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REGULATION OF DNA SYNTHESIS IN THE LIVER OF TUMOR-BEARING RAT

I. DNA SYNTHESIS IN TUMOR-BEARING RATS

PURIFICATION OF LIVER THYMIDINE KINASE STIMULATING FACTOR FROM YOSHIDA SARCOMA

II. GROWTH FACTOR FROM YOSHIDA SARCOMA AND AH-130 TUMOR CELLS

Nobuyuki Harada

CONTENTS

ABBREVIATION

ATP: adenosine triphosphate

BHK: baby hamster kidney

CDP: cytidine diphosphate

CTP: cytidine triphosphate

Cyd: cytidine

dATP: deoxyadenosine triphosphate

dCMP: deoxycytidine monophosphate

dCTP: deoxycytidine triphosphate

dCyd: deoxycytidine

DEAE: diethylaminoethyl

dGTP: deoxyguanosine triphosphate

DME: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleoside triphosphate

dThd: thymidine

dTMP: thymidine monophosphate

DTT: dithiothreitol

dTTP: thymidine triphosphate

dUMP: deoxyuridine monophosphate

dUrd: deoxyuridine

GTP: guanosine triphosphate

HEL: human embryonic lung

ip: intraperitoneal

MEM: minimum essential medium

PBS: phosphate buffered saline

PBS$^-$: PBS without Ca^{++} and Mg^{++}

Tris: tris(hydroxymethyl)-aminomethane

TSF: liver thymidine kinase stimulating factor

Ura: uracil

Urd: uridine

UTP: uridine triphosphate

I. DNA SYNTHESIS IN TUMOR-BEARING RATS

PURIFICATION OF LIVER THYMIDINE KINASE STIMULATING FACTOR FROM YOSHIDA SARCOMA.

SUMMARY

Yoshida sarcoma cells contain a factor that stimulates thymidine kinase activity in the liver of mice *in vivo*; intraperitoneal injection of an extract from Yoshida sarcoma into normal mice stimulated their liver thymidine kinase activity 2- to 3-fold, whereas injection of a crude extract of normal rat liver did not stimulate the activity at all.

A factor that stimulates the *de novo* synthesis of thymidine kinase in the liver was partially purified from Yoshida sarcoma by ammonium sulfate fractionation, DEAE-cellulose column chromatography and gel filtration. It appeared to be thermolabile. These results suggested that it was high-molecular weight protein. Intraperitoneal injection of this factor into 67% hepatectomized rats stimulated thymidine kinase activity 2- to 3-fold. Increase of liver thymidine kinase activity after injection of the factor into mice was blocked by simultaneous injection of actinomycin-D. Activities of other DNA synthesizing enzymes; cytidine triphosphate (CTP) synthetase, ribonucleoside diphosphate reductase, deoxycytidine monophosphate (dCMP) deaminase, thymidine monophosphate (dTMP) synthase and DNA polymerase, were increased by injection of this factor. These results suggest that this factor stimulates *de novo* synthesis of thymidine kinase and stimulates DNA synthesis in the liver.

INTRODUCTION

A number of *in vivo* studies on the host-tumor relationship have been reported[1,2,3,4,7,13,16,22,23,24]. Several investigators[7,13,16,22] have shown an increase of the incorporation of labeled precursors into DNA of normal tissues in tumor-bearing animals. Increases of size of various organs[16], and particularly the spleen[2,24], of tumor-bearing animals have also been reported. Cerecedo *et al.*[3,4] observed increase of the nucleic acid content of the liver and lung in mice with transplanted tumors. Morgan and Cameron[13] reported increases of the DNA content and of DNA synthesis in the liver and spleen of tumor-bearing mice, the latter being at least twice that in control animals. Previously, we reported that incorporation of thymidine into DNA increased in the liver and spleen of rats bearing Yoshida sarcoma (solid type) or AH-130 (solid type), and that the thymidine kinase and DNA polymerase activities increased in the liver and spleen of these rats[20].

Rounds[17] and Rubin[18] found that culture media of Rous sarcoma cells and human cancer cells contained growth stimulating substances. Nair and DeOme[14,15] reported that a soluble fraction, prepared from spontaneous primary mouse mammary solid tumors, stimulated the growth of density-inhibited mouse embryo cells in monolayer culture. These results suggested that some substances that affect DNA synthesis in various tissues may be released from tumor tissue.

This paper reports the existence of the factor in Yoshida sarcoma cells that causes the increase of liver thymidine kinase

activity and the partial purification of this factor. The character of this factor is also reported.

MATERIALS AND METHODS

Animals Male ddY mice and male Donryu strain rats, both 3 to 4 weeks old, were obtained from Kitayama LABES Co., Kyoto. They were housed in a constant temperature room with lighting from 8 a.m. to 8 p.m., and were fed on a commercial solid feed MF (Oriental Yeast Co., Tokyo).

Tumors Yoshida ascites tumor cells were maintained in the peritoneal cavity of Donryu strain rats by weekly transfer. Ascites fluid was harvested 7 days after inoculation of cells. Tumor cells were collected by centrifugation at 1,500 x g for 1 min, washed 3 times with 0.9% NaCl solution and stored at -20° until used. Solid type Yoshida sarcomas were induced by transplanting 5×10^7 ascites cells into the subepidermal tissues of the back of Donryu strain rats.

Chemicals [^{14}C]-Thymidine was obtained from New England Nuclear, Boston, Mass. Unlabeled thymidine and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, U.S.A. DEAE-cellulose (DE-23) was obtained from Whatman, and Sephadex G-200 was obtained from Pharmacia Fine Chemical Co. Carrier ampholines were purchased from LKB-Produkter AB, Stockholm, Sweden. DEAE-cellulose paper was obtained from Toyo Roshi Co., Osaka. All chemicals used were of the highest commercial grade available.

Liver of Tumor-bearing Rats Male Donryu strain rats were inoculated subcutaneously with 5×10^7 ascites cells. They were killed at the time indicated and their liver was thoroughly

perfused with 0.9% NaCl solution and removed. Samples of 1 g of liver were homogenized in 5 ml of 0.1M Tris-HCl buffer (pH 8.0) containing 5mM 2-mercaptoethanol in a Potter homogenizer. The homogenates were centrifuged at 8,000 x g for 30 min, and the supernatants were used for estimation of enzyme activities. Bioassay of the Factor Affecting Thymidine Kinase Activity and Activities of Other DNA Synthsizing Enzymes in the mouse Liver Samples were injected intraperitoneally into normal ddY mice every 12 hr, and the mice were killed 48 hr after the first injection. The liver was rapidly removed and washed with cold 0.9% NaCl solution.

Whole liver was homogenized in 5 ml of 0.1M Tris-HCl buffer (pH 8.0) containing 5mM 2-mercaptoethanol in a Potter homogenizer. The homogenates were centrifuged at 8,000 x g for 30 min, and the supernatants were used for estimation of thymidine kinase activity. The supernatants were further centrifuged at 105,000 x g for 1 hr, and the resultant supernatants were used for assay of CTP synthetase, dCMP deaminase and DNA polymerase. For assay of dTMP synthase and ribonucleoside diphosphate reductase, three samples of the supernatants obtained after ultracentrifugation were collected and treated with streptomycin sulfate at 1% final concentration and centrifuged at 8,000 x g for 20 min. The resulting supernatants were fractionated with 0-35% ammonium sulfate (ribonucleoside diphosphate reductase) and were centrifuged at 8,000 x g for 20 min. The resultant supernatant were further fractionated with 35-65% ammonium sulfate (dTMP synthase) and were centrifuged at 8,000 x g for 20 min. The pellets obtained

by centrifugation were dissolved in 50mM potassium phosphate buffer (pH 7.0) containing 25mM KCl and 5mM $MgCl_2$ (ribonucleoside diphosphate reductase) or 50mM Tris-HCl buffer (pH 8.0) containing 5mM 2-mercaptoethanol and 1mM EDTA (dTMP synthase), respectively, and dialyzed against the same buffer over night, and used for enzymatic assay. Partial hepatectomy was performed by the procedure of Higgins and Anderson[6].

Assay of Thymidine Kinase Thymidine kinase was assayed as reported previously[20]. The reaction mixture contained, in a final volume of 0.25 ml, 6.25 μmoles of Tris-HCl buffer (pH 8.0), 12.5 nmoles of [2-^{14}C]-thymidine (300,000 cpm), 1.25 μmoles of $MgCl_2$, 1.25 μmoles of ATP, 1.5 μmoles of α-glycerophosphate and 0.2 ml of enzyme solution. After incubation for 30 min at 37°, the reaction was terminated by heating the mixture in a boiling water bath for 3 min and the mixture was centrifuged at 3,000 rpm for 10 min. Then 100 μl of the supernatant was applied to DEAE-cellulose filter paper discs. Thymidine was washed off with 1mM ammonium formate and the radioactivity remaining on the paper was counted in a toluene scintillator [POPOP (0.1 g/liter) and PPO (4.0 g/liter) in toluene] with a Packard liquid scintillation spectrometer.

Assay of CTP Synthetase The assay conditions were essentially as described by Williams et al.[25]. The reaction mixture in total volume of 0.20 ml consisted of 70 mM glycyl-glycine buffer (pH 7.4), 18 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM L-glutamine, 8 mM ATP, 1 mM GTP, 8 mM phosphoenolpyruvate, 10 mM NaF, 0.2 mM

[4-^{14}C] UTP (0.04 μCi/tube) and enzyme solution (0.1 ml). After incubation at 37° for 30 min, the reaction was terminated by heating the mixture in a boiling water bath for 3 min. The amount of CTP formed was determined by converting UTP and CTP to their corresponding nucleosides, i.e., Urd and Cyd, by adding 50 μl of apyrase (20 mg/ml) and 50 μl of alkaline phosphatase (20 mg/ml), and separating the nucleosides on a column of Dowex 50W x 8 (H^+form, 0.5 x 3 cm): ^{14}C-Urd was eluted with 1 mM HCl (5 ml) and ^{14}C-Cyd with 3M lithium hydroxide (LiOH, 3 ml). Then 1 ml of the eluate with 3M LiOH was places in a vial, mixed with the toluene-Triton X-100 scintillator (10 ml) [toluene and Triton X-100 were in the ratio 2:1, containing POPOP (0.1 g/liter) and PPO (4.0 g/liter) in toluene] and 60% perchloric acid (0.3 ml) and kept at room temperature for at least 1 hr, and then the radioactivity was counted in a scintillation counter.

Assay of Ribonucleoside Diphosphate Reductase Assay of ribonucleoside diphosphate reductase activity was based on the principle described by Steeper and Steuart[21]. The reaction mixture, consisting of 10 mM DTT, 4 mM ATP, 1.3 mM $FeCl_3$, 5 mM NaF, 3 mM magnesium acetate, 50 mM potassium phosphate buffer (pH 7.0), 0.125 mM [(U)-^{14}C] CDP (0.4 μCi/tube) and 0.1 ml of enzyme solution in a total volume of 0.3 ml, was incubated at 37° for 60 min. The reaction was terminated by heating the mixture in a boiling water bath for 3 min. Then 50 μl each of 4 mM dCyd, apyrase solution (20 mg/ml) and alkaline phosphatase solution (20 mg/ml) were added successively and incubation was continued for 60 min. The solution was then boiled at 100° for 3 min and centrifuged at 3,000 rpm for 10 min. A sample of 250 μl of the

supernatant was loaded onto a 0.5 x 3 cm Dowex 1 x 4 (borate form) column. [^{14}C]dCyd was eluted with 3 ml of water, and the radioactivity of 1 ml of the eluate was counted as described above.

