper, we report the taxonomy of the producing strain, fermentation, isolation and biological activities of cytostatin.

Results and Discussion

Taxonomy of the Producing Strain

The producing microorganism, strain MJ654-NF4, was isolated from a soil sample collected in Tsukuba-shi, Ibaraki Prefecture, Japan. Taxonomic studies were carried out mainly according to the procedures of the International Streptomyces Project[5]. The cultural and physiological characteristics of strain MJ654-NF4 are shown in Tables 1 and 2, respectively. Strain MJ654-NF4 has branched substrate mycelia, and spore chains in the form of spirals. Matured spores occurred as chains of more than 30 cylindrical spores. Sporangium and whirl-formation were not observed. The spore surface was smooth and about $0.7 \sim 0.8$ by $1.1 \sim 1.3$ μm in size. Vegetative mass color was pale yellow to pale yellowish brown on the various media. Aerial mass color of the colony was light gray as described in Table 1. Moist, black, liquefied (hygroscopic) areas are found in the aerial mycelium in 10 to 21 days. Soluble pigment and melanoid pigment were not formed. The whole cell hydrolysates contained LL-diaminopimelic acid.

Table 1. Cultural characteristics of strain MJ654-NF4.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Colorless	Natural (3dc)	None
Glucose-asparagine agar	Lt lemon yellow (1ga) to Lt tan (3gc)	Silver gray (3fe)	None
Glycerol-asparagine agar (ISP-5)	Lt tan (3gc) to cinnamon (3le)	Natural (3dc) to silver gray (3fe), hygroscopic	None
Inorganic salts-starch agar (ISP-4)	Bamboo (2gc)	White to silver gray (3fe), hygroscopic	None
Tyrosine agar (ISP-7)	Nude tan (4gc) to cork tan (4ie)	Natural (3dc) to silver gray (3fe), hygroscopic	Faint, brownish
Nutrient agar	Bamboo (2gc)	None	None
Yeast extract-malt extract agar (ISP-2)	Camel (3ie) to cinnamon (3le)	Silver gray (3fe), hygroscopic	None
Oatmeal agar (ISP-3)	Lt ivoly (2ca) to bamboo (2gc)	Silver gray (3fe), hygroscopic	None
Glycerol-nitrate agar	Colorless to pale yellow	White	None
Starch agar	Pale yellow (1ca) to canary yellow (1ea)	None	None

Color names and numbers from Color Harmony Manual, Container Corporation of America. Observation after incubation at 27°C for 21 days.

Table 2. Physiological characteristics of strain MJ654-NF4.

Temperature range for growth (°C)	20~37	Utilization of L-Arabinose	+
Optimum temperature (°C)	27~30	D-Xylose	d
Formation of melanoid pigment	Negative	D-Glucose	+
Liquefaction of gelatin	Weak	D-Fructose	+
Coagulation of milk	Positive	Rhamnose	−
Peptonization of milk	Weak	Sucrose	(−)
Hydrolysis of starch	Positive	Raffinose	−
Reduction of nitrate	Probably positive	Inositol	−
		D-Mannitol	−

+: Utilized, d: doubtful, −: not utilized (−): probably not utilized.

From these taxonomic properties, strain MJ654-NF4 was identified as belonging to the genus *Streptomyces*. Among the known species of *Streptomyces*, strain MJ654-NF4 was considered to resemble *S. humidus*[6], *S. endus*[6], *S. hygroscopicus*[6] and *S. cirratus*[6]. Of the four nomen species cited above, strain MJ654-NF4 was most closely related to *S. humidus*. The taxonomic characteristics of MJ654-NF4 were compared with *S. humidus*. As shown in Table 3, strain MJ654-NF4 is different from *S. humidus* in utilization of rhamnose, inositol and D-mannitol.

Therefore, strain MJ654-NF4 was designated *Streptomyces* sp. MJ654-NF4. This strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-13600.

Fermentation

Fermentations for the production of cytostatin were carried out as follows. Strain MJ654-NF4 on an agar slant was inoculated into 500-ml Erlenmeyer flasks containing 110 ml of an autoclaved seed medium consisting of soluble starch 4%, Ebios 4%, polypeptone 0.5%, KH_2PO_4 0.1%, K_2HPO_4 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% (adjusted to pH 7.4 before sterilization) and cultured on a rotary shaker (180 rpm) at 30°C for 3 days. For the production of cytostatin, 2.2 ml of the mature

Table 3. Comparison of taxonomic characteristics of strain MJ654-NF4 and *Streptomyces humidus* IMC S-0145 (ISP 5263).

