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Fumio Takahashi[*] Makoto Kuramitsu[†] Masaaki Tokuda[‡]

Hideki Matsui[**] Toshifumi Itano[††] Tetsu-Hide Murakami[‡‡]

Osamu Hatase[§] Isamu Nishida[¶]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,
[‡‡]Okayama University,
[§]Okayama University,
[¶]Okayama University,

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Fumio Takahashi, Makoto Kuramitsu, Masaaki Tokuda, Hideki Matsui, Toshifumi Itano, Tetsu-Hide Murakami, Osamu Hatase, and Isamu Nishida

Abstract

Cellular stimulating factors on cell proliferation in the supernatants of chick embryo carcases and adult muscles were studied. There were plural stimulating factors in embryonic and adult muscular supernatants that promoted cell proliferation without any supplement of sera and other materials. Salting-out methods with ammonium sulfate, ethanol fractionation, and isoelectric precipitation were used to isolate the stimulating factors, and these three methods proved the presence of plural stimulants on cell proliferation in the supernatants of chick embryo and adult muscles. The stimulants had altered physico-chemical properties and biological activities due to embryological development. The embryonic stimulants enhanced the synthesis of DNA and protein remarkably, and RNA synthesis in whole cell systems slightly. The muscular stimulants enhanced protein synthesis without any stimulation of DNA and RNA synthesis. Partial purification of the stimulants from the ethanol fractions was performed by DEAE-cellulose chromatography and Sephadex gel chromatography.

KEYWORDS: chick growth factors, cell proliferation, growth regulation, DNA and RNA synthesis, protein synthesis

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PARTIAL PURIFICATION AND BIOLOGICAL ACTIVITIES AND PROPERTIES OF CHICK GROWTH FACTORS

Fumio TAKAHASHI[1], Makoto KURAMITSU, Masaaki TOKUDA, Hideki MATSUI, Toshifumi ITANO, Tetsu-Hide MURAKAMI, Osamu HATASE and Isamu NISIDA

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

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Abstract. Cellular stimulating factors on cell proliferation in the supernatants of chick embryo carcases and adult muscles were studied. There were plural stimulating factors in embryonic and adult muscular supernatants that promoted cell proliferation without any supplement of sera and other materials. Salting-out methods with ammonium sulfate, ethanol fractionation, and isoelectric precipitation were used to isolate the stimulating factors, and these three methods proved the presence of plural stimulants on cell proliferation in the supernatants of chick embryo and adult muscles. The stimulants had altered physicochemical properties and biological activities due to embryological development. The embryonic stimulants enhanced the synthesis of DNA and protein remarkably, and RNA synthesis in whole cell systems slightly. The muscular stimulants enhanced protein synthesis without any stimulation of DNA and RNA synthesis. Partial purification of the stimulants from the ethanol fractions was performed by DEAE-cellulose chromatography and Sephadex gel chromatography.

Key words: chick growth factors, cell proliferation, growth regulation, DNA and RNA synthesis, protein synthesis

In early studies of tissue culture, extracts of animal organs or embryos were used as nutrient supplements (1–6). As cellular or organic regulatory factors for cell proliferation, Coon (7, 8) and Cahn (9) independently reported the presence of active components in chick embryo extract which could modulate cell differentiation and growth. However, they did not succeed in purifying the active components from chick extract. As stimulating factors of cell proliferation, a fibroblast growth factor (10), epidermal growth factor (11), and a nerve growth factor (12) were purified from certain organs; we also tried to isolate cell proliferation stimulatory factor from chick embryo carcases and adult muscles and found

1, Present Address: Division of Foods and Nutrition, Sanyo-Gakuen Women's Junior College, 1-14-1 Hirai, Okayama 703, Japan

that there were plural stimulants in their supernatants. The chick embryo stimulants showed about 3 times the active stimulating effect on cell proliferation of that from adult muscles without any supplement, and the muscular stimulants showed different physico-chemical properties from the embryonic ones. Partial purification of the embryonic and muscular stimulants was performed, and their properties were also investigated. Compared with the preceeding investigation of humoral stimulating factors for cell proliferation, the roles and properties of the cellular and organic stimulants are still ambiguous. So it was thought relevant to study the action mechanism of cellular stimulants on cell growth that responds to changes in the humoral information induced by physiological events such as animal growth, partial hepatectomy, administration of chemical agents, and so on.

