Journal of Microbiology and Biotechnology
Vol. 5. No. 5, 264-268, 1995

Screening for Inhibitor of c-*myc* Expression and Identification of Isolate No.2303

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Sulforhodamine B(SRB) assay was performed on the human lung carcinoma, A549 cell line to screen soil microorganisms for production of anti-cancer agent. Among 4,265 microorganisms, 45 isolates were selected for their cytotoxicity and tested for their effects on the expression of c-*myc* by RNA slot blot and Northern blot analysis resulting in selection of No.2303 isolate. This No.2303 was identified as *Streptomyces* sp. by ISP classification and the chemotaxonomic analysis method. N0.2303 inhibited the expression of c-*myc* in Colo320 DM and A549 cell lines. The culture extract of No. 2303 also inhibited the progression of the cell cycle of G_0 in NIH 3T3 cells, implying that the extract also inhibited the expression of c-*myc* in NIH 3T3 cell.

Various anticancer drugs have been isolated from soil microorganisms (especially *Streptomyces* sp). since sarkomycin was discovered by Umezawa *et al.* at 1953 (3). Cancer cell specific anticancer drugs, however, have not yet been developed. Many existing drugs inhibit DNA synthesis by interacting with DNA or by blocking synthesis of a DNA precursor. Therefore, they also exert cytotoxic effects on normal cells as well (17). Recently many researchers have focused on the signalling pathways stimulated by polypeptide growth factors to develop the efficient anticancer drugs. The inhibitors of these signalling pathways are thought to be good candidates for anticancer drugs. The inhibitors of several enzymes which include phospholipase C (PLC), protein kinase C (PKC) and protein tyrosine kinase (PTK) have already been developed (5). However, these enzymes are also important in signalling pathways of normal cells. Therefore, to develop a specific anticancer drug, it is necessary to understand the mechanism by which cancer cells develop.

Many oncogenes have been known to be involved in carcinogenesis. Among these, c-*myc* is one of the best known oncogenes. The product of c-*myc* oncogene is nuclear protein involved in cell proliferation (1). The c-*myc* gene is found in many human cancer cells where it is translocated, amplified and overexpressed thus appears to play a critical role in tumorigenecity (4, 7, 11). If the specific inhibition of c-*myc* gene expression is possible, it would block the proliferation of cancer cells in which the over expression of c-*myc* gene is the major cause of carcinogenesis. In this study, we screened soil microorganisms for anticancer agents which inhibit the expression of c-*myc* oncogene. To ensure efficient screening, we first isolated strains which have cytotoxicity on human cancer cell lines then selected strains inhibiting the expression of c-*myc* oncogene. One selected microorganism was named as No.2303 and identified using numerical analysis.

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Key words: screening of microorganism, c-*myc* expression inhibitor, *Streptomyces* sp., cell cycle

MATERIALS AND METHODS

Preparation of Microbial Extract and Diagnosis of Taxonomic properties

Microorganism was grown on medium: 2.0% sucrose, 0.5% yeast extract, 0.5% polypeptone, 0.5% KH_2PO_4, 0.5% $Na_2HPO_4 \cdot 12H_2O$, 2.0% vegetable juice (V8, Campbell Co.) at 30°C for 7 days on a shaking incubator. The cultured broth was treated with acetone of equal volume and left at 4°C for 24 hours. The disrupted cells were then removed by centrifugation and supernatant was saved. The supernatant was evaperated to give the microbial extract.
Cultural and morphological properties were studied

on solid media according to the recommentation of the International *Streptomyces* Project (ISP) (15). Analyses of total sugars (16) and diaminopimelic acid (14) were carried out using thin layer chromatography. Selected strain was identified by index of Bergey's Manual Of Systematic Bacteriology (10).