Assay of dCMP Deaminase dCMP deaminase was assayed by a slight modification of the method of Maley and Maley[11]. The reaction mixture in a final volume of 0.25 ml consisted of 20 mM potassium phosphate buffer (pH 7.0), 2 mM $MgCl_2$, 20 mM NaF, 0.05 mM dCMP, 2 mM [5-^{3}H]dCMP (1 μCi/tube) and the enzyme solution (0.20 ml). After incubation for 30 min at 37°, the reaction was terminated by heating the mixture in a boiling water bath for 3 min. Then 50 μl of apyrase solution (20 mg/ml) and 50 μl of alkaline phosphatase solution (20 mg/ml) were added and the mixtures were incubated further for 60 min at 37°. Then the reaction was stopped by heating and the mixture was centrifuged at 3,000 rpm for 10 min. The dUrd and Ura in the supernatant (0.2 ml) were separated from dCyd on a column of Dowex 50 x 8 [H^+form] (0.5 x 3 cm) eluted with 0.1M HCl (2 ml). A sample of 1 ml of the eluate containing dUrd and Ura was mixed with scintillator (10 ml) and its radioactivity was counted as described above.

Assay of Thymidylate Synthase The assay method was essentially as described by Dunlap *et al.*[5]. The assay mixture in a total volume of 0.5 ml consisted of 0.1M potassium phosphate buffer (pH 7.0), 0.2 mM dl,L-5,10-methylenetetrahydrofolate, 5 mM NaF, 5 mM sodium hydrogen carboxide, 7 mM formaldehyde, 25 mM 2-mercaptoethanol, 0.1 mM [5-^{3}H]dUMP (0.16 μCi/tube) and enzyme solution. A solution of dl,L-5,10-methylenetetrahydrofolate

was prepared under a nitrogen atmosphere just before the assay. After incubation for 15 min at 37° under nitrogen, the reaction was terminated by adding 10% perchloric acid (0.1 ml). The mixture was centrifuged (3,000 rpm, 10 min), 0.2 ml of charcoal suspension in water (80 mg/ml) was added and the mixture was recentrifuged (3,000 rpm, 10 min). Then 0.2 ml of the supernatant containing released tritiated water was mixed with scintillator (10 ml) and its radioactivity was measured as described above.

Assay of DNA polymerase The reaction mixture in a final volume of 25 µl consisted of 50 mM Tris-HCl (pH 7.9), 6 mM magnesium acetate, 1 mM DTT, 16% glycerol, calf serum albumin (10 µg), calf thymus activated DNA (10 µg), 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM [Methyl-^{3}H] dTTP (5 µCi/tube) and enzyme solution[12]. After 30 min incubation at 37°, the reaction was stopped by cooling in an ice bath, and the whole reaction mixture was applied to Whatman DEAE-cellulose (DE81) filter paper discs. Free dNTPs were washed off with 5% aqueous dipotassium hydrogen phosphate and the radioactivity remaining on the paper was counted in a toluene scintillator system as described above.

Protein Assay Protein was determined by the method of Lowry et al.[10] using bovine serum albumin as standard.

Purification Methods Unless otherwise indicated, all procedures were performed at 0-5°. Frozen Yoshida sarcoma ascites tumor cells (80 g) were homogenized with a Potter homogenizer in 180 ml of 50 mM Tris-HCl buffer (pH 8.0). The homogenate

was sonicated (60 W for 2 min) and then centrifuged at 105,000 x g for 1 hr. The resulting supernatant was slowly mixed with solid ammonium sulfate to give 60% saturation. The mixture was stirred for an additional 1 hr, stood for 1 hr, and then centrifuged at 10,000 x g for 20 min. The precipitate was suspended in 50 ml of 50 mM Tris-HCl buffer (pH 8.0), and dialyzed against the same buffer with frequent changes of the buffer. The supernatant obtained after addition of 60% saturation of ammonium sulfate was also dialyzed against the same buffer.

DEAE-cellulose Chromatography The ammonium sulfate fraction (0-60%) was applied to a DEAE-cellulose column (3.5 x 37 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was washed with 10 volumes of the same buffer at a flow rate of about 100 ml/hr, and then eluted stepwise. Most of TSF activity was eluted with 0.2M NaCl in 50 mM Tris-HCl buffer (pH 8.0). This fraction was dialyzed against 50 mM Tris-HCl buffer (pH 8.0), and then lyophilized and stored at -20° until used.

Sephadex G-200 Gel Filtration The lyophilized preparation of TSF from the DEAE-cellulose column (250 mg protein) was dissolved in 5 ml of 50mM Tris-HCl buffer (pH 8.0) and applied to a Sephadex G-200 column (2.7 x 52 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was then eluted with the same buffer at a flow rate of 30 ml/hr.

Isoelectric Focusing The fraction containing TSF from the

Sephadex G-200 column was subjected to isoelectric focusing as described by Sedwick et al.[16] using on LKB column (110 ml). A mixture of 40 ml of Fraction I, and 0.5 ml of 40% carrier ampholyte was introduced into a 0-50% sucrose gradient containing a gradient of 0.5-1.5% carrier ampholyte. Isoelectric focusing was carried out at 450 V for 2 days at 0°, and then the pH value of each fraction (2 ml) was measured at 4° with a Hitachi-Horiba pH-meter.

RESULTS

Effect of Actinomycin-D on Thymidine Kinase Activity in the Liver of Tumor-bearing Rats We reported previously that incorporation of thymidine into DNA increased in the liver and spleen of rats bearing Yoshida sarcoma or AH-130, and that the thymidine kinase and DNA polymerase activities were also increased in the liver and spleen of these rats. Liver thymidine kinase decreased after removal of the Yoshida sarcoma[20]. The mechanism of increase of thymidine kinase activity on implantation of the tumor was studies using actinomycin-D, a potent inhibitor of mRNA synthesis. As shown in Fig. 1, a single injection of actinomycin-D at this dose did not affect the enzyme activity in the tumor, but reduced the enzyme levels in the liver by 70 and 100% after 5 and 12 hr, respectively. These results suggest the existence in tumor cells of some substance that can stimulate thymidine kinase activity in the liver. Therefore, attempts were made to isolate this substance from Yoshida sarcoma cells.

Effect of Injection (ip) of Cell-free Preparations from Yoshida Sarcoma and Normal Rat Liver on Thymidine Kinase Activity in the Liver of Normal Mice A 30% homogenate of Yoshida sarcoma in 50 mM Tris-HCl buffer (pH 8.0) was sonicated (60 W for 2 min) and then centrifuged at 105,000 x g for 1 hr, and the effect of the resultant supernatant on liver thymidine kinase was compared with that of a similar preparation from normal liver. As shown in Table I, liver thymidine kinase activity of normal mice was

increased by intraperitoneal injection of the supernatant from Yoshida sarcoma, but not affected by injection of the supernatant from Normal rat liver or Tris-HCl buffer alone. Thymidine kinase activity in the liver of normal mice injected with homogenate from normal rat liver decreased more than that of control mice injected with Tris-HCl buffer. This difference may be because normal adult rat liver contains a growth inhibitor[9]. The active substance in Yoshida sarcoma was designated as the "liver thymidine kinase stimulating factor (TSF)".

Purification of Liver Thymidine Kinase Stimulating Factor (TSF)

As shown in Table I, intraperitoneal injection of a crude extract of Yoshida sarcoma into mice increased their liver thymidine kinase activity. Therefore, we attempted to purify the factor responsible for this increase from Yoshida sarcoma. Table II, shows the results of ammonium sulfate fractionation of TSF. As shown in Table II, TSF was precipitated with 60% saturation of ammonium sulfate. The ammonium sulfate fraction was subjected to the DEAE-cellulose chromatography and gel filtration according to "Materials and Methods". Fig. 2 shows the elution pattern of protein and the liver thymidine kinase stimulating factor (TSF) obtained from chromatography on DEAE-cellulose. TSF eluted with 0.2M NaCl in 50 mM Tris-HCl buffer (pH 8.0). TSF from DEAE-cellulose was applied to Sephadex G-200 column. Fig. 3 shows the results obtained from gel filtration. As shown in Fig. 3, TSF was found in the void volume on gel filtration. The results at each step of purification are summarized in Table III. One

unit of TSF is defined as that quantity inducing an increase in specific activity of liver thymidine kinase of 0.1 nmol/mg protein. Fig. 4 shows the dose-response curve of TSF in DEAE-cellulose fraction. The increase of thymidine kinase activity was linear up to 10 mg proteins.

Characteristics of TSF As shown in Table IV, TSF was inactivated by heating at 100° for 5 min. This property indicated that it is probably a thermolabile protein. Addition of TSF to the reaction mixture for assay of thymidine kinase did not affect the activity. The isoelectric point of TSF was about 6.0 (Fig. 5).

Increase in Liver Thymidine Kinase Activity in Mice after Injection of Partially Purified TSF A single injection of a crude extract of tumor containing TSF did not affect the liver thymidine kinase activity of normal mice. However, a single injection of partially purified TSF from the DEAE-cellulose column increased their liver thymidine kinase activity. As shown in Fig. 6, the increase of thymidine kinase activity in the liver of mice was detectable 36 to 40 hr after a single injection of this fraction. This increase in enzyme activity was inhibited by simultaneous injection of actinomycin-D (Table V).

Effect of TSF on Thymidine Kinase Activity during Liver Generation TSF also increased thymidine kinase activity during liver generation. As shown in Fig. 7, when TSF was injected intraperitoneally into normal rats 12 hr before

partial hepatectomy, their liver thymidine kinase activity increased slightly by 19 hr after partial hepatectomy and became 2 to 3 times the level of the control group at 24 hr after partial hepatectomy. Injection of bovine serum albumin or Tris-HCl buffer did not cause similar stimulation of thymidine kinase activity.

Effect of TSF on Activities of Other DNA Synthesizing Enzymes in the Liver of Normal Mice Activities of CTP synthetase ribonucleoside diphosphate reductase, dCMP deaminase, dTMP synthase and DNA polymerase were assayed simultaneously with thymidine kinase. As shown in Table VI, activities of CTP synthetase and dCMP deaminase increased about 2.4 times compared with the values from control experiments. Activities of dTMP synthase and DNA polymerase increased about 1.2-1.3 times compared with the values from control experiments. The activity of ribonucleoside diphosphate reductase were detected in the samples from mice injected TSF, though the activity of this enzyme in the control group were not detected.

DISCUSSION

Studies on the effects of tumors on the metabolism of host tissues are of particular importance in understanding the deleterious effects of tumors on the host. Several investigators have reported that the presence of tumors in the body markedly affected the metabolism of host tissues[1, 2, 3, 4, 7, 13, 16, 22, 23, 24].

Previously we found[20] that the activities of thymidine kinase, TMP kinase and DNA polymerase in the liver of tumor-bearing rats increased markedly during tumor growth, and that removal of the tumors resulted in rapid decrease of liver thymidine kinase activity. We also found that injection of actinomycin-D into tumor-bearing rats caused a rapid decrease of liver thymidine kinase activity. These results suggest that the change in enzyme activity in the host tissues may be caused by some substance released from the tumor cells, the results on the effect of actinomycin-D on thymidine kinase suggest that this substance stimulates de novo synthesis of thymidine kinase in tumor-bearing rat liver. In the present study, we partially purified this factor (TSF) by ammonium sulfate precipitation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. The purified TSF was recovered in 120% yield and its specific activity was about 55 times that of the high speed supernatant. At ammonium sulfate fraction, yield increased remarkably. Although this reason is not clear, there is a possibility that at this step some factor which inhibited the increase of liver thymidine kinase separated from TSF. We showed that TSF was eluted in the void volume on gel filtration

on a Sephadex G-200 column and that its isoelectric point was about pH 6.0. These results indicate that TSF is an acidic high molecular weight protein. As shown in Table V, the increase of liver thymidine kinase activity of mice after injection of TSF was inhibited by treatment with actinomycin-D, suggesting that TSF increases de novo synthesis of thymidine kinase in the liver. It seems that the increase of liver thymidine kinase of tumor-bearing rats that we observed previously[20] was a similar phenomenon to the increase of liver thymidine kinase in mice after injection of TSF. Activities of other DNA synthesizing enzymes also increased by injection of TSF. This result suggests that TSF stimulates DNA synthesis in the liver.