MATERIALS AND METHODS

Chicken eggs (N-101) were purchased from Fukuda hatchery, Okayama. The embryo carcases (10-day-old) and adult thigh muscles were well washed and homogenized with STE solution consisting of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 1 mM ethylendiaminetetraacetic acid (EDTA). Homogenates were prepared by a Warring blender. After filtration through 4 layers of gauze and 2 layers of tetron cloth the homogenates were centrifuged at 8,000×g for 10 min. The supernatants were centrifuged at 104,000×g for 1 h at 0°-4°. The final supernatants (S_2) from embryo carcasses (Em) and adult muscles (M) were named Sol-EmS_2 and Sol-MS_2, respectively. Sol-S_2 were lyophilized after intensive dialysis, and were named EmS_2 and MS_2.

Ethanol fractionation. Sol-EmS_2 and Sol-MS_2 were slowly saturated up to 50% and 70% with chilled ethanol (−15°C) in the presence of 0.5 mM 2-mercaptoethanol (2-Met) with continuous stirring. The precipitates were centrifuged at 6,000 rpm for 10 min after being left to stand for 1 h. The precipitates at 50% ethanol saturation from Sol-EmS_2 and Sol-MS_2 were named Em 50 and M 50, respectively: the precipitates at 70% saturation were named Em 70 and M 70.

Salting out method. Each Sol-S_2 was slowly saturated by the addition of solid ammonium sulfate up to 30% and 60% in the presence of 0.5 mM 2-Met, and the precipitates were stepwisely centrifuged. The precipitates at 30% ammonium sulfate saturation from Em S_2 and M S_2 were named EmA30 and MA30, respectively; the precipitates at 60% saturation were also named EmA60 and MA60 and unprecipitated fractions were named EmSup and MSup.

Isoelectric precipitating method. The pH of Sol-S_2 was slowly adjusted to pH 4.0 by the addition of 0.5 M H_2PO_4 in the presence of 0.5 mM 2-Met. The precipitates were centrifuged. The pH of the supernatants after centrifugation was re-adjusted to pH 7.0 with 4 M NaOH. The residues were also centrifuged. These fractions which were precipitated at pH 4.0 from Sol-EmS_2 and Sol-MS_2 were named EmpH4 and MpH4. The supernatants after centrifugation of EmpH7 and MpH7 were named Em7Sup and M7Sup. All fractions were in-

tensively dialysed against 1 mM Tris-HCl (pH 7.8) containing 0.5 mM 2-Met and then lyophilized before stocking. These fractions were dissolved with phosphate buffered saline [PBS (−)] and were used for the assay of biological activity after centrifugation at 10,000 rpm for 10 min.

