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Heat stable cell growth inhibiting factor isolated from rat liver microsomes.

Kozo Inaba* Akitaka Doi† Isamu Nishida‡

*Okayama University,
†Okayama University,
‡Okayama Unversity,

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Kozo Inaba, Akitaka Doi, and Isamu Nishida

Abstract

A heat stable cell growth inhibiting factor was isolated from rat liver microsomes by hot salt extraction, ethanol fractionation and the hot phenol method. The factor was contained in the RNA fraction (designated as mhRNA). mhRNA inhibited the growth of mouse fibroblast (L-929) cells at a relatively low concentration (55 microgram/ml of culture medium). The molecular weight of mhRNA was about 27,000 and the base composition was guanine and cytosine rich.

KEYWORDS: cell growth inhibitor, liver, microsomes, RNA

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HEAT STABLE CELL GROWTH INHIBITING FACTOR ISOLATED FROM RAT LIVER MICROSOMES

Kozo INABA, Akitaka DOI and Isamu NISIDA[1]

Department of Physiology, Okayama University Medical School, Okayama 700, Japan

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Abstract. A heat stable cell growth inhibiting factor was isolated from rat liver microsomes by hot salt extraction, ethanol fractionation and the hot phenol method. The factor was contained in the RNA fraction (designated as mhRNA). mhRNA inhibited the growth of mouse fibroblast (L-929) cells at a relatively low concentration (55 μg/ml of culture medium). The molecular weight of mhRNA was about 27,000 and the base composition was guanine and cytosine rich.

Key words: cell growth inhibitor, liver, microsomes, RNA

It has been reported that several substances extracted from mammalian livers suppress the growth of cultured mammalian cells (1-4). Liberman and Ove (5) and Holley (6) clarified that arginase is one of the rat liver cytosol inhibitors of the growth of cultured mammalian cells. Goutier and Bologna (7) reported that rat liver microsomes inhibit DNA synthesis in a cell free extract system from regenerating rat liver. It has been reported that a cell growth inhibitory factor which was isolated from rat liver microsomes showed a strong arginase activity (8, 9) and the injection of the growth inhibitory factor into the peritoneal cavity of tumor-bearing mice prolonged the survival time of the animals (9). It was recently observed that hot salt extract from rat liver microsomes inhibits the growth of the cultured mouse fibroblasts, and the purification of the inhibiting factor from the extract has been examined. The present paper reports on the purification and partial characterization of a growth inhibiting factor from rat liver microsomes.

METHODS

Assay for inhibition of cell growth. L-929 cells (mouse fibroblast cell line) at the logarithmic growth phase were subcultured at a density of 4×10^4 cells per tube in 1 ml of Eagle's minimum essential medium with 10% calf serum at 37°C. At 24 h after subculture, the used medium was replaced with fresh medium (1 ml) containing the test material. Usually at 48 h after subculture the cells were detached from the tube wall by treatment with 0.05% trypsin-0.025% EDTA in phosphate-buffered saline, pH 7.2. The cells were counted by a Coulter counter. The specific activity was indicated as inhibitory dose 50% (ID_{50}, μg/ml of medium)

1. Present Address: Kagawa Medical School, Kagawa, Japan.

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which is the amount of test material necessary to inhibit cell growth by 50% in this assay system during incubation for 24 h at 37°C.

Preparation of microsomal fraction. Adult male rat (Wister strain) livers were used. The livers (usually, 100 g) were homogenized with 4 volumes of cold 0.25 M sucrose by teflon-pestle-glass homogenizer. The homogenate was filtered through 4 layers of gauze, and the filtrate was centrifuged at 800×g for 10 min. The supernatant was then centrifuged at 10,000×g for 10 min and from the resulting supernatant microsomes were precipitated by centrifugation at 105,000×g for 60 min.

