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Abstract

A factor, cornin, inhibiting the growth of L cells cultured in monolayer was extracted from bovine liver with boiling water and was partially purified by gel filtration with Sephadex G-200. The factor was (1) precipitable with ethanol at the concentration between 70% and 90%, (2) impermeable through dializing memo brane, (3) eluted as the last peak at the gel filtration and (4) containing protein and RNA but no DNA.

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STUDIES ON THE CORNIN EXTRACTED FROM BOVINE LIVER
I. PURIFICATION OF THE CORNIN AND ITS PHYSICO-CHEMICAL PROPERTIES

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Abstract: A factor, cornin, inhibiting the growth of L cells cultured in monolayer was extracted from bovine liver with boiling water and was partially purified by gel filtration with Sephadex G-200. The factor was (1) precipitable with ethanol at the concentration between 70% and 90%, (2) impermeable through dializing membrane, (3) eluted as the last peak at the gel filtration and (4) containing protein and RNA but no DNA.

"Cornin", a growth regulating substance in adult animal tissues, has been studied in this laboratory for the last ten years. NISIDA and MURAKAMI (1) have reported that cornin, extracted from cornea and skeletal muscle, has retarding effects on mitosis of sea-urchin eggs. The substance inhibited the synthesis of DNA and of RNA in sea-urchin embryos as well as the incorporation of ^{32}P into regenerating rat liver nucleotids (2). Moreover, cornin extracted from smooth muscle of canine intestine has marked inhibitory effects on the growth of Ehrlich ascites carcinoma cells inoculated into mice (3). These effects of cornin have been considered to be due to the inhibition of oxidative phosphorylation (2, 4).

Chemical analysis has shown that cornin extracted by the method of NISIDA and MURAKAMI (1) contains inorganic substances, polypeptides and nucleotides (5).

In the present experiments, cornin extracted from bovine liver was fractionated by gel filtration and the inhibitory effect of each fraction on the growth of L cells cultured in monolayer was tested attempting to purify the cell growth inhibiting factor in cornin.

MATERIALS AND METHODS

Cornin was prepared from bovine liver by the method of NISIDA and MURAKAMI (1). Minced liver was boiled in 3 volumes (v/w) of distilled water for 10 min. After cooling, boiled liver was filtered to remove debris. To the filtrate was added cold ethanol, adjusting the final concentration to 70% and

90% (v/v). Precipitates appearing at 90% of ethanol were collected by centrifugation at 12,000 rpm using a continuous type centriguge (Kubota, KCF-62) and were washed with ethanol, methanol, acetone and ether. Crude liver cornin powder was then obtained by evaporating ether.

Aqueous crude liver cornin solution was dialyzed overnight against about 10 volumes of distilled water at 2°C. The outer fluid of dialyzing tube was lyophilized. The inner one was further dialyzed for 2 days with several changes of the outer fluid and was lyophilized. The two fractions obtained after lyophilization were designated as dialyzable- (D-) and undialyzable- (U-) fraction.

The U-fraction was gel filtered by a Sephadex G-200 column (3×42cm), eluted with distilled water and was fractionated to 4 ml each. Optical densities of each fraction at 260 and 280 mμ were measured by a photoelectric spectrophotometer (Hitachi, EPU-2A). The fractions from elution volume of 80 ml through 400 ml were divided into 5 groups, and after lyophilization they were stored in a desiccator until use.

For chemical analysis nucleic acids were extracted by the method of SCHNEIDER (6), and protein was obtained as the residue after extraction by trichloroacetic acid and was dissolved in 1 N NaOH. DNA, RNA and protein were estimated by the method of BURTON (7), WEBB (8) and LOWRY, *et al.* (9), respectively.

L cells originating from mouse fibroblasts were cultured in monolayer with Eagle's minimal essential medium, supplemented with 10% bovine serum. The simplified replicate culture method by KATSUTA, *et al.* (10) was employed for the examination of inhibitory effects on the growth of the cells. Test materials dissolved in the culture medium were sterilized by Millipore filtration and were added into culture tubes two days after inoculation of the cells. They were scraped off with a policeman and were counted by a hematocytometer.

RESULTS

Crude liver cornin possessed inhibitory effects on the growth of L cells cultured in monolayer at concentration higher than 0.4% (Fig. 1). The inhibition paralleled with dosage. After dialyzation, undialyzable- (U-) fraction inhibited more markedly the growth of the cells than dialyzable- (D-) fraction at the same concentration of 0.2% (Fig. 2).

The U-fraction was therefore separated further into 5 subfractions, by gel filtration as shown in Fig. 3. Among these subfractions, fraction IV (F-IV) and V (F-V), of which optical densities at 260 mμ were higher than at 280 mμ, markedly inhibited the cell growth (Fig. 3, upper figures).

Activities of U-fraction and F-IV at 0.2% were compared with that of crude liver cornin at 1%. Fig. 4 shows that the inhibitory activity of F-IV is higher than that of crude liver cornin and U-fraction. It indicates that the inhibitory factor(s) were more concentrated in F-IV than in U-fraction

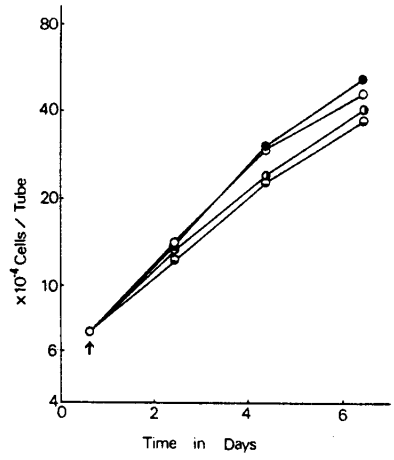

Fig. 1. Inhibitory effects of crude liver cornin on the growth of L cells cultured in monolayer. Cornin dissolved in the medium was added into the culture tubes two days after inoculation of the cells. The time adding test materials is shown by an arrow in this and the following figures. Concentration of test materials added; ○ : control (0%), ● : 0.2%, ◑ : 0.4% and ◒ : 0.6%.