Cells and Assay of cell proliferation. Primary cultured cells of chick embryo (CEF) were prepared from 10-day-old chick embryo carcases of White leghorn strain, N-101, by the method of Vogt (13). The assay procedures were performed as follows: The secondary CEF were seeded in a resting medium (RM) that contained 2% triptose phosphate broth (DIFCO, abbreviated TPB) and Eagle's minimum essential medium (MEM). The assay was started at (48 to 72 h) after seeding by replacing the RM with experimental medium consisting of fresh RM and the sample fractions. The RM of the resting cell system as a control was replaced with fresh RM without the addition of the sample fractions. The number of cells was counted by a Coulter counter after trypsinization (13) after 48 h of culture in a CO_2 gas incubator at 37°C. The biological activity in percentage was culculated by the formula: [(Cell number in RM+Samples)−(Cell number in RM)]÷(Cell number in RM)×100. The standard error of each system was less than 7.5% at each assay point calculated from 3 identical plastic dishes. Treatment of Em 50 in PBS (−) was by 500 nmoles of N-ethylmaleimide (NEM), 10 μg of trypsin, 25 μg of RNase containing 1 mM $MgCl_2$ under 37°C for 2 h. In the case of heat treatment, Em 50 (1 mg/ml) in PBS (−) was heated in a boiling water bath for 1 min and rapidly chilled down. After being centrifuged at 2,000 rpm for 20 min, the supernatant containing a certain concentration of sample was applied to the CEF system. The treatment concentrations of NEM, trypsin, and RNase are shown in the legend of Table 4. The control system contained the same final concentrations of NEM, trypsin, and RNase in the assay dishes in the absence of the Em samples.

Assay of DNA, RNA, and protein synthesis. Secondary cultured cells were seeded in the RM and after 48 to 72 h the RM was replaced with the GM to turn-on the resting cells (14), and 17 h later radioactive precusors were added. The assay of DNA synthesis was performed by the method of Schaefer (15) with (methyl-^{3}H) thymidine and RNA and protein synthesis with (5, 6-^{3}H) uridine and dl-leucine-1-^{14}C by the method of Rudland (16). Averaged radioactivity from 4 identical experimental dishes (18.1 ^{2}cm) was calculated in count per min (C. P. M.) per mg protein. Radioactivity on glass filter was counted in a toluene based scintillation solution (17) by a Scintillation counter.

Chromatography with DEAE-cellulose and Sephadex G-100. DEAE-cellulose column was conditioned with a medium consisting of 10 mM Tris-HCl, pH 7.8 and 0.5 mM 2-Met. The samples, Em 70 or M 70 ethanol fraction, were dissolved in the conditioning medium (CM) and were centrifugated at 10,000×g, for 10 min before charging. The samples were charged on the columns (1.0 cm⌀ ×12.6 cm) and were step-wisely eluted by eluting solutions, first step by CM (fraction 1; abbreviated Fr. 1), and 2nd step by CM plus 0.2 M NaCl (Fr. 2), 3rd step by the 2nd step solution plus 1.0 M NaCl (Fr. 3). All fractions were intensively dialysed against 1 mM Tris-HCl, PH 7.4 and 0.5 mM 2-Met and were lyophilized.

Sephadex G-100 column (2.6 cm ⌀ × 44 cm) was conditioned with CM and the samples charged were fractionated in 4 ml eluant. To calibrate the averaged molecular weight of the samples, a standard kit of proteins, Combithek, Protein Calibration Kit, Size I, Boehringer Mannheim GmbH, was used.

RESULTS

In the supernatant of chick embryo and adult muscles, the presence of plural stimulating factors of cell growth was proved by three different methods; ammonium sulfate salting-out, ethanol precipitation, and isoelectric precipitation. Table 1 shows that there were two or more stimulating factors precipitated by

TABLE 1. GROWTH PROMOTING EFFECTS OF EMBRYONIC AND MUSCULAR FRACTIONS PRECIPITATED BY AMMONIUM SULFATE

Systems	Stimulation in Percent[a]
Resting Medium (RM)	0
RM+Em S2	55.4
RM+M S2	19.0
RM+Em A30	26.5
RM+M A30	19.0
RM+Em A60	25.4
RM+M A60	15.2
RM+Em Sup	54.6
RM+M Sup	11.4

a Assay procedure and calculation formula are described in Materials and Methods. The concentration of samples used was 50 μg/ml, and 0% in resting cell system indicated 5.2×10^4 cells per plate.