LaBrecque et al.[8] reported a factor in weanling rat liver that stimulated DNA synthesis during hepatic regeneration. They found that extracts of weanling rat liver contained a higher level of the hepatic regenerative stimulator substance (SS) than extracts of adult liver. However this substance (SS) probably differs from TSF because it was stable on heat-treatment at 100° for 15 min and its molecular weight was 10,000, whereas TSF was heat-labile and had a higher molecular weight.

Several investigators[14, 15, 17, 18] have reported the existence of a growth-stimulating substance in tumor cells. In most of these studies, the activity of the growth stimulating factor has been assayed on cultured normal cells. Although

it is impossible to compare these factors and TSF because of the difference of the assay system, the relation of these factors may be clear, since we intend to purify TSF using the cell culture system. Further purification and characterization of TSF are in progress.

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Table 1. Effect of Injection of Cell-Free Preparations from Yoshida Sarcoma and Normal Rat Liver on Liver Thymidine Kinase Activity of Normal Mice

Material injected	Thymidine kinase activity (nmol/mg protein)
50 mM Tris-HCl buffer (pH 8.0) (control)	0.161 ± 0.009
Normal rat liver extract (20 mg protein/mouse)	0.117 ± 0.018
Yoshida sarcoma extract (20 mg protein/mouse)	0.228 ± 0.081

Values are means ± S.D. for 5 mice.

Table II. Effect of Injection of Ammonium Sulfate Fractions of Yoshida Sarcoma on Liver Thymidine Kinase Activity of Normal Mice

Ammonium sulfate fraction	Thymidine kinase activity (nmol/mg protein)
50 mM Tris-HCl buffer (pH 8.0) (control)	0.16 ± 0.02
0-60% Ammonium sulfate precipitate	0.44 ± 0.10
0-60% Ammonium sulfate supernatant	0.23 ± 0.06

The ammonium sulfate fractions indicated (5 mg protein) were injected (ip) into mice. Values are means ± S.D. for 5 mice.

Table III. A Summary of Purification of TSF from Yoshida Sarcoma

Fractions	Protein (g)	Specific activity (unit/mg protein)	Purification	Total activity
105,000 x g Supernatant	8.14	0.047	1	382.6
0-60% Ammonium sulfate fraction	5.23	0.560	11.9	2928.8
DEAE-cellulose	1.15	0.860	18.3	989.0
Sephadex G-200	0.18	2.573	54.7	462.6

Each fraction was injected (ip) into mice and activity was assayed as described in "Materials and Methods".

Table IV. Characterization of TSF

Sample	Thymidine kinase activity (nmol/mg protein)
50 mM Tris-HCl buffer (pH 8.0) (control)	0.15 ± 0.08
Untreated TSF	0.99 ± 0.18
Heated TSF (100°, 5 min)	0.23 ± 0.08

The effects of heat on TSF were tested on the preparation from DEAE-cellulose. TSF (10 mg protein/ml) was boiled at 100° for 5 min and then centrifuged at 3,000 rpm for 10 min, and the clear supernatant was tested for thymidine kinase stimulating activity. Samples (1 ml) were injected (ip) into mice.
Values are means ± S.D. for 5 mice.

Table V. Effect of Actinomycin-D on Thymidine Kinase Activity in the Liver of Mice after Treatment with TSF

Treatment	Thymidine kinase activity (nmol/mg protein)
50 mM Tris-HCl buffer (pH 8.0) (control)	0.169 ± 0.067
TSF (4 mg protein/mouse)	0.426 ± 0.117
Actinomycin-D (40 μg/kg)	0.104 ± 0.023
TSF (4 mg protein/mouse) + actinomycin-D (40 μg/kg)	0.180 ± 0.076

Actinomycin-D (40 μg/kg) was injected (ip) into mice at the same time as TSF (4 mg protein/mouse), and mice were killed 40 hr later for measurement of thymidine kinase activity. Values are means ± S.D. for 5 mice.

Table VI. Effect of Injection of TSF on Activities of DNA Synthesizing Enzymes in the Liver of Mice

Enzyme	Enzyme Activities		
	control	Treatment with TSF	
dThd kinase	0.10 ± 0.04	0.60 ± 0.20	(nmol/mg protein/30 min)
CTP synthetase	0.12 ± 0.01	0.27 ± 0.09	(nmol/mg protein/hr)
R. Reductase	N. D.	6.43 ± 1.87	(pmol/mg protein/hr)
dCMP deaminase	0.036 ± 0.015	0.085 ± 0.016	(μmol/mg protein/hr)
dTMP synthase	1.65 ± 0.50	1.90 ± 0.38	(nmol/mg protein/hr)
DNA polymerase	4.78 ± 0.60	6.18 ± 1.50	(pmol/mg protein/hr)

N.D.: not detectable

Each enzyme was prepared and assayed as described in "Materials and Methods". Values of dThd kinase, CTP synthetase, dCMP deaminase and DNA polymerase are means ± S.D. for 6 mice. Values of ribonucleoside diphosphate reductase and dTMP synthase are means ± S.D. for 3 samples.

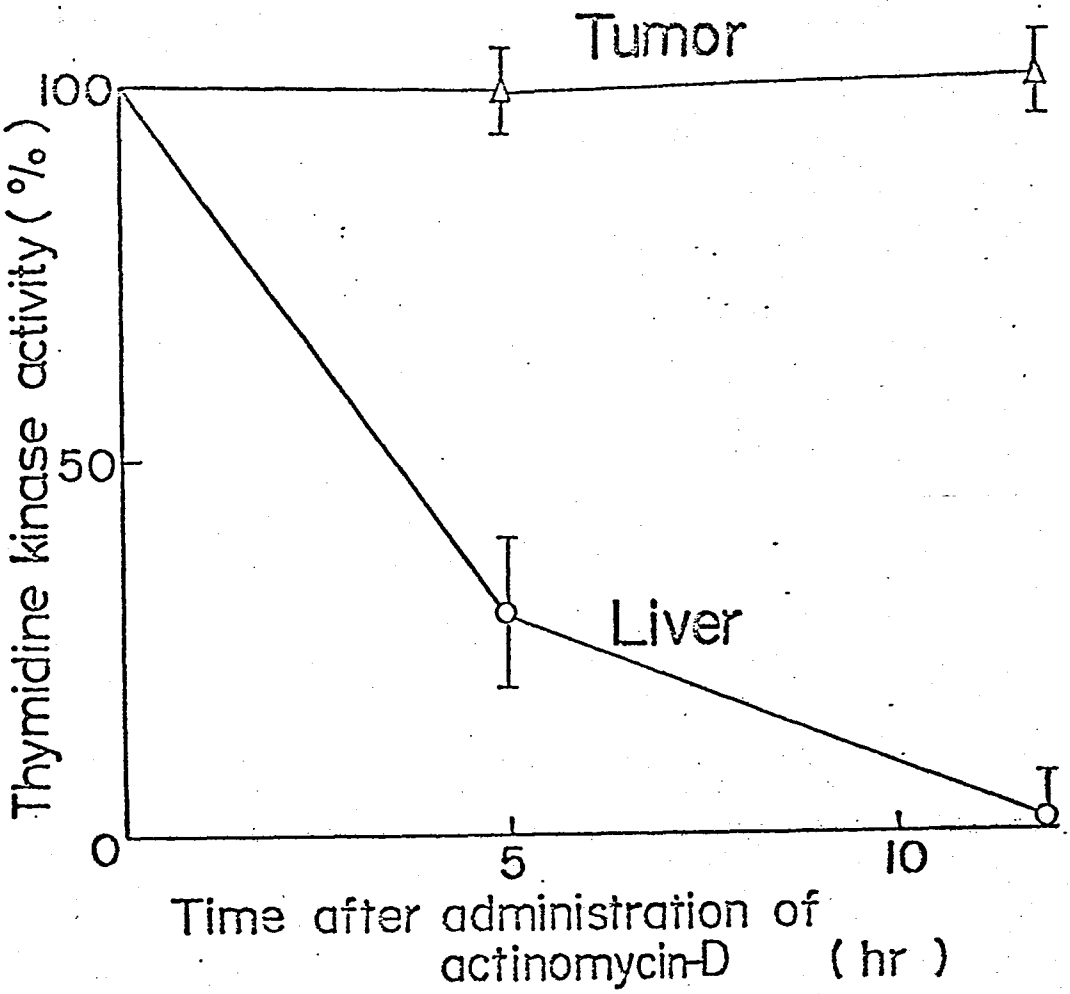

Fig. 1. Effect of intraperitoneal administration of actinomycin-D on liver and tumor thymidine kinase activities of rats bearing Yoshida sarcoma (solid type)

Actinomycin-D (40 μg/kg) was administered 7 days after tumor transplantation and then thymidine kinase activity in the tumor (-Δ-) and liver (-O-) were determined at intervals. The enzyme activity of controls injected with saline was taken as 100%. Points and bars are means and S.E. of values in 8 to 10 animals.

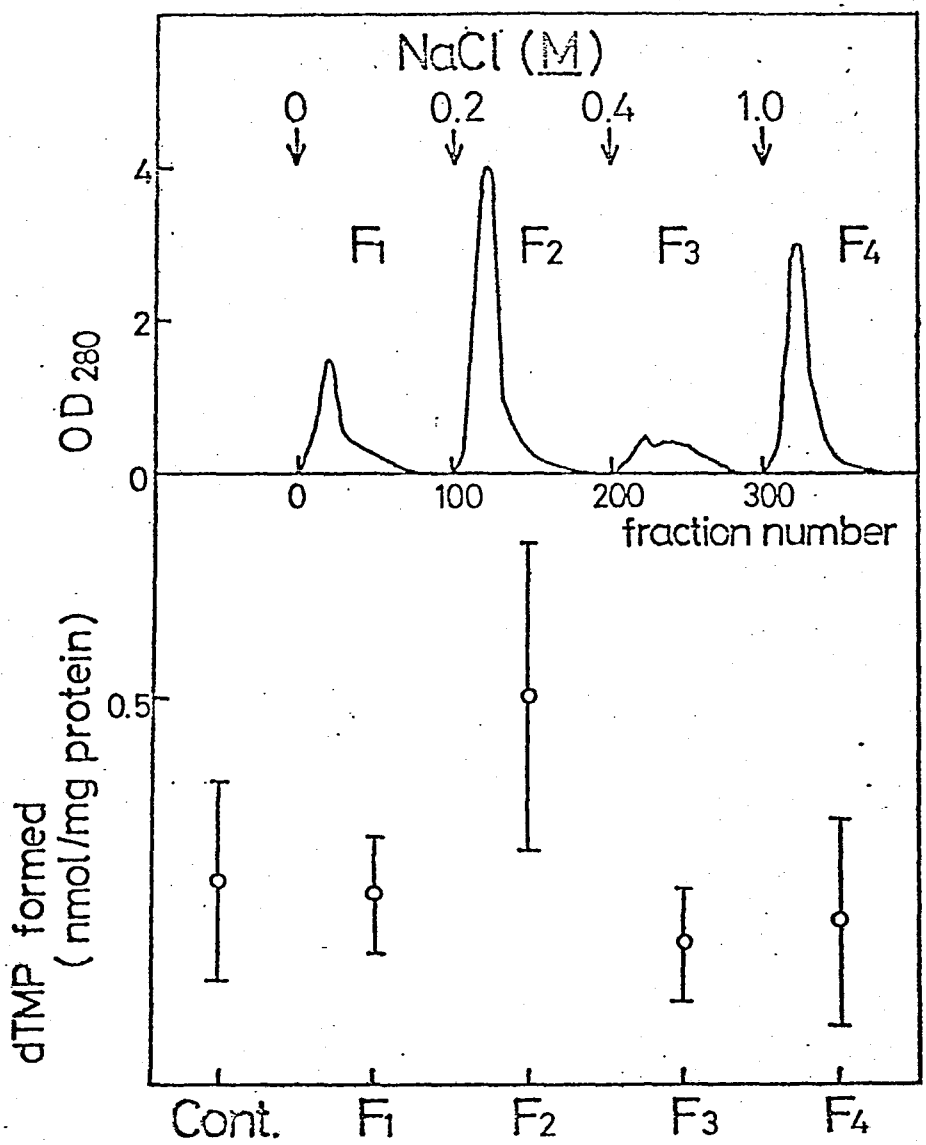

Fig. 2. DEAE-cellulose column chromatography of TSF isolated from Yoshida sarcoma

An aliquot (200 ml) of the ammonium sulfate fraction (0-60%) containing 5 g protein was applied to a DEAE-cellulose column. The column was eluted stepwise with 0.2M, 0.4M and 1.0M NaCl in 50 mM Tris-HCl buffer (pH 8.0). Fractions of 15 ml were collected. Portions of each fraction (5 mg protein) were injected (ip) into mice. The upper panel shows the elution pattern of protein from the column and the lower panel shows the liver thymidine kinase activity of mice. Points and bars are means and S.D. of values in 15 animals.