Cell Culture and Cytotoxicity Analysis

Human adenocarcinoma Colo320 DM (ATCC CCL 220) and lung carcinoma A549 (ATCC CCL 185) cell lines were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum and penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2/95% air. NIH 3T3 fibroblast (ATCC CRL 1658) was grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% bovine calf serum. The cells were passaged twice a week. For the SRB assay, A549 cells were plated in a 96 well plate at densities of 1×10^4 cells per well. After treatment of microbial extracts, cells were incubated at 37°C for 24 hours in a CO_2 incubator. The cultures were fixed with 10% TCA at 4°C for 1 hour then washed five times with tap water. The plate was dried under air and stained with 0.4% SRB in 1% acetic acid for 30 minutes. The plate was washed with 1% acetic acid after staining. Bound dye was dissolved in 10 mM of Tris then absorbance was was measured at 520 nm (12).

Isolation of total RNA, and Slot and Northern Blot Analysis

Total RNA was isolated from the cancer cells by using the single step guanidium isothiocyanate method (2). RNA was denatured by heating at 68°C for 10 minutes and blotted onto nylon membrane by using slot blot kit (Hoefer) or electrophoresed on 0.8% formaldehyde agarose gel. Twenty μg of RNA was loaded on the gel, which was confirmed by staining with ethidium bromide. After electrophoresis, the RNA was transferred to the nylon membrane and fixed on it using a UV transilluminator. Prehybridization of the membrane was carried out in hybridization solution (5X SSC [pH7.0], 50% formamide, 0.1% N-laurylsarcosine, 7% SDS, 50 mM sodium phosphate [pH 7.0]) containing 50 μg/ml of denatured yeast RNA at 37°C for 6~12 hours. The blotted RNA was hybridized with denatured digoxigenin (DIG)-labeled or ^{32}P-labeled c-*myc* DNA at 37°C overnight. The used c-*myc* probe was 1.4 kb of *ClaI*/*Eco*RI DNA fragment containing the third exon of the human c-*myc* gene. After hybridization, the membrane was washed twice for 5 minutes in washing solution I (1X SSC, 0.1% SDS) at room temperature, then twice for 15 minutes in washing solution II (0.2X SSC, 0.1% SDS) at 68°C. Detection of hybridized RNA was performed with alkaline phosphatase (AP)-labeled anti-DIG antibody using nitroblue tetrazolium (NBT) and bromo- chloro-indolylphosphate (X-phosphate) solution as substrates.

RESULTS AND DISCUSSION

Screening of Soil Microorganisms for Anticancer agent

Cytotoxicity tests were performed on the human lung carcinoma A549 cell line for screening soil microorganism producing anticancer agent. Among 4,265 microorganisms tested, 45 microorganisms were selected for their stronger cytotoxicity to A549 cell compared to NIH 3T3 cell and tested for their inhibitory effect on c-*myc* expression by RNA slot blot analysis. Finally, one microorganism, named No. 2303, was screened for its inhibitory effect on the expression of c-*myc* gene (Fig. 1). In this screening experiment, a 24 well plate was used, so it was impossible to measure the amount of RNA because of low concentration of RNA. Instead, to normalize the cell numbers, SRB assay was also performed. As shown in Fig. 1, the total amount of protein in treated cell was not decreased, indicating that the amount of cell numbers was not changed in this condition. Also, the expression of β-actin was not changed in the same condition, suggesting that the inhibition was specific for c-*myc* gene. Also, the level of c-*myc* mRNA was measured by Northern blot in Colo320 DM cells which contained amplified c-*myc* oncogene. The culture extract of No.2303 inhibited the expression of c-*myc* gene in cancer cell line Colo320