ammonium sulfate which promote the growth of chick embryo cells (CEF) in the supernatant of chick embryos and adult muscles. The stimulants of embryo enhanced CEF growth about 25 to 55% in the absence of supplementary sera and the adult muscular stimulants showed about 10 to 20% stimulation. Table 2 showed by ethanol fractionation that there are at least two stimulants in the same supernatant. The 50% precipitant of embryos stimulated about 210% and the 70% fraction showed about 140% stimulation. The muscular stimulants showed about 60% stimulating effect in both fractions of 50% and 70% precipitants. The presence of plural stimulants was also confirmed by isoelectric precipitation method. Table 3 shows that there were two stimulants in pH 4 fraction and pH unprecipitating fraction (Em-Sup and M-Sup). Because of variations in biological activity of S_2 in each experiment (Table 1–3), it was difficult to compare the stimulatory activity in each fraction. The embryonic stimulants were more active than the muscular ones in each method, and this suggests that embryonic

TABLE 2. GROWTH PROMOTING EFFECTS OF EMBRYONIC AND MUSCULAR ETHANOL FRACTIONS

Systems	Stimulation in Percent[a]
Resting Medium (RM)	0
RM+Em 50	210
RM+M 50	60
RM+Em 70	139
RM+M70	64

[a] Assay procedure and calculation formula are described in Materials and Methods. The concentration of samples used was 50 μg/ml, and 0% in resting cell system indicated 8.8×10^4 cells per plate.

TABLE 3. GROWTH PROMOTING EFFECTS OF EMBRYONIC AND MUSCULAR FRACTIONS OBTAINED BY ISOELECTRIC PRECIPITATION

Systems	Stimulation in Percent[a]
Resting Medium (RM)	0
RM+Em S2	130
RM+M S2	83
RM+Em pH4	115
RM+M pH4	95
RM+Em 7Sup	192
RM+M 7Sup	68

[a] Assay procedure and calculation formula are deseribed in Materials and Methods. The concentration of samples used was 50 μg/ml, and 0% in resting cell system indicated 3.76×10^4 cells per plate.

development induced the changes in the functional and physico-chemical properties of the stimulants.

The embryonic ethanol fraction (Em 50) was used to elucidate physico-chemical properties of the stimulants. Trypsin treatment completely inactivated the stimulating activity of Em 50, N-ethylmaleimide inactivated 20% activity of Em 50, and RNase treatment showed no inactivating effect on Em 50 stimulatory activity. Em 50 lost about 60% stimulating activity by heat treatment (Table 4). These results suggest that the active component in Em 50 is a protein.

To study the stimulatory mechanism of the embryonic and muscular stimulants, the effects of Em S_2 and M S_2 on the synthesis of DNA, RNA, and protein were assayed. The embryonic stimulants (Em S_2, Em pH4, Em Sup) enhanced the synthesis of DNA and protein remarkably, but RNA synthesis was slightly stimulated (Table 5). The muscular stimulants stimulated protein synthesis only. These results suggest that the embryonic stimulants enhanced cell proliferation directly by stimulation of DNA synthesis and possibly indirectly by

TABLE 4. LOSS OF STIMULATION ACTIVITY OF EM 50 TREATED WITH TRYPSIN, RNASE, NEM, AND HEAT

Systems	Stimulation in Percent[a]
Resting Medium (RM)	0
RM+Em 50	115
RM+Trypsin-treated Em 50[b]	0
RM+RNase-treated Em 50[b]	120
RM+NEM-treated Em 50[b]	92
RM+Heat-treated Em 50[b]	53

a Assay procedure and calculation formula are described in Materials and Methods. The amount of samples used after treatment was 50 μg/ml.

b Em 50 (500 μg/ml) was treated by 10 μg of trypsin, 25 μg of RNase, 25 μM of NEM per ml system for 2 h at 37°, then heated at 100° for 60′.