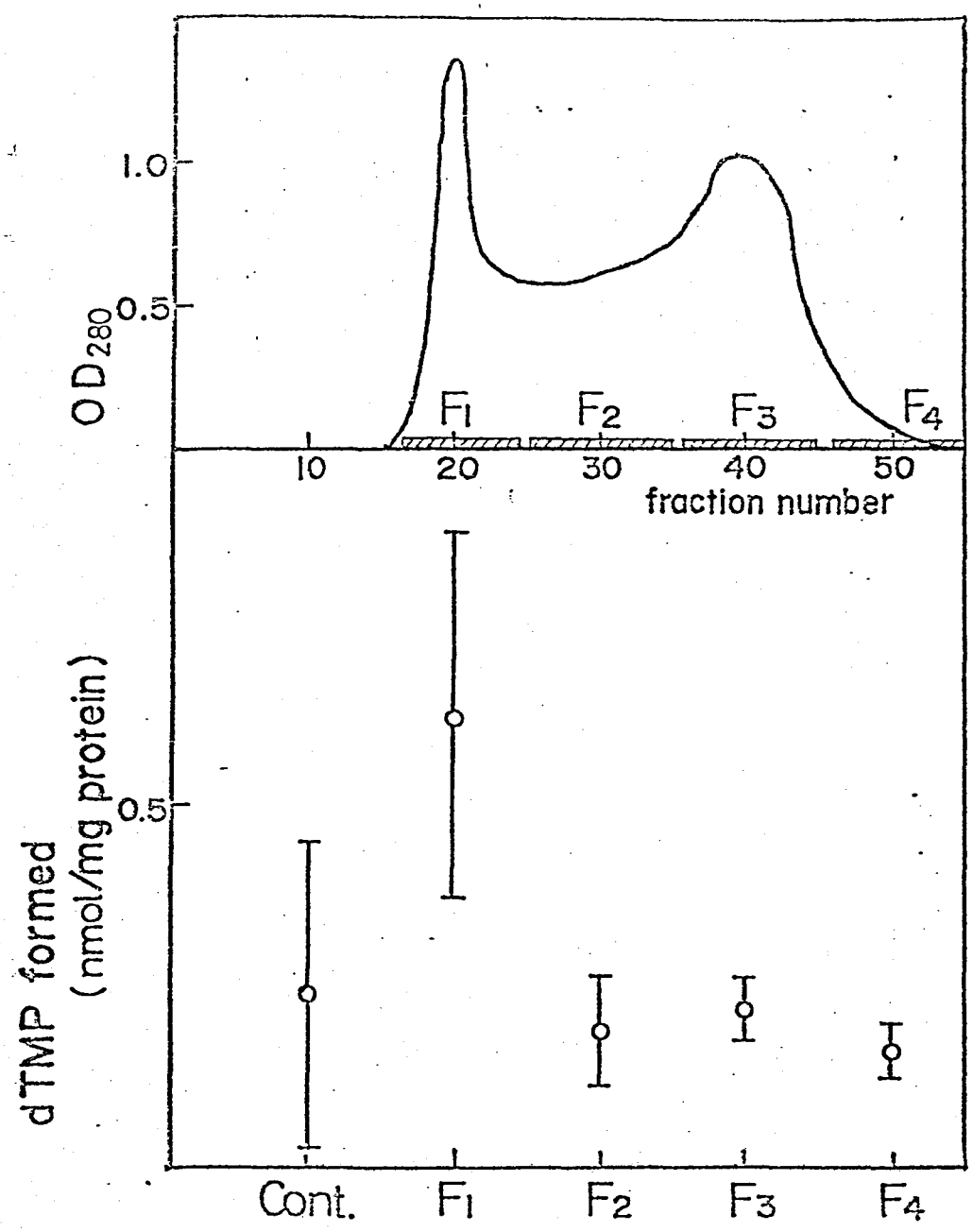

Fig. 3. Gel filtration on Sephadex G-200 of TSF from Yoshida sarcoma

The fraction containing TSF from the DEAE-cellulose column (Fig. 2; Fraction 2) was applied to a Sephadex G-200 column. Fractions of 5 ml were collected and the total eluate was divided to four fractions (F_1-F_4) as shown in the figure. Each fraction (1.5 mg protein) was assayed for stimulating activity. The upper panel shows the elution pattern of protein and the lower panel shows liver thymidine kinase activity. Points and bars are means and S.D. of values in 15 animals.

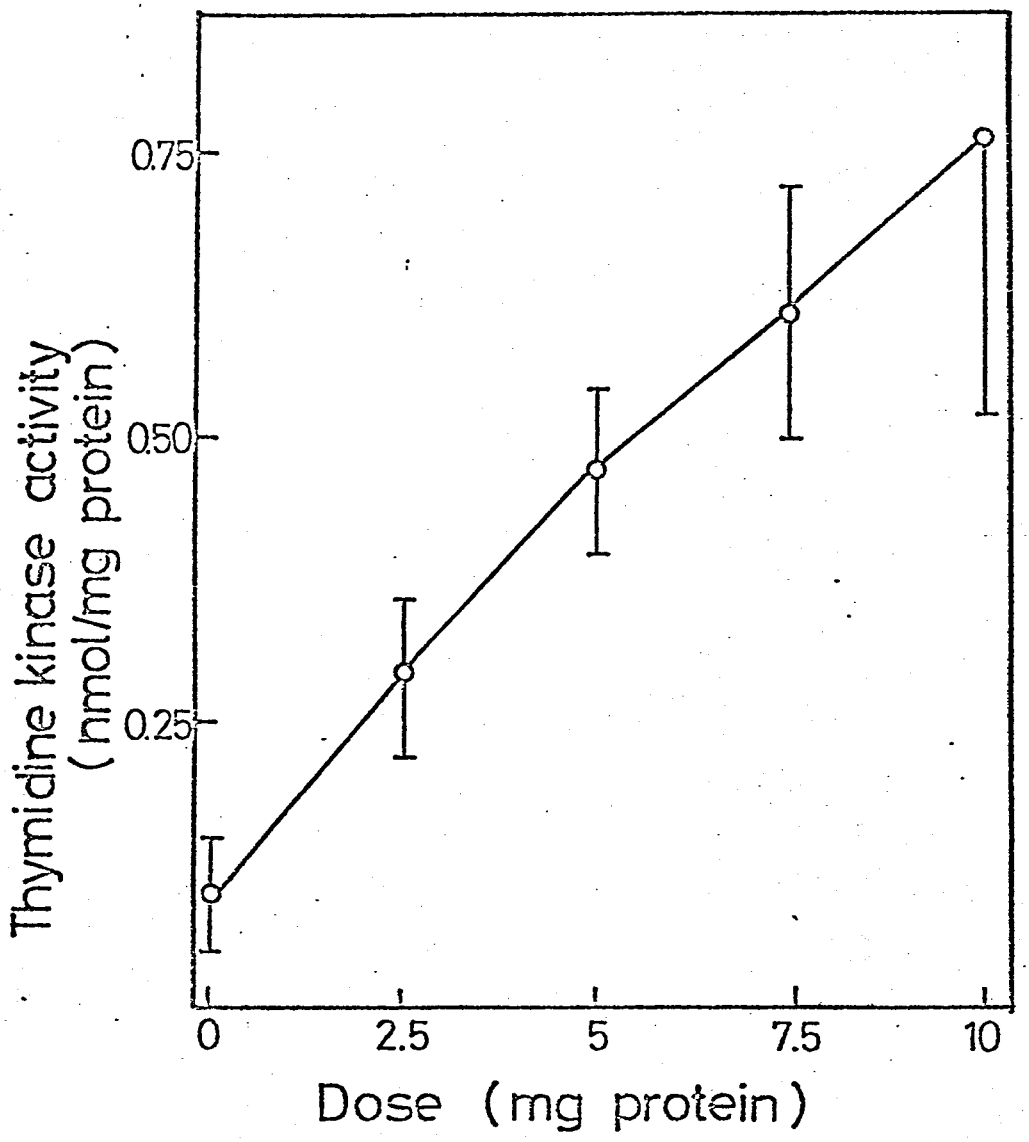

Fig. 4. Dose-response curve of partially purified TSF
Mice were injected (ip) with different dose of TSF (DEAE-cellulose fraction). Points and bars are means and S.D. of values of 5 mice.