Extraction and ethanol fractionation of cell growth inhibiting factor from microsomes. Rat liver microsomes were suspended in 20 volumes of cold 0.1 M NaCl and boiled for 3 min. After cooling, the microsomal suspension was centrifuged at 10,000×g for 10 min and to the supernatant (hot salt extract), cold ethanol was added to a final concentration of 40%. After standing for 2h at 0°C, the mixture was centrifuged at 10,000×g for 10 min, and the precipitate was lyophilized (or mixed with 10 volumes of cold acetone and washed with cold ether and then dried in air). The precipitate fraction was designated as mh-40.

Isolation of RNA from mh-40. Usually 100 mg of mh-40 was dissolved in 50 ml of 0.01 M acetate buffer containing 0.05 M NaCl (pH 5.1), and RNA portion of mh-40 was separated by a slight modification of the hot phenol method (10). The aqueous phase was washed five times with cold ether and 2.5 volumes of cold ethanol added. After standing for 2 h at 0°C, the mixture was then centrifuged and the precipitate was dissolved in distilled water and dialyzed against distilled water at 0 to 4°C overnight. The dialyzed solution was lyophilized (or treated with 10 volumes of cold acetone and washed with cold ether, and dried in air). The lyophilized (or acetone powder) fraction was designated as mhRNA.

Base analysis of mhRNA. An appropriate amount of mhRNA was hydrolyzed with 0.3 N KOH for 20 h at 37°C. After neutralization and centrifugation, the hydrolysate (supernatant) was charged on a Dowex-1 (×2, formate form, 0.8× 24 cm column, 200 to 400 mesh). Elution was performed by passing the following fluids in succession into a 100 ml mixing chamber containing 50 ml of distilled water: 30 ml of distilled water, 50 ml of 1 N formic acid and 240 ml of 4 N formic acid. The elution rate was 12 ml/h, and 3 ml fractions were collected.

Gel elctrophoresis of RNA. Electrophoresis of mhRNA in formamide-polyacrylamide gel was carried out by the method of Duesberg and Vogt (11). $tRNA^{Val}$ of E. coli was purchased from Sigma Chemical Co.

Chemical analysis. RNA was estimated by the method of Brown (12). Protein was estimated by the method of Lowry *et al.* (13) using bovine serum albumin as standard.

RESULTS

Extracts from liver microsomes and cytosol with hot salt solution have an inhibitory activity on the growth of L-929 cells, and the inhibitory activity (ID_{50}) of the former was about 3 times higher than that of the latter (Table 1).

TABLE 1. CELL GROWTH INHIBITORY ACTIVITY OF HOT SALT EXTRACTS FROM MICROSOMAL AND CYTOSOL FRACTIONS OF RAT LIVER

Fraction	Cell number (cells/ml)	ID_{50} (μg/ml)	Yield (mg/100 g liver)
Initial[a]	58170	—	—
Control	113430	—	—
Microsomal[b]	59980	680	260
Cytosol[b]	108750	2200	244

a, Cell number at 24 h after subculture; *b*, The culture medium was replaced with one ml of fresh culture medium containing 1 mg of acetone powder of microsomal or cytosol extract with hot 0.1 M NaCl at 24 h after subculture. Values are the average of three experiments.

Ethanol fractionation was examined for the purification of the growth inhibiting factor in the salt extract. The highest inhibitory activity on cell growth was observed when the precipitate fraction (mh-40) obtained by 40% saturation of cold ethanol and this activity was five times higher than that observed using microsomal hot salt extract (Table 2). The mh-40 fraction was mainly composed of RNA and protein in the ratio of about 3 : 1, respectively. The RNA component (mhRNA) was isolated from mh-40 fraction by the hot phenol method and assayed for its inhibitory activity on cell growth. The inhibitory activity of mhRNA was about two and ten times higher in comparison with that of mh-40 and microsomal hot salt extract, respectively (Table 2). The ID_{50} of mhRNA on the growth of L-929 cells was about 55 μg/ml. To confirm the efficacy of the salt extraction from microsomes dose-effect relations of three RNA preparations which were isolated from liver microsomes or cytosol were observed (Fig. 1). The inhibitory activity of total microsomal RNA or total cytosol RNA fraction was very low in comparison with that of mhRNA.