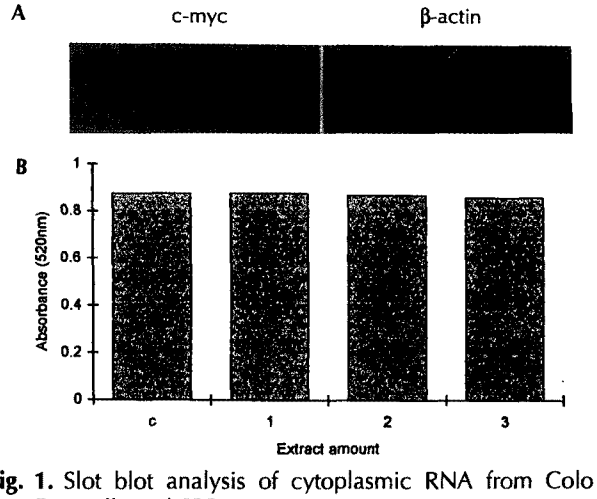

Fig. 1. Slot blot analysis of cytoplasmic RNA from Colo 320 DM cells and SRB assay.

Cytoplasmic RNA was prepared from Colo320 DM cells treated with various amount of NO.2303 extract for 2 h and the blotted RNA was hybridized with DIG-labeled c-*myc* and β-actin DNA probe (A). At the same condition, SRB assay was performed (B): c, untreated; 1, 4 μl; 2, 20 μl; 3, 40 μl extract was treated on the 24-well plate.

DM in a dose dependent manner (Fig. 2). Therefore, we selected this microorganism and identified it.

Identification of No.2303

No.2303 strain produced a rectiflexible spore chain. The spores were elliptical form with 0.8 to 1.0 μm in length and their surface was smooth. Mature aerial mycelia corresponded to the gray color series (Fig. 3). While sporulation was good on various solid media, it was poor on sucrose nitrate agar. Soluble pigment of a pink color was observed on oatmeal agar (ISP-3) and inorganic salt-starch agar (ISP-4). Aerial mycelium was formed in all media, and reverse color was brown to black on all media but dark pink on inorganic salt-starch agar (Table 1). No.2303 used all carbon sources with a few exceptions, inuline, sodium acetate, and sodium citrate and used all nitrogen sources except L-valine (Table 2). The type of diaminopimelic acid in the cell wall was determined as LL-DAP and alanine, glutamic acid, and glycine were detected from the cell wall hydrolysate (Fig. 4). This indicates the strain is cell wall chemotype I (8) and peptidoglycan type A3 (10). No diagnostic sugars were

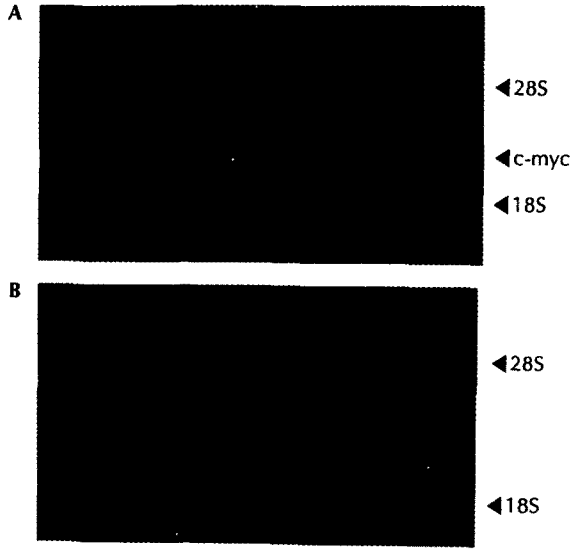

Fig. 2. Northern blot analysis.
The effect of No.2303 extract on c-*myc* gene expression in Colo320 DM cell. Colo320 DM cells were incubated with extract for 3 hours and total RNA was prepared. Twenty μg of RNA was loaded per lane, electrophoresed on 0.8% agarose gel and hybridized with ^{32}P-labeled human c-*myc* DNA probe. (A): c, untreated; 1, 50 μl; 2, 200 μl; 3, 500 μl; 4, 1 ml; 5, 2 ml (B) Before transferring the RNA into nylon membrane, the gel was stained with ethidium bromide.