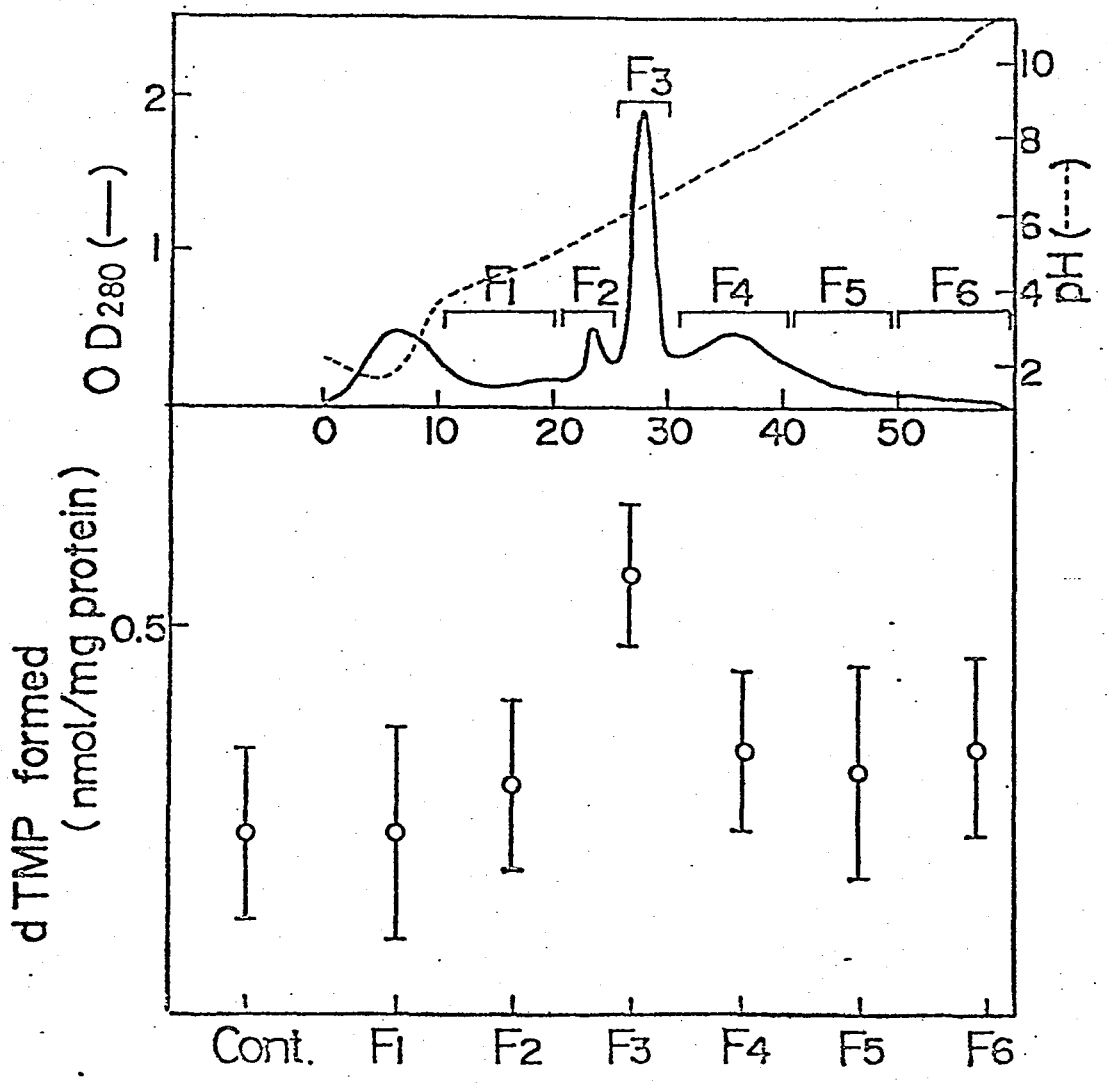

Fig. 5. Isoelectric focusing of TSF isolated from Yoshida sarcoma

The fraction from Sephadex G-200 gel (Fig. 3; Fraction 1) containing 22 mg protein was directly subjected to isoelectric focusing. Fractions of 2 ml were collected and the total eluate was divided to six fractions as shown in the figure. Each fraction was dialyzed against 50 mM Tris-HCl buffer (pH 8.0), and assayed for stimulating activity. The upper panel shows the elution pattern of protein (——) and the pH (---), and the lower panel shows liver thymidine kinase activity of mice. Points and bars are means and S.D. of values in 15 animals.

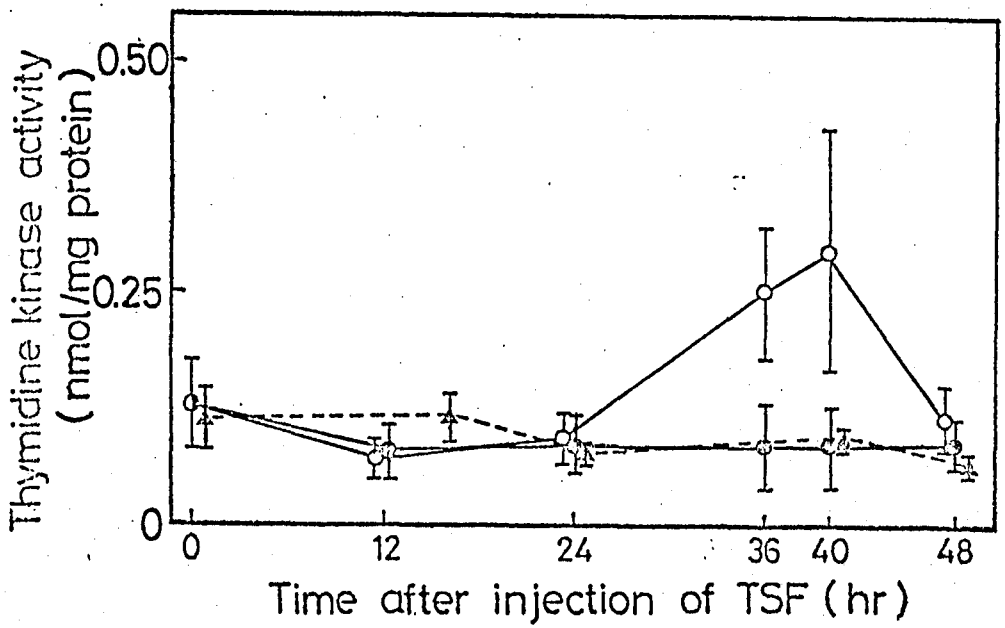

Fig. 6. Increase in thymidine kinase activity in the liver of mice after treatment with partial purified TSF

Mice were injected (ip) with TSF (2 mg protein) (-O-), 50 mM Tris-HCl buffer (pH 8.0) (-▲-) or crude extract from Yoshida sarcoma cells (10 mg protein) (-●-), and thymidine kinase activity in the liver was determined at intervals. The partially purified TSF used was the fraction from DEAE-cellulose, and the crude extract was a homogenate of Yoshida sarcoma cells in 50 mM Tris-HCl buffer (pH 8.0). Points and bars are means and S.D. of values of 5 mice.

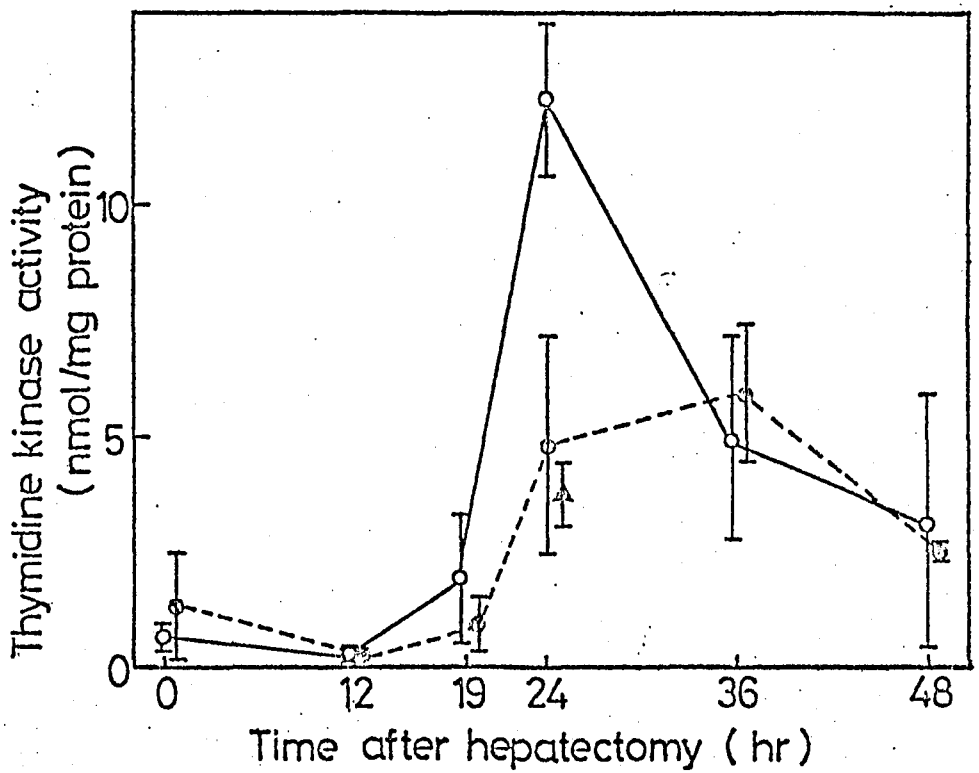

Fig. 7. Effect of TSF on thymidine kinase activity during liver generation

TSF (DEAE-cellulose fraction) (-△-) or bovine serum albumin (-▲-) was injected (ip 20 mg protein) into rats 12 hr before partial hepatectomy. Thymidine kinase activity in the liver was determined at intervals. Control rats (-○-) were treated with 50 mM Tris-HCl buffer (pH 8.0). Samples of 1 g of rat liver were used for assay. Points and bars are means and S.D. of values of 5 rats.

II. GROWTH FACTOR FROM YOSHIDA SARCOMA AND AH-130 TUMOR CELLS

SUMMARY

Suitable conditions for assay of the growth factor from Yoshida sarcoma and AH-130 tumor cells were investigated using three different cell lines: baby hamster kidney (BHK) cells, human embryonic lung (HEL) cells and Swiss Albino mouse 3T3 embryo fibroblasts cells. Results indicated that the 3T3 cell line was more sensitive to the growth stimulating activity than the other two lines. A fraction of Yoshida sarcoma tumor cells containing the growth factor showed less growth stimulating activity, than a fraction of AH-130 tumor cells prepared by the same method. Using the 3T3 cell line for assay of activity, the growth factor was partially purified from AH-130 tumor cells by ammonium sulfate fractionation, DEAE-cellulose column chromatography and gel filtration on a Sepharose-4B column. It appeared to be thermolabile, and its molecular weight was estimated as 900,000 by gel filtration on Sepharose-4B. These findings suggest that it is a high-molecular weight substance.

INTRODUCTION

Growth-stimulating factors capable of initiating proliferation of most animal cells *in vitro* have been obtained from various organs. For instance, EGF (epidermal growth factor)[3] and NGF (nerve growth factor)[14] were obtained from submaxillary gland, FGF (fibroblast growth factor) was found in pituitary and brain[9], a growth factor was isolated from cartilage[13] and ovarian growth factor was isolated from bovine pituitary[10]. In addition, growth-stimulating factors were isolated from conditioned medium of cultured tumor cells[15,19,20] and tumor tissues[8,16]. Furthermore, Todaro *et al.* found an EGF-like growth factor in serum-free medium from transformed cell cultures[6], an IGF (insulin-like growth factor)-like growth factor in serum-free medium of human fibrosarcoma cells[5], and an NGF-like growth factor in human melanoma cells[22]. Nishikawa *et al.*[17] also observed FGF-like activity in an extract of Rhodamine fibrosarcoma.

We previously reported the partial purification from tumor tissues of a factor stimulating the activities of DNA synthesizing enzymes in the liver *in vivo*[11]. The fact that the purifications of growth factors from various tumor tissues have frequently been followed by assays in cell cultures led us to reexamine the conditions for assay of our factor *in vitro*. In this work we examined conditions for assay of the growth factor from Yoshida sarcoma and AH-130 tumor cells using three different cell lines.

MATERIALS AND METHODS

Materials Eagle's minimum essential medium (MEM) and glutamine were purchased from Nissui Seiyaku Co. Dulbecco's modified Eagle's medium (DME) was purchased from Flow Laboratories, Inc. Heat inactivated calf serum was obtained from Nakarai Chemicals. Trypsin was obtained from Miles-Seravac (Pty). Tissue culture flasks (75 cm^2) were from Lux Scientific Corporation. Tissue culture dishes of 10 cm and 3.5 cm diameters were from Falcon and Corning Glass Works, respectively. [^{3}H]-Thymidine was obtained from New England Nuclear, Boston, Mass. Epidermal growth factor (EGF) was from Bethesda Research Laboratories, Inc. Pronase E was from Kaken Chemicals. DEAE-cellulose (DE-23) was from Whatman, and Sepharose-4B from Pharmacia Fine Chemicals. All chemicals used were of the highest commercial grade available.

Cells and Growth Conditions Baby hamster kidney cells (BHK-21/clone 13) and Swiss Albino mouse 3T3 embryo fibroblasts were obtained from Flow Laboratories, Inc. Human embryonic lung cells (HEL) were kindly provided by Prof. Isao Yamane of Tohoku University. BHK cells were grown in 20 ml of MEM containing 10% calf serum and 60 µg/ml of kanamycin in tissue culture flasks. 3T3 cells were grown in 20 ml of DME containing 10% calf serum and 60 µg/ml of kanamycin in tissue culture flasks. When the BHK and 3T3 cells had grown to confluency, they were collected with

0.01% trypsin in Dulbecco and Vogt's phosphate-buffered saline without divalent cations (PBS^-) and inoculated into new tissue culture flasks at a dilution of about one in ten. HEL cells were grown in 10 ml of MEM containing 10% calf serum and 60 μg/ml of kanamycin in 10 cm tissue culture dishes. Confluent HEL cells, obtained after 5 days, were collected with 0.01% trypsin and inoculated into new 10 cm tissue culture dishes at a dilution of one in two. HEL cells were used between passages 10 and 25. All cells was incubated at 37° in a CO_2 incubator with a humidified atmosphere of 5% CO_2 in air.