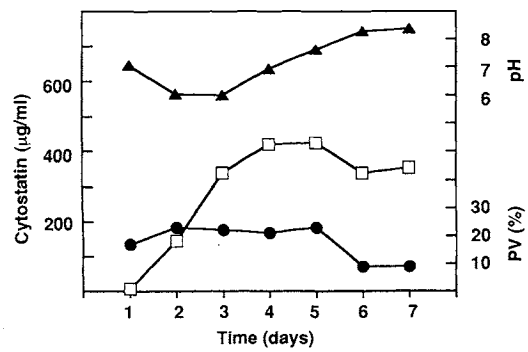
	MJ654-NF4	*S. humidus* IMC S-0145 (ISP5263)
Spore chain morphology	Spirals	Spirals
Spore surface	Smooth	Smooth
Aerial mass color	Light gray	Light gray
Color of vegetative growth	Pale yellow to pale yellowish brown	Pale yellow to pale yellowish brown
Soluble pigment	None	None
Melanin formation		
ISP-medium 1	Negative	Negative
6	Negative	Negative
7	Negative	Negative
Liquefaction of gelatin	Weak	Weak
Coagulation of skim milk	Positive	Positive
Peptonization of skim milk	Weak	Weak
Nitrate reduction	Doubtful	Positive
Hydrolysis of starch	Positive	Positive
Carbon utilization		
L-Arabinose	+	+
D-Xylose	(+)	(+)
D-Fructose	+	+
D-Glucose	+	+
Rhamnose	−	+
Sucrose	(−)	−
Raffinose	−	−
Inositol	−	(+)
D-Mannitol	−	+

+: Utilized, d: doubtful, −: not utilized, (+): probably utilized, (−): probably not utilized.

seed fermentation was transferred to 500-ml flasks containing 110 ml of an autoclaved production medium consisting of fish meal 2%, soybean oil 2%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.1%, yeast extract .0.5% (pH of the medium was not adjusted). The fermentation for production of cytostatin was performed at 27°C for 4 days on a rotary shaker (180 rpm).

Fig. 1. Time course of cytostatin production.

□ Cytostatin, ● PV, ▲ pH.



The time course of the production of cytostatin is shown in Fig. 1. Production was monitored by the B16 cells adhesion assay *in vitro*. The amount of cytostatin in the cultured broth was determined by HPLC using a Capcell Pak C_{18} column (Shiseido) with mixture of MeOH and 0.01 N HCl (65:35) as an eluent.

Isolation and Purification

The cultured broth (10 liters) was centrifuged and the supernatant was adjusted to pH 4 with 1 N HCl. The resulting solution was applied on a column of Diaion HP-20 packed with water. The column was washed with water and then eluted with MeOH. Methanol was removed *in vacuo*, and the residue

was dissolved with 4% $NaHCO_3$. The solution was washed with EtOAc. The aqueous layer was adjusted to pH 4 with 1 N HCl, and then extracted with EtOAc. The organic layer was concentrated under reduced pressure to afford 2.0 g of oily crude material. This crude material was dissolved in 4% $NaHCO_3$ and applied on a Diaion HP-20 column. After washing the column with water, the active substance was eluted with 80% MeOH. The eluate was concentrated under reduced pressure to give an oily material (694 mg). This was subjected to Sephadex G-25 column chromatography using H_2O as an eluent. Each fraction containing cytostatin was collected and concentrated *in vacuo*. The residue was dissolved in a small amount of MeOH and chromatographed by reverse phase HPLC (Capcell Pak C_{18}, 20 × 250 mm, Shiseido) using MeOH - H_2O (55 : 45) as an eluent at a flow rate of 3 ml/minute. The active fractions were concentrated *in vacuo*. Cytostatin was further purified by Sephadex LH-20 chromatography with MeOH. Active eluates were concentrated to afford 255 mg of cytostatin as a yellowish powder.

Biological Activities

Inhibitory Activity of Cytostatin on Adhesion of B16 Melanoma Cells and EL-4 Cells to ECM Components

The cell adhesion assay was performed by the same method described as previous report[4]. Briefly, B16 cells which were maintained by serial cultures were plated in a microplate coated with an ECM component, laminin, fibronectin or collagen type IV and incubated with cytostatin for 1 hour at 37°C in a CO_2 incubator. After the incubation, the plates were washed with phosphate buffered saline (PBS) thoroughly, fixed with glutaraldehyde and stained with crystal violet. After washing, the density of cells adhesing to the plate was determined by spectroscopy. As shown in Fig. 2, cytostatin at 0.39 to 6.25 μg/ml inhibited the adhesion of B16 melanoma cells to laminin and collagen type IV in a dose dependent manner but not to fibronectin. The IC_{50} values were 1.3 μg/ml to laminin and 1.4 μg/ml to collagen IV. On the other hand, it did not inhibit the adhesion of Con A-activated EL-4 cells to laminin and fibronectin.