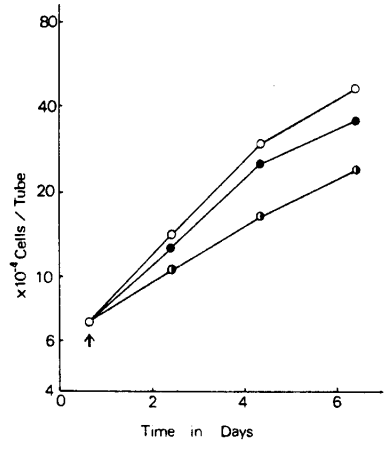

Fig. 2. Inhibitory effects of dialyzable and undialyzable fraction of liver cornin on the growth of L cells cultured in monolayer. ○ : control, ● : 0.2% dialyzable fraction, ◑ : 0.2% undialyzable fraction.

and that the inhibitory activity of F-IV was at least five times higher than that of crude liver cornin. Quantities of crude liver cornin, U-fraction and F-IV, obtained from 1 kg of original tissue, were 6.4 g, 2.7 g and 0.67 g, respectively.

From the elution curves in Fig. 3, it may be suggested that F-IV includes

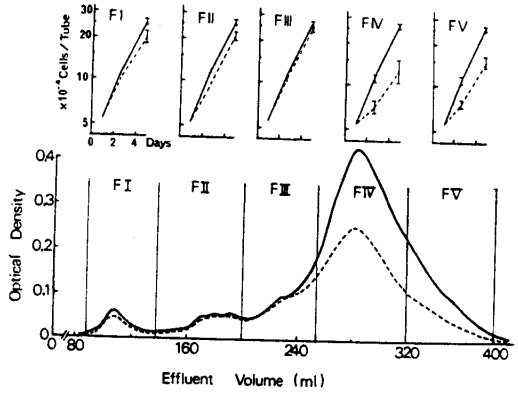

Fig. 3. Elution pattern of undialyzable liver cornin upon gel filtration by Sephadex G-200 and the inhibitory activities of five subfractions on the growth of L cells cultured in monolayer. Bottom: Elution pattern of undialyzable liver cornin, obtained by optical densities at —— 260 mμ and ····· 280 mμ. The filtrates were divided into five subfractions (F-I to F-V) as shown by vertical bars. Top: Inhibitory effects of each subfraction (mean values with standard errors).

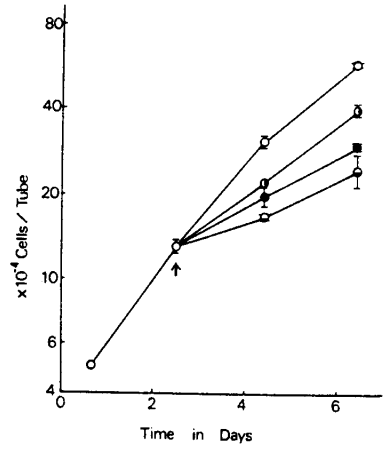

Fig. 4. Inhibitory effects of liver cornin fractions on the growth of L cells cultured in monolayer. ○ : control, ◑ : 0.2% undialyzable fraction, ◒ : 0.2% F-IV fraction, ● : 1% crude liver cornin. (mean values with standard errors).

nucleic acids, because the ratio of OD 260/OD 280 was nearly 2. The absorption maximum of F-IV in the UV-region of the spectrum was found at 260 mμ. Chemical analysis of U-fraction and F-IV showed that DNA was not detectable in both fractions and that the ratio of protein/RNA in U-fraction was higher than in F-IV (Table 1).

TABLE 1 PROTEIN, RNA AND DNA CONTENTS IN μg IN UNDIALYZABLE- AND F-IV FRACTION.

	Undialyzable-fraction	F-IV fraction
Protein	338±14.9	281±13.7
RNA	49.7±4.5	99.8±2.9
DNA	<1.0	<1.0
Protein/RNA	7.01±0.67	2.97±0.21

μg/mg of original materials (mean value ± standard error)

DISCUSSION

It has been reported that cell proliferation in adult animal tissues can be inhibited by a factor, such as "chalone" (BULLOUGH, 11) or "retin" (SZENT-GYÖRGYI, *et al.*, 12). Inhibitory factors of the cell growth have been found in the liver, in addition to chalone which has recently been extracted (13), arginase in liver was found to inhibit the growth of cultured cells *in vitro* (14, 15). Another factor inhibiting the DNA synthesis in Sarcoma 180 cells was obtained by OTSUKA (16). All these factors mentioned above are, however, different from cornin in respect to heat stability and/or chemical composition.

Considering the heat stability and a parallel relationship between the inhibitory activity and RNA content, it may be suggested that RNA is a plausible candidate inhibiting the growth of cells. Indeed, it has been reported that small mollecular RNAs in the chromatin are different depending on tissues and may regulate the gene activies in the tissue (17).

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