Cell Counts Cell numbers for inoculation were determined in hemocytometer. For cell numbers in experiments, cells were harvested with 1 ml of a solution of 0.0075% pronase E in PBS^-containing 0.02% EDTA and then 3 ml of 0.9% NaCl solution was added. Cells were counted with a Sysmex microcellcounter CC-108.

Assay of Growth Factor with BHK Cells Confluent cultures grown in 10% serum were treated with 0.01% trypsin solution. Trypsinization was stopped with MEM containing 10% calf serum, and the cell suspension was centrifuged at 1,000 rpm for 3 min. The pellet was resuspended in the same medium and cells were plated at 1-2 x 10^4 cells per 3.5 cm tissue culture dish in 2 ml of MEM containing 10% calf serum. Then 100 μl samples of test fractions were promptly added to the dishes. After 48 hr, the same volume of the same fractions was again

added, and one day later, cells were counted.

Assay of Growth Factor with HEL Cells Cells were plated in 3.5 cm tissue culture dish as described above. After 24 hr, the medium was removed and 2 ml of MEM containing 0.1% calf serum was added. After another 24 hr, the medium was removed and 2 ml of MEM containing 0.1% calf serum and 100 µl of test samples was added. After a furthers 48 hr, the same volume of the samples was again added. Cells were counted at the indicated times.

Assay of Growth Factor with Swiss 3T3 Cells Cells were plated at 2 x 10^4 cells per 3.5 cm tissue culture dish in 2 ml of DME containing 10% calf serum. After 6 hr, the cells were washed once with PBS^-, and 2 ml of DME containing 0.4% calf serum was added. After incubation for 24 hr, 100 µl of samples was added and the cells were incubated for 6 days and then counted. For measurement of incorporation of [^{3}H]-thymidine into DNA, the cells were plated at 20 x 10^4 cells per 3.5 cm tissue culture dish in 2 ml of DME containing 10% calf serum. After 48 hr, the medium was replaced by DME containing 0.5% calf serum and after 24 hr, test samples in 100 µl were added to the cultures. The cultures were incubated for 16 hr and then [^{3}H]-thymidine was added (20 µCi/ml, 100 µl/dish). After incubation for 1 hr, cells were washed twice with PBS and then treated with 10% trichloroacetic acid. Cells were collected with a rubber policeman, washed once with 10% trichloroacetic acid with centrifugation at

3,000 rpm for 5 min. The precipitated cells were mixed with 1 ml of 5% perchloric acid, boiled for 20 min and centrifuged at 3,000 rpm for 5 min. The whole supernatant was mixed with 10 ml of toluene-Triton X-100 scintillator (toluene and Triton X-100 were in the ratio 2:1, containing 1,4-bis[2-(5-phenyl-oxazolyl)]-benzene (0.1 g/liter) and 2,5-diphenyloxazole (4.0 g/liter) in toluene) and radioactivity was measured in a Beckman liquid scintillation spectrometer.

Tumor Cells Yoshida sarcoma cells and AH-130 tumor cells were maintained by intraperitoneal transfer every 7 days and 10 days, respectively, in Donryu strain rats. Tumor cells were washed with 0.9% NaCl solution and frozen at -20° until used.

Animals Male Donryu strain rats of 5 weeks old were obtained from Kitayama LABES Co., Kyoto. They were housed in a constant temperature room with lighting from 8 a.m. to 8 p.m., and were fed on a commercial solid feed MF (Oriental Yeast Co., Tokyo).

Preparation of Growth Factor Tumor cells were homogenized with 3 volumes of 50 mM Tris-HCl buffer (pH 8.0) and sonicated for 2 min. The homogenates were centrifuged at 10,000 x g for 30 min and the supernatants were further centrifuged at 105,000 x g for 60 min. The resulting supernatants were fractionated with 0-60% ammonium sulfate and the pellet obtained by centrifugation at 8,000 x g for 20 min was dissolved in homogenizing buffer and dialyzed against the same buffer with frequent changes of the buffer. The ammonium

sulfate fraction was applied to a DEAE-cellulose column equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Material was eluted with stepwise increasing concentrations of NaCl (0M NaCl, DE-F_1; 0.2M NaCl, DE-F_2; 1.0M NaCl, DE-F_3) in the same buffer and the eluates were collected, lyophilized, dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and stored at -20° until used. Further purification of growth factor from AH-130 tumor cells by DEAE-cellulose column chromatography was carried out with a linear concentration gradient of NaCl. The DE-F_2 fraction from AH-130 tumor cells was purified further on a Sepharose-4B column (2.2 x 50 cm). All samples was sterilized by filtration with a millipore filter (pore size; 0.3 μm).

RESULTS

Effects of DEAE-cellulose Fractions from Tumor Tissues on the Growth of BHK Cells

Fractions of Yoshida sarcoma and AH-130 tumor cells from DEAE-cellulose were added to medium of BHK cell cultures and cells were counted 3 days after the first addition of samples. As shown in Fig. 1, the fractions from Yoshida sarcoma and AH-130 tumor cells that were eluted with 0.2M NaCl from a DEAE-cellulose column ($DE\text{-}F_2$) stimulated growth of BHK cells. Though fractions of Yoshida sarcoma and AH-130 tumor cells eluted with 1.0M NaCl ($DE\text{-}F_3$) also stimulated growth of BHK cells, they were less effective than $DE\text{-}F_2$. Fractions of Yoshida sarcoma and AH-130 tumor cells eluted with 0M NaCl had no detectable effect on cell growth. $DE\text{-}F_2$ from AH-130 tumor cells had more effect on the growth of BHK cells than $DE\text{-}F_2$ from Yoshida sarcoma.

Effects of $DE\text{-}F_2$ Fractions from Tumor Tissues on the Growth of HEL Cells

The effects of the $DE\text{-}F_2$ fractions from Yoshida sarcoma and AH-130 tumor cells on the growth of HEL cells are shown in Fig. 2. Cells were counted 4 days after the first addition of these fractions. As in the case of BHK cells, the $DE\text{-}F_2$ fractions from Yoshida sarcoma and AH-130 tumor cells both stimulated the growth of HEL cells, and $DE\text{-}F_2$ from AH-130 tumor cells was more effective than $DE\text{-}F_2$ from Yoshida sarcoma.

EGF is known to be a potent mitogen for cultured human fibroblasts[12]. So we compared its growth stimulating effect on HEL cells with that of the growth factor from AH-130 tumor cells. As shown in Fig. 2, the growth stimulating activity of EGF was slightly higher than that of DE-F_2 from AH-130 tumor cells.

Effects of DE-F_2 Fractions from Tumor Tissues on the Growth of 3T3 Cells

The effects of the DE-F_2 fractions from Yoshida sarcoma and AH-130 tumor cells on the growth of 3T3 cells were investigated. DE-F_2 fractions from Yoshida sarcoma and AH-130 tumor cells were added to the medium of 3T3 cell cultures and cells were counted 6 days after addition of the fractions. As shown in Fig. 3, both fractions stimulated growth of 3T3 cells, as in the case of BHK and HEL cells, and DE-F_2 from AH-130 tumor cells was more effective than DE-F_2 from Yoshida sarcoma tumor cells. As shown in Fig. 3, DE-F_2 from AH-130 tumor cells was inactivated by heating at 100° for 1 min, indicating that the growth factor from AH-130 tumor cells is probably a thermolabile substance.

The sensitivities of the three cell lines, BHK, HEL and 3T3 cells, to the growth factor (DE-F_2) from AH-130 tumor cells were compared. As shown in Table 1, the assay system with 3T3 cells was more sensitive to the growth factor (DE-F_2) from AH-130 tumor cells than the systems with BHK and HEL cells.

The time dependency of the effect of DE-F_2 from AH-130 tumor cells on the growth of 3T3 cells was examined. As shown in Fig. 4, DE-F_2 from AH-130 tumor cells increased growth of 3T3 cells to 4 to 5 times that of the control on day 6 or 7 after its addition.

Effects of the DE-F_2 Fraction from AH-130 Tumor Cells on Incorporation of dThd into DNA of 3T3 Cells

The effect of DE-F_2 from AH-130 tumor cells on incorporation of dThd into DNA of 3T3 cells was investigated. Since EGF is known to stimulate incorporation of dThd into DNA in 3T3 cells[1)], its effect was compared with that of the DE-F_2 from AH-130 tumor cells. Table 2 shows that DE-F_2 from AH-130 tumor cells stimulated incorporation of dThd into DNA as well as growth of the cells. The effect of the DE-F_2 from AH-130 tumor cells on incorporation of dThd into DNA was similar to that of EGF, but was less sensitive than that of the assay system with cell numbers. Therefore, further purification of the growth factor from AH-130 tumor cells was carried out by measuring effects of fractions on the cell number of 3T3 cells in the assay system.

Effects of DEAE-cellulose Fractions Eluted with a Linear Gradient of Salt Concentration

The ammonium sulfate fraction from AH-130 tumor cells was applied to a column (2 x 20 cm) of DEAE-cellulose previously equilibrated with 50mM Tris-HCl buffer (pH 8.0).

The column was eluted with a linear concentration gradient formed with 250 ml of 50mM Tris-HCl buffer (pH 8.0) in the mixing chamber and 250 ml of 50mM Tris-HCl buffer (pH 8.0) containing 0.4M NaCl in the reservoir. After dialysis against 50mM Tris-HCl buffer (pH 8.0), samples of each fraction were added to the medium of 3T3 cell cultures, and cell numbers were measured 6 days later. As shown in Fig. 5, the main peak of the growth stimulating activity was eluted at 0.19M NaCl concentration (fraction number 80) and it increased growth of 3T3 cells to 4 to 5 times that of the control culture.

Sepharose-4B Gel Filtration of the DE-F_2 Fraction from AH-130 Tumor Cells

The DE-F_2 fraction of AH-130 tumor cells was subjected to gel filtration on Sepharose-4B to estimate the molecular weight of the growth factor. The Sepharose-4B column (2.2 50 cm) was calibrated with ferritin, (M.W., 540,000) and human γ-globulin (M.W., 160,000). Fractions eluted from the column were added to 3T3 cell cultures, and cell numbers were measured 6 days later. As shown in Fig. 6, the elution volume of the growth factor from AH-130 tumor cells was about 84 ml, indicating that the molecular weight of this factor is about 900,000.

DISCUSSION

Growth-stimulating effects of preparations from various tumor cells have been reported[8,15,16,19,20]. Fenselau and Mello[8] found that growth of endothelial cells of fetal bovine heart was increased by addition of a crude cell homogenate of Walker 256 adenocarcinoma to the culture medium. Nair and DeOme[16] reported that a soluble fraction of a solid spontaneous primary mouse mammary tumor stimulated the growth of density-inhibited mouse embryo cells in monolayer culture, and that the growth-stimulating factor precipitated with 60% saturation of ammonium sulfate was similar to preparations of growth factors from the culture media of Rous sarcoma cells[20] and mouse mammary tumor cells[15,16]. However, further purification of their growth factors obtained from various tumor cells have not been reported. In this work, we used three different cell lines - baby hamster kidney (BHK) cells, human embryonic lung (HEL) cells and Swiss Albino mouse 3T3 embryo fibroblasts (3T3) cells - for the assay and purification of the growth factors from tumor cells. The growth factor from AH-130 tumor cells stimulated the growth of all three cell lines, but it had most effect in the 3T3 cell system. A similar preparation from Yoshida sarcoma cell showed some growth stimulating activity, but less than that of the preparation from AH-130 tumor cells. The growth factor of AH-130 tumor cells from a DEAE-cellulose column with a linear concentration

gradient of NaCl (Fig. 5) seemed to be the same fraction as that eluted stepwise (0.2M NaCl) from the results in Figs. 3 and 5. The fraction of growth factor obtained by gel filtration on a Sepharose-4B column was a high molecular weight substance (M.W., about 900,000) (Fig. 6).