Fig. 2. Inhibition of adhesion of B16 melanoma cells to ECM components by cytostatin.

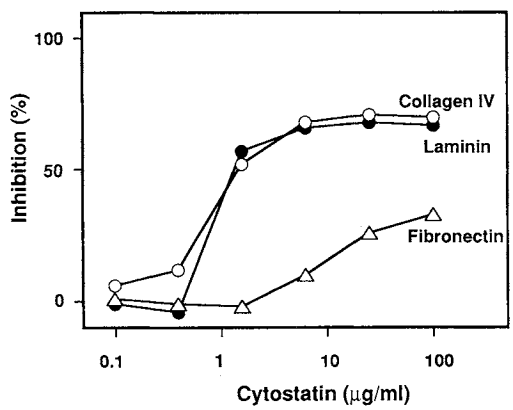


Table 4. Inhibitory effects of cytostatin on experimental lung metastasis of B16-F10 melanoma cells.

Dose (mg/kg, ip)	Schedule (Day)	No. of lung metastases[a]		Inhibition (%)	*t*-test[b]
		Mean ± S.D.	(range)		
0		338.1 ± 63.8	(262 ~ 431)	0	
1.25	1 ~ 9	154.6 ± 84.2	(70 ~ 278)	54.3	$P<0.01$
0.31	1 ~ 9	273.0 ± 67.2	(185 ~ 354)	19.3	ns
1.25	1 ~ 19	79.6 ± 19.7	(50 ~ 98)	76.5	$P<0.001$
0.31	1 ~ 19	191.8 ± 25.1	(169 ~ 226)	43.3	$P<0.001$

BDF_1 mice were inoculated with B16-F10 melanoma cells (5×10^5, i.v.).

[a] Mean number or lung metastases cells were counted 20 days after inoculation.

[b] Compared with untreated control mice by STUDENT's *t*-test.

ns: Not significant.

Inhibitory Effect of Cytostatin on Experimental Metastases of B16-F10 Melanoma in BDF_1 Mice

The effect of cytostatin on metastases of B16-F10 melanoma cells was investigated. B16-F10 cells were maintained by serial cultures and 5×10^5 cells were inoculated to mice iv. Cytostatin was given ip days 1 to 9 or to 19 and metastatic foci in lung were counted day 20. As shown in Table 4, the administration of cytostatin inhibited metastases of B16-F10 markedly. The inhibitory ratio was about 60 to 70% at 1.25 mg/kg/day.

Table 5. Cytotoxicity of cytostatin on tumor cells *in vitro*.

Tumor cells	IC_{50} (μg/ml)
L1210 lymphoid leukemia cells	0.042
P388 monocytic leukemia cells	0.065
EL-4 thymoma cells	0.178
B16 melanoma cells	0.572
IMC carcinoma cells	0.071
Meth A fibrosarcoma cells	0.164
A431 cells	0.082

Cytotoxicity of Cytostatin *In Vitro* and Antitumor Activity against L1210 and B16-F10 Melanoma

The cytotoxicity of cytostatin to tumor cells was examined. Tumor cells maintained in our institute by serial cultures were cultured with cytostatin for 48 hours in a CO_2 incubator and cytotoxicity was determined by the MTT assay[7]. As shown in Table 5, cytostatin showed cytotoxicity to various tumor cell lines at 0.042 to 0.57 μg/ml. The cytotoxicity to B16 cells was lower than to other cells.

The antitumor activities of cytostatin against L1210 and B16-F10 melanoma were tested. Although cytostatin was not effective in inhibiting B16 melanoma in BDF_1 mice in 0.15~1.25 mg/kg daily for 9 days, it prolonged survival of L1210 bearing CDF_1 mice at 2.5 mg/kg/day for 9 days but not effective at lower than 1.25 mg/kg/day. The T/C % at 2.5 mg/kg was 181%.

Cytostatin did not show antimicrobial and antifungal activities at 100 μg/ml.

The toxicity of cytostatin by single injection ip to ICR mice was tested. The LD_{50} was estimated at doses 5.0 to 10.0 mg/kg.

Acknowledgments

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