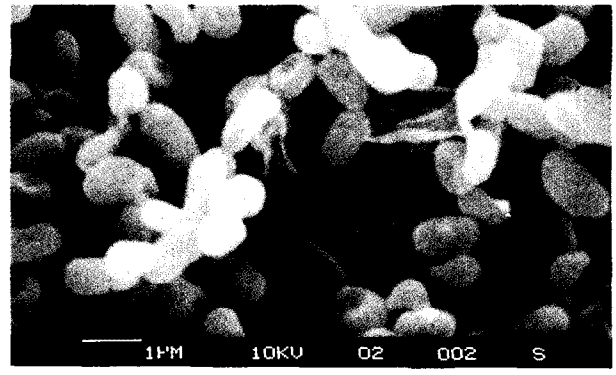

Fig. 3. Scanning electron microphotography of No.2303 grown on ISP4 medium (x 8,000).

Table 1. Cultural characteristics of isolate No.2303 on various solid media.

Media	Growth	Aerial mycelium	Reverse color	Soluble pigment
Yeast extract-malt extract agar (ISP2)	Good	Gray	Dark brown	Orange
Oatmeal agar (ISP3)	Good	Gray	Dark brown	Pink
Inorganic salt-starch agar (ISP4)	Good	White	Dark pink	Pink
Glycerol-asparagine agar (ISP5)	Good	Gray	Dark brown	Yellow
Tyrosine agar (ISP7)	Good	Gray	Black	None
Glucose-asparagine agar	Good	Gray	Black	None
Sucrose-nitrate agar	Poor	Gray	Brown	None
Nutrient agar	Good	White	Brwon	None

Table 2. Utilization of energy sources of isolate No.2303.

Energy source	utilization	Energy source	Utilization	Energy source	Utilization
Carbon source		Inuline	-	Nitrogen source	+
Sucrose	+	Salicin	+	Potassium nitrate	-
D-Xylose	++	Dextran	+	L-Valine	++
Mannitol	++	Cellobiose	++	L-Threonine	++
Raffinose	+	Sodium acetate	-	L-Serine	++
D-Mannose	++	Sodium citrate	-	L-Phenylalanine	++
D-Lactose	++	Sodium propionate	+	L-Methionine	++
D-Galactose	++			L-Histidine	++
				L-Arginine	

found in the whole cell hydrolysate (Fig. 5). This suggested that cell sugar pattern was type C (9). Thus, the No.2303 strain was identified as *Streptomyces* from our chemotaxonomical and morphological characteristics and named as *Streptomyces* sp. N0.2303.

Effect of *Streptomyces* sp. No.2303 on the NIH 3T3 Cell Cycle Progression

Studying the effect of an anticancer agent from cultured extracts of *Streptomyces* sp. N0.2303 on the progression of the cell cycle, we synchronized NIH 3T3 cells by serum starvation (0.5% BCS) then added fresh media containing 10% serum and *Streptomyces* sp. N 0.2303 extract at different time intervals (0, 0.5, 1, 3, 6, 12 hours). The extract inhibited the progression of cell cycle of G_0 in NIH 3T3 cells within 1 to 3 hours after serum stimulation (Fig. 6). Judith *et al.* reported that *c-myc* gene expression is required for cell proliferation of NIH 3T3 cells out of G_0 phase (6). Rozengurt has shown that notable expression of c-*myc* gene is detected within 1 to 2 hours after serum stimulation (13). These results indicate that c-*myc* gene expression is increased at G_0 to G_1 transition of the cell cycle. Therefore, our results imply that the anticancer

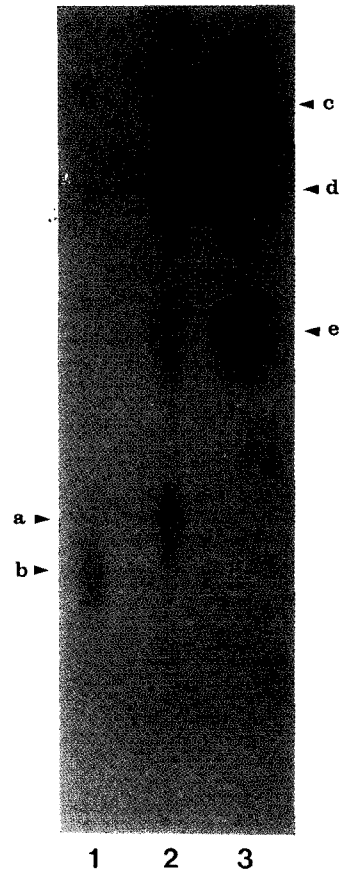

Fig 4. Cellulose thin layer chromatography of cell wall diaminopimelic acid (DAP) isomers and amino acids of No. 2303.