The epidermal growth factor (EGF)[3] and platelet derived growth factor (PDGF)[21] were reported to be heat-stable polypeptides. Recently, Todaro et al. reported that the transforming growth factors produced by certain human tumor cell lines had molecular weights of 20,000-23,000 and were heat-stable polypeptides[24]. The growth factor from AH-130 tumor cells differed from these factors because DE-F_2 from AH-130 tumor cells was inactivated by heating at 100° for 1 min.

Heat-labile growth factors, such as fibroblast growth factor (FGF)[2], nerve growth factor (NGF)[4] and insulin-like growth factor (IGF)[21] have been reported, but the molecular weights were 13,000 for FGF[9], 26,000 for NGF[25] and 7,000 for IGF[18], suggesting that they also differ from the growth factor obtained from AH-130 tumor cells. However, it is possible that the growth factor obtained from AH-130 tumor cells might to be a complex like EGF[23] or NGF[25], and its further purification and characterization are necessary for comparison with these growth factors.

The growth factor obtained from Yoshida sarcoma and AH-130 tumor cells seems similar to the liver dThd kinase

stimulating factor (TSF) described in Part 1 of this paper [11], since intraperitoneal injection of an extract of Yoshida sarcoma into normal mice stimulated their liver dThd kinase, and, like TSF, the growth factor is a high molecular, heat labile substance.

It is important to purify the growth factor and investigate the mechanism of its growth stimulation, to obtain information on how cell growth is controlled.

Further purification of this growth factor using the 3T3 cell system for assay of activity is in progress.

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Table 1. Sensitivities of cell lines to growth stimulating activity of DE-F_2 from AH-130 tumor cells

Cells	% of control
BHK	140 ± 23 (6)[a]
HEL	178 ± 23 (6)
3T3	452 ± 117 (8)

a. Numbers in parentheses show numbers of experiments

Values are means ± S.D.

Table 2. Incorporation of dThd into DNA of 3T3 cells.

Sample	% of control
Control	100 (6)[a]
AH-130 DE-F_2 (200 µg protein/ml)	285 ± 87 (6)
EGF (10 ng/ml)	287 ± 97 (6)

a. Numbers in parentheses are numbers of experiments Samples of 50 µl of AH-130 DE-F_2 or EGF were added to cultures of 3T3 cells and 16 hr later 0.2 µCi/dish of dThd was added. The reaction was stopped 1 hr later. The radioactivity in DNA was measured as described in "Materials and Methods". Values are means ± S.D. for six experiments.

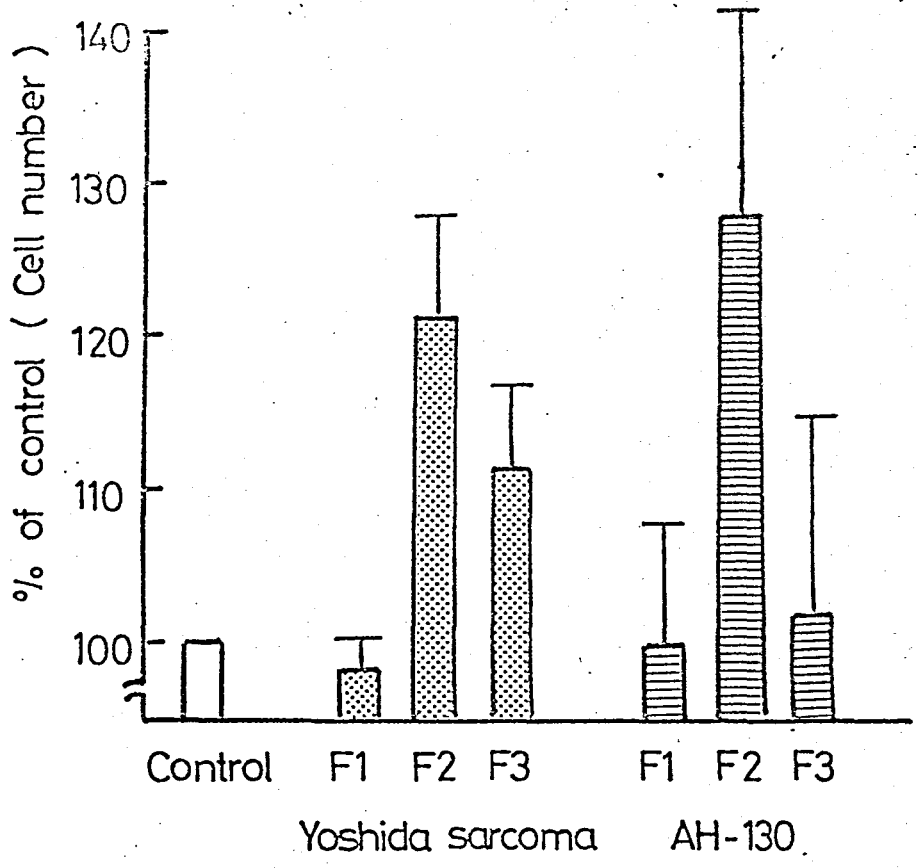

Fig. 1. Effects of DEAE-cellulose fractions from Yoshida sarcoma and AH-130 tumor cells on growth of BHK cells

DEAE-cellulose fractions from Yoshida sarcoma and AH-130 tumor cells eluted with 0M NaCl (F_1), 0.2M NaCl (F_2) and 1.0M NaCl (F_3) in a volume of 100 µl were added at final concentrations of 200 µg protein/ml to BHK cell cultures. 100 µl of 50mM Tris-HCl buffer (pH 8.0) was added to control cultures. Cell numbers were measured as described in "Materials and Methods".

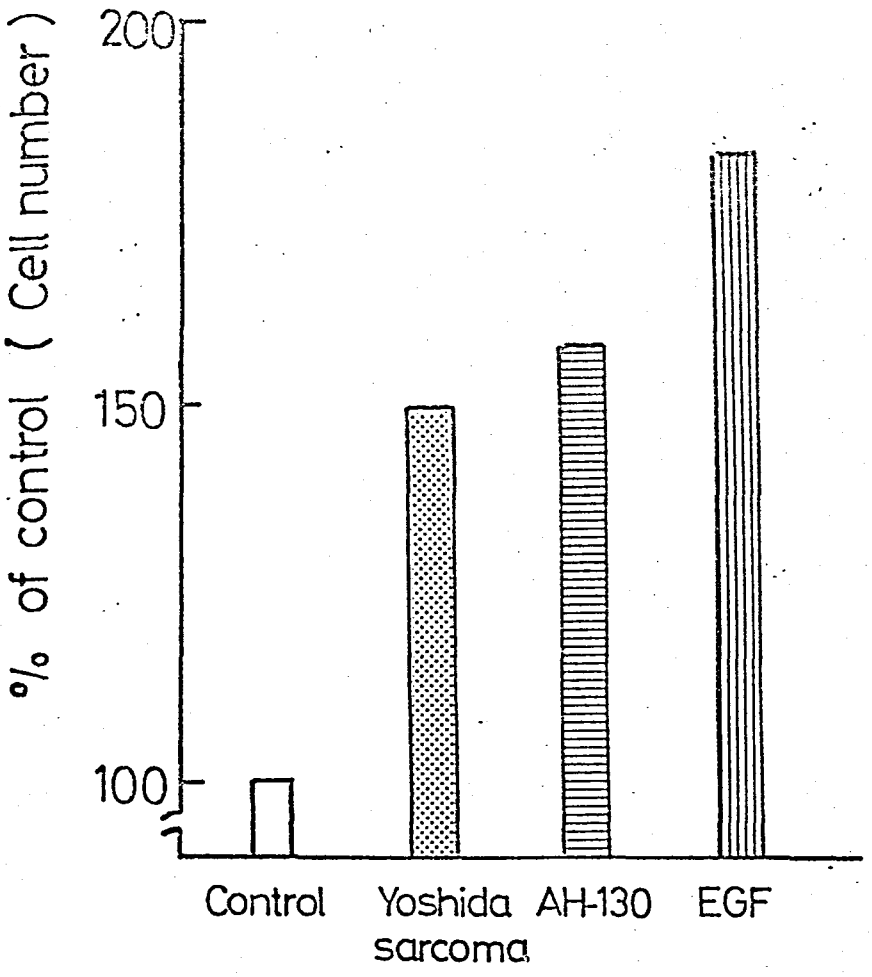

Fig. 2. Effects of EGF and DE-F_2 fractions from Yoshida sarcoma and AH-130 tumor cells on growth of HEL cells

EGF and DE-F_2 fractions of Yoshida sarcoma and AH-130 tumor cells were added to HEL cell cultures. The sample volume was 100 µl. The final concentration of EGF and DE-F_2 fractions was 10 ng/ml and 200 µg protein/ml, respectively, and 100 µl of 50mM Tris-HCl buffer (pH 8.0) was added to control cultures. Cell numbers were measured 4 days after the first addition of fractions.

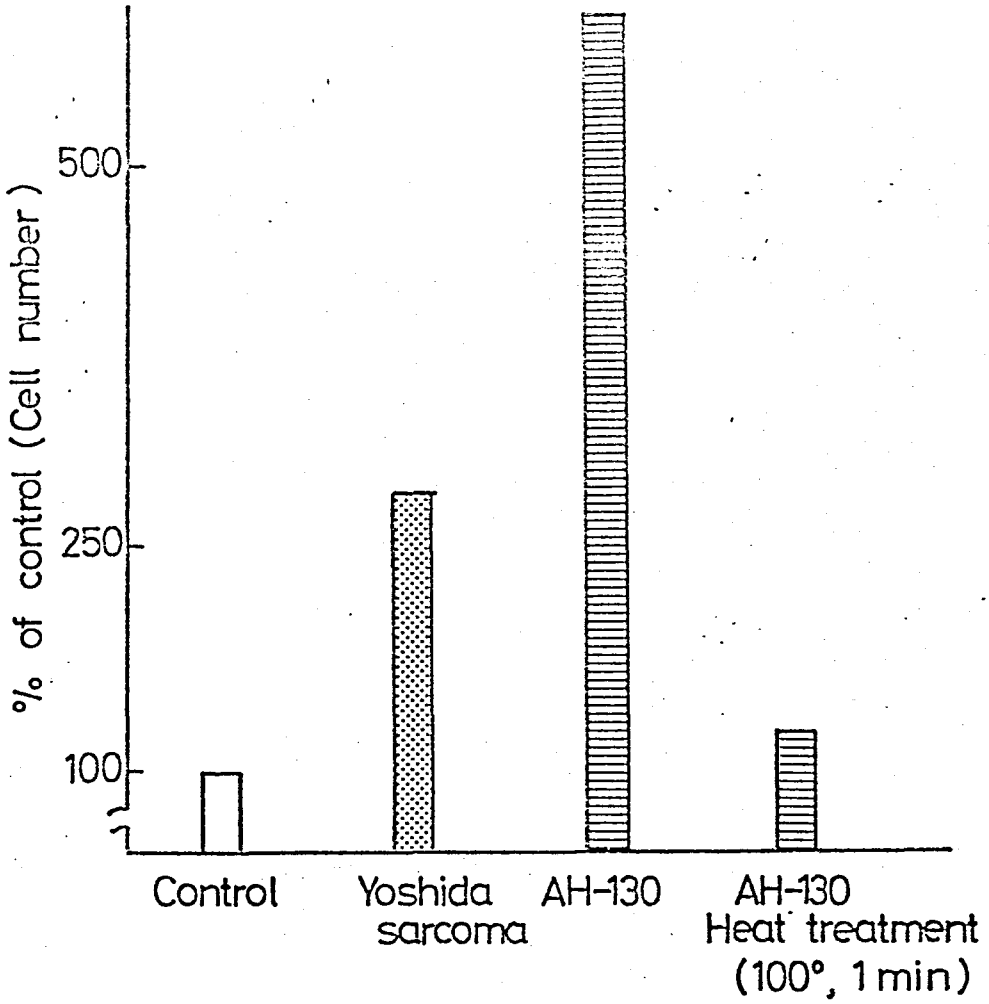

Fig. 3. Effects of DE-F_2 fractions from Yoshida sarcoma and AH-130 tumor cells on growth of 3T3 cells

DE-F_2 fractions of Yoshida sarcoma and AH-130 tumor cells were added in a volume of 100 µl to 3T3 cell cultures at a final concentration of 200 µg protein/ml. The fraction from AH-130 tumor cells was heated at 100° for 1 min and centrifuged at 3,000 rpm for 5 min and the resulting supernatant was used for assay. Cell numbers were measured 6 days after addition of samples as described in "Materials and Methods".