TABLE 5. EFFECTS OF EMBRYONIC AND MUSCULAR STIMULANTS ISOLATED BY ISOELECTRIC PRECIPITATION ON DNA, RNA, AND PROTEIN SYNTHESIS

Systems	Stimulation in Percent[a]		
	DNA	RNA	Protein
Resting Medium (RM)[b]	0	0	0
RM+Em S2	33	6	57
RM+Em pH4	38	17	45
RM+Em Sup	52	14	63
RM+M S2	0	0	0
RM+M pH4	2.6	2.3	37
RM+M Sup	0	0	44

a Assay procedure and calculation formula are described in Materials and Methods. The amount of samples used was 50 μg/ml.

b Zero percent control indicated 5890±748 c. p. m./mg protein in DNA synthesis, 3840±245 c. p. m./mg protein in RNA synthesis, and 1680±103 c. p. m./plate in the protein synthesis systems.

enhancement of protein synthesis; moreover, that the muscular stimulants possibly worked by stimulation of protein synthesis. Partial purification of the stimulants in Em 70 ethanol fraction (Table 2) was performed by DEAE-cellulose chromatography and Sephadex gel filtration. Fig. 1 shows the step wise elution pattern of Em 70 ethanol fraction (Table 2) from DEAE-cellulose column (1.0×12.6 cm): The stimulatory activity on cell proliferation was eluted in the first fraction only (Fr. 1), and not in Fr. 2 or Fr. 3 (Table 6). Gel chromatography of the active fraction Fr. 1 with Sephadex G-100 revealed one major fraction and a minor one. The average apparent molecular weight of the major fraction was

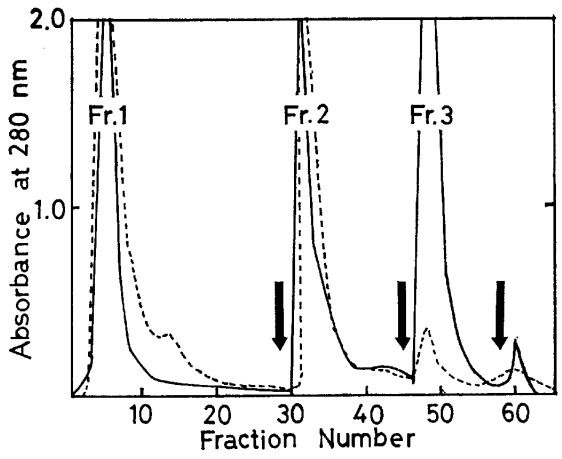

Fig. 1. Step-wise fraction of Em 70 and M 70 ethanol fraction by DEAE-cellulose chromatography. Fractionation procedure and nomenclature are described in Materials and Methods. The solid line (——) indicates the fraction pattern of Em 70 fraction, and the dotted line (·····) indicates that of M 70 fraction.

TABLE 6. GROWTH PROMOTING ACTIVITY OF EM 70 AND M 70 FRACTIONS FRACTIONATED BY DEAE-CELLULOSE CHROMATOGRAPHY

Systems		Stimulation in Percent[a]
Exp. I	Resting Medium (RM)	0
	RM+Em Fr. 1[b]	73
	RM+Em Fr. 2	0
	RM+Em Fr. 3	0
Exp. II	Resting Medium (RM)	0
	RM+M Fr. 1[b]	64
	RM+M Fr. 2	83
	RM+M Fr. 3	52

a Assay procedure and calculation formula are described in Materials and Methods. The concentration of samples used was 40 μg/ml, except for 20 μg/ml in M Fr. 3 system. The zero percent in the resting cell system of Exp. I was 6.0×10^4 cells per plate and Exp. II was 5.3×10^4 cells per plate.

b DEAE-cellulose chromatography is described in Materials and Methods. The fractionated names are also described in the same section.

about 2.7×10^5 (Fig. 2). Step-wise elution from DEAE-cellulose column of M 70 ethanol fraction (Table 2) shows a different elution pattern from that of Em 70; two major fractions, Frs. 1 and 2, and two minor ones (Fig. 1). The stimulatory activities were recognized in Frs. 1, 2, and 3 (Table 6). Gel chromatography with Sephadex G-100 of Fr. 2 in Fig. 1 showed one major peak (P-I) and a minor

one (P-II) (Fig. 2). The averaged apparent molecular weight of the major fraction was about 1.25×10^5 and that of the minor one was about 7.5×10^3. The stimulatory activity of P-I in Fig. 2 is shown in Table 7.