1; standard DAP isomer (a: LL-DAP, b: meso-DAP), 2; No.2303 cell wall hydrolysate, 3; standard amino acids (c: alanine, d: glutamic acid, e: glycine).

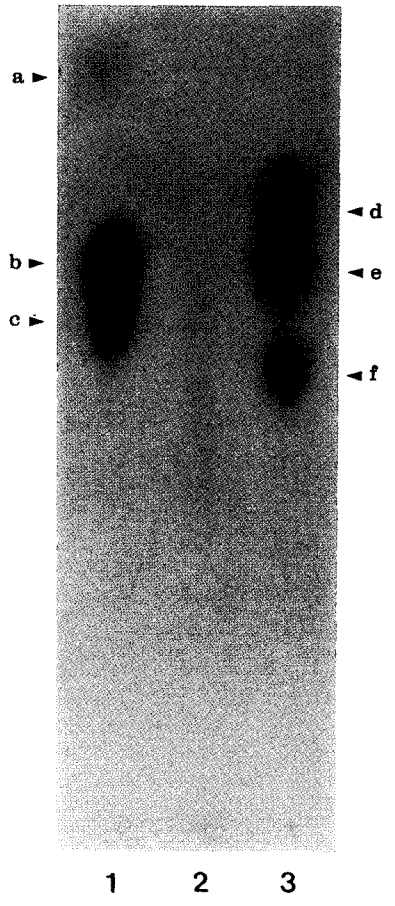

Fig. 5. Cellulose thin layer chromatography of whole cell sugar extract of NO.2303.

1; standard sugar (a: rhamnose, b: arabinose, c: glucose), 2; No.2303 cell extract, 3; standard sugars (d: xylose, e: mannose, f: galactose).

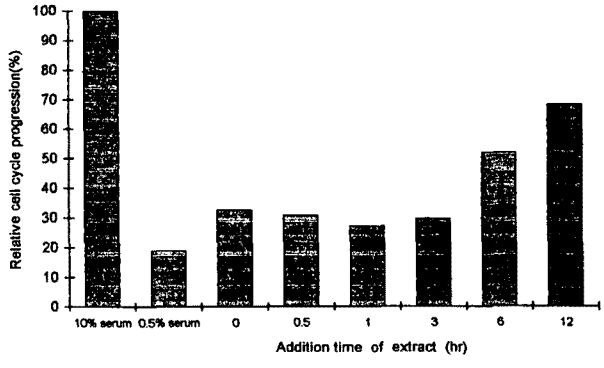

Fig. 6. The effect of No.2303 extract on the cell cycle.

NIH 3T3 cells were synchronized by serum starvation then fresh media containing 10% BCS was added. The extract was treated at different time intervals.

agent in *Streptomyces* sp. N0.2303 inhibits early stage (G_0 to G_1) of cell cycle progression which may be related to the inhibition of c-*myc* gene expression in NIH 3T3. This result suggested that the extract from *Streptomyces* No.2303 inhibited the growth of the normal cell. But the selected microorganism exerted less cytotoxic effect on NIH 3T3 cells than on A549 cells.

To elucidate the mechanism of the inhibitory effect of this microbial extract, purification of this anticancer agent is being performed. In a future study, the mechanism by which the expression of c-*myc* is inhibited should be also elucidated.

Acknowledgement

This work was supported by the Ministry of Science and Technology, Korea.

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(Received May 11, 1995)