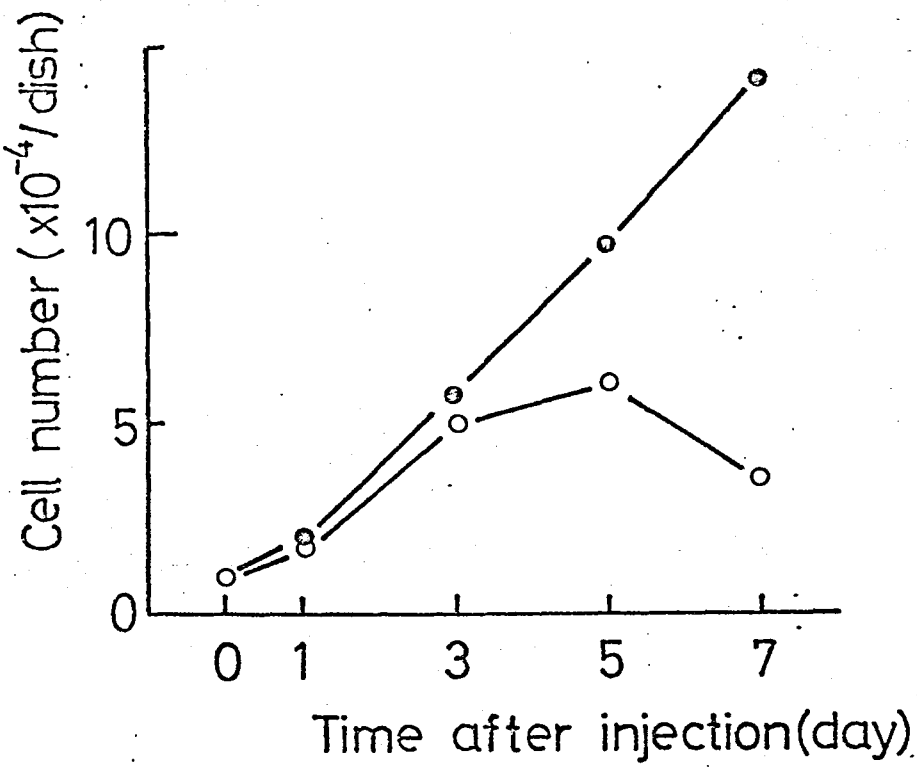

Fig. 4. Growth curve of 3T3 cells in the presence and absence of the DE-F_2 fraction of AH-130 tumor cells

The DE-F_2 fraction of AH-130 tumor cells (-●-) or 50mM Tris-HCl buffer (pH 8.0) (-O-) was added to 3T3 cell cultures on day 0. The volume of samples was 100 µl and their final concentration was 200 µg protein/ml. After addition of the sample, cell numbers were measured at intervals.

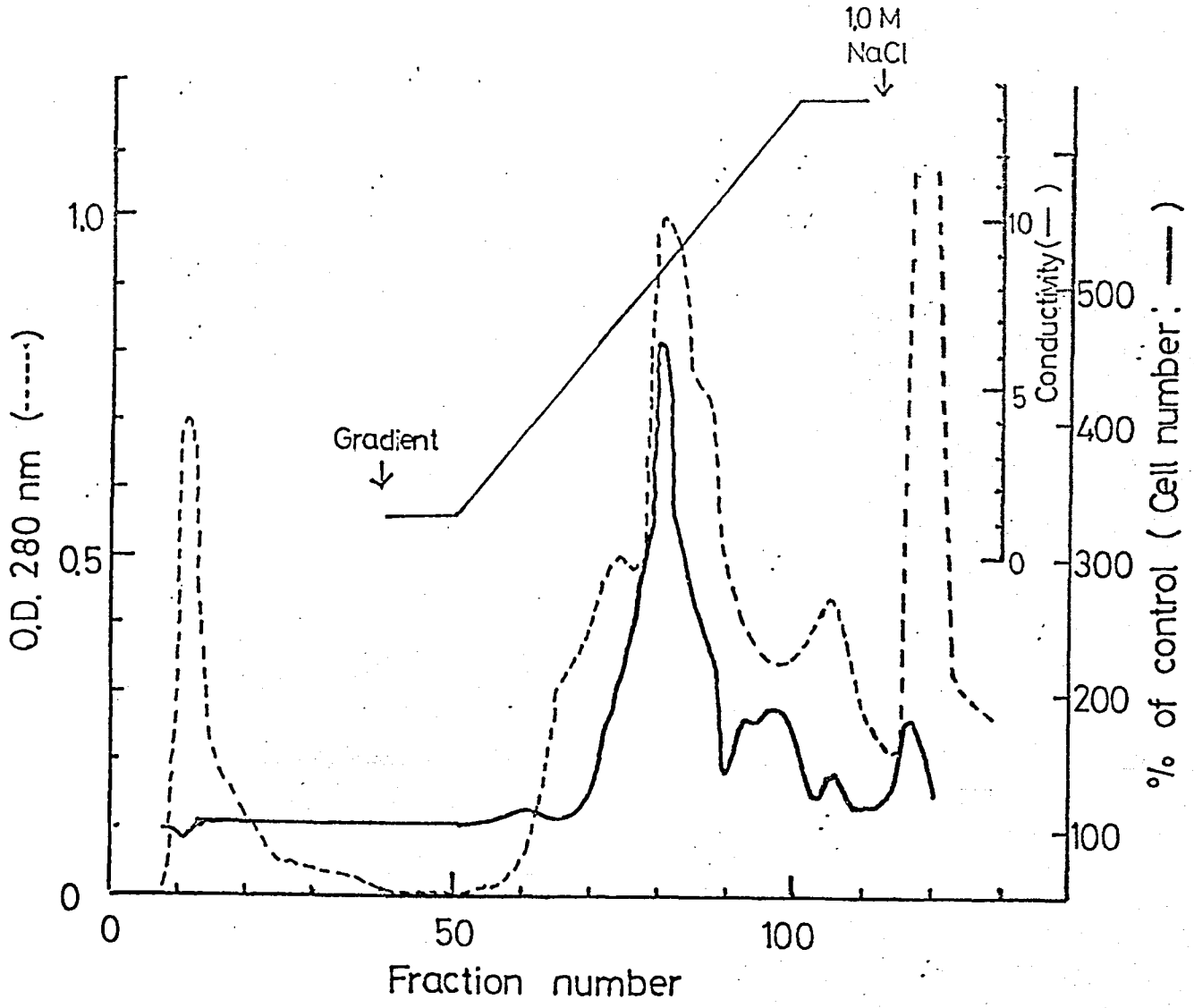

Fig. 5. DEAE-cellulose column chromatography of the growth factor from AH-130 tumor cells

The ammonium sulfate fraction (0-60%) was applied to a DEAE-cellulose column (2 x 20 cm) and material was eluted with a 500 ml linear salt gradient (0 to 0.4M NaCl in 50mM Tris-HCl buffer, pH 8.0). Fractions of 8 ml were collected. Each fraction was dialyzed against 50mM Tris-HCl buffer (pH 8.0) and 100 µl of each fraction was added to 3T3 cell cultures. Cell numbers were measured 6 days after addition of samples as described in "Materials and Methods".

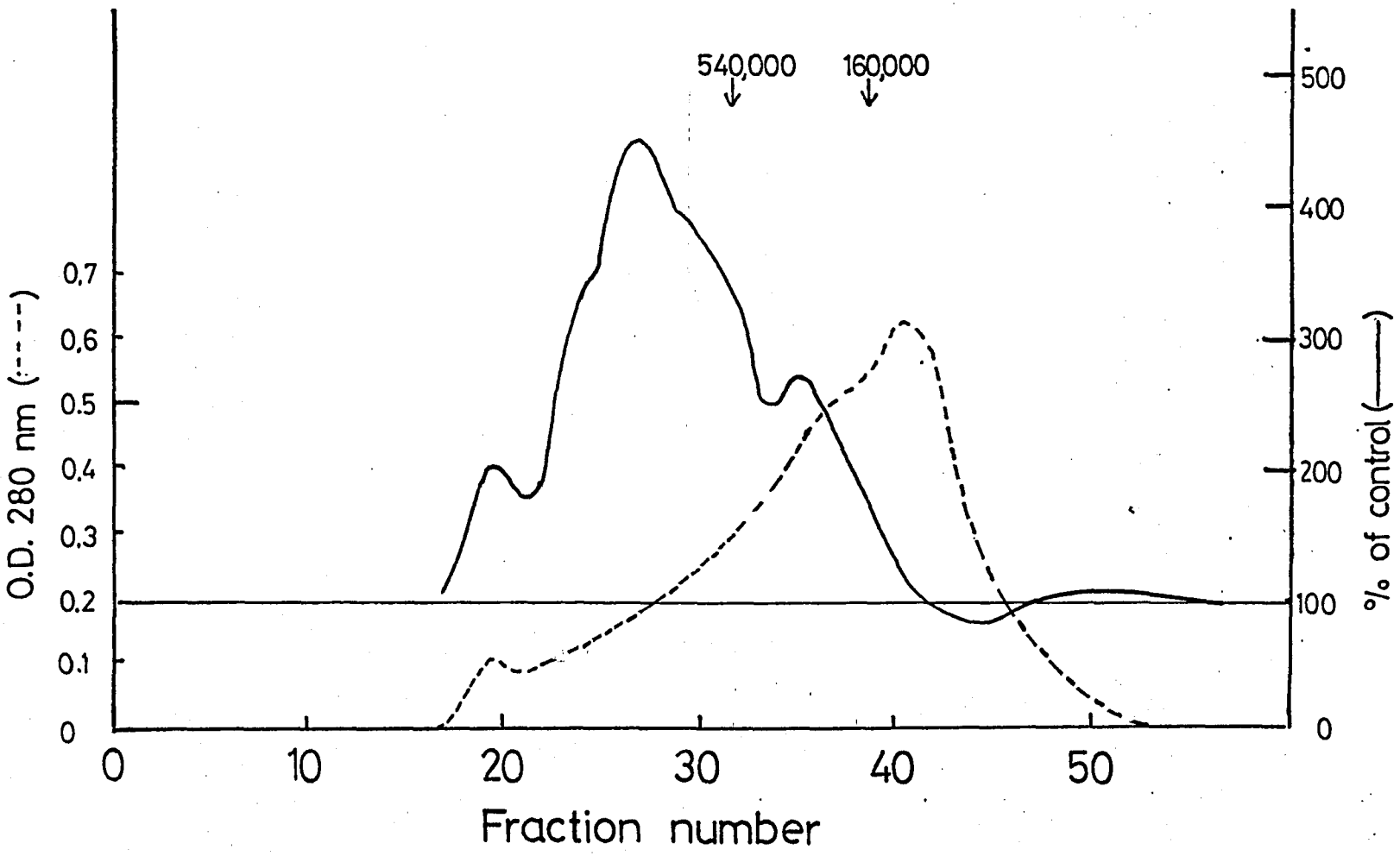

Fig. 6. Sepharose-4B gel filtration of DE-F_2 of AH-130 tumor cells

The DE-F_2 fraction of AH-130 tumor cells was applied to a Sepharose-4B column (2.2 x 50 cm). Material was eluted with 50mM Tris-HCl buffer (pH 8.0). Fractions of 4 ml were collected and 100 µl of each fraction was added to 3T3 cell cultures. Cell numbers were measured as described in "Materials and Methods".

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