TABLE 2. PARTIAL PURIFICATION OF CELL GROWTH INHIBITING FACTOR FROM HOT SALT EXTRACT OF RAT LIVER MICROSOMES

Fraction	ID_{50} (μg/ml)	Yield (mg/100 g liver)
Hot salt extract	610	232
mh-40[a]	125	87
mh-70[a]	1110	42
mh-90[a]	880	23
mhRNA[b]	55	43

a, Microsomal hot salt extract was subjected to differential precipitation with ethanol, and the precipitate at 40%, 70% and 90% ethanol was designated as mh-40, -70 and -90, respectively. *b*, RNA fraction from mh-40 using the hot phenol method.

For the determination of the molecular weight of mhRNA, polyacrylamide gel electrophoresis of mhRNA (acetone powder) fraction was carried out. The molecular weight was calculated to be about 27,000 (Fig. 2).

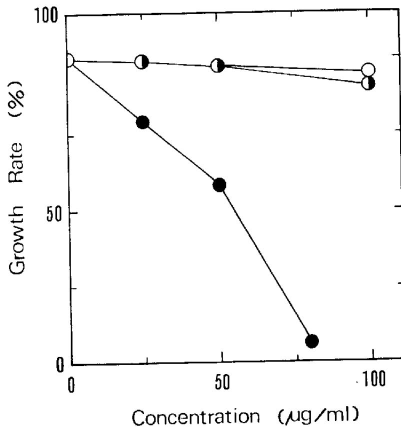

Fig. 1. Dose-effect relations of RNA's on the growth of L-929 cells. Open circles, RNA isolated from rat liver cytosol without hot salt extraction; Half solid circles, RNA isolated from rat liver microsomes without hot salt extraction; Solid circles, mhRNA.

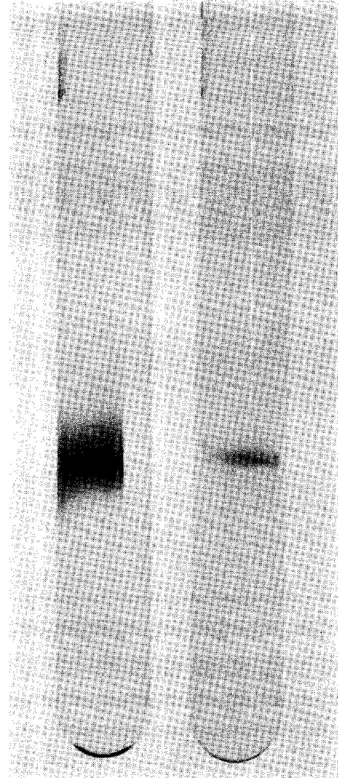

Fig. 2. Gel electrophoresis of mhRNA in formamide. Right, $tRNA^{Val}$ of E. coli; Left, mhRNA.

For the determination of base composition, mhRNA was hydrolyzed and then analyzed by Dowex-1 column chromatography. The base composition of mhRNA was guanine and cytosine rich (Table 3).

TABLE 3. BASE COMPOSITION OF mhRNA ISOLATED FROM HOT SALT EXSTRACT OF RAT LIVER MICROSOMES

AMP	GMP	CMP	UMP	G+C
13.9%	37.4%	29.0%	19.7%	66.4%

The values are the average of mole % from three experiments.

DISCUSSION

The cell growth inhibiting factor seems to be a heat stable substance complexed with RNA rather than RNA itself, since the digestion of mhRNA fraction with RNase or alkaline treatment did not affect significantly on the cell growth

inhibitory activity in preliminary experiments. It has been elucidated that there are at least two kinds of cell growth inhibiting factors in rat liver microsomes, namely, one of the factors is heat labile and has a arginase activity (8, 9) and the other is a heat stable substance complexed probably with guanine and cytosine rich RNA (mhRNA). It was recently reported that fibroblast chalone which was an aqueous extract from calf lung inhibited the growth of 3T3 cells and the substance was complexed with RNA in tissue extract and it was thought to be a polypeptide (14).

The action mechanism of mhRNA and further purification of the inhibitory factor from mhRNA fraction are under investigation.

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