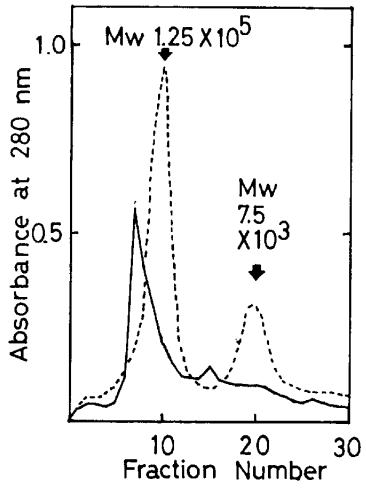

Fig. 2. Sephadex G-100 chromatography of Fr. 1 prepared from the DEAE-cellulose column of Em 70 and Fr. 2 prepared from same column as M 70. The procedure is described in Materials and Methods. The solid line (——) indidates the elution pattern of Fr. 1 (Em 70) and the dotted line (·····) indicates that of Fr. 2 (M 70). Fr. 1 and Fr. 2 were obtained by DEAE-cellulose chromatography (see Fig. 1). P-I was a major fraction of Fr. 2 (M 70), and P-II was a minor fraction of it.

TABLE 7. GROWTH POMOTING ACTIVITY OF P-I FRACTION

Systems	Stimulation in Percent[a]
Resting Medium (RM)	0
RM+P-I (40 μg/ml)	11
RM+P-I (80 μg/ml)	58

a Assay procedure and calculation formulae are described in Materials and Methods. P-I was prepared from M Fr. 2 by Sephadex G-100 Chromatography (see Fig. 2). Zero percent in resting cell system indicated 2.84×10^4 cells per plate.

Although these stimulants have not been identified, the results indicate that there are two or more cytoplasmic protein stimulants in the supernatants of embryo carcases and adult muscles, and that embryological development induced differences in their stimulatory activity and physico-chemical properties.

DISCUSSION

Many cytoplasmic protein factors have been studied as cell proliferation stimulating factors; Some are chick embryo extract (7-9), fibrobrast growth factor (FGF, 10), epidermal growth factor (EGF, 11), and nerve growth factor (NGF, 12). The role of these stimulating factors is still ambiguous; however, these factors are useful in investigating cellular or organic responses against humoral informations *in vivo* and changes in culture media *in vitro*.

In the present paper we reported the presence of plural growth stimulants in the supernatant of chick embryo and adult muscles. These stimulants had altered biological activity and physico-chemical properties due to embryological development. It would be relevant to purify these stimulants and to study changes in their physico-chemical properties for the investigation of regulatory mechanism of chick embryo development. Although it was difficult to purify and identify each of the stimulants, we attempted to purify one of the embryonic and muscular stimulants. One of the muscular stimulants was about 1.25×10^5 in molecular weight and one of the embryonic ones was about 2.7×10^5 in molecular weight. Both were different from FGF, NGF, EGF, in molecular weight. These chick stimulants showed stimulating effects on cell proliferation of chick embryo cells without any supplements such as sera and hormones. The stimulating activity of the embryonic stimulants was more active than that of the muscular ones, as might be expected from embryological development.

Identification of the stimulants and investigation of their biological activities or cellular regulatory mechanisms are yet to be completed. These stimulants would be useful in analysis of the roles of cytoplasmic factors in cell proliferation and differentiation.

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