STUDIES ON THE BEATING PROPERTIES OF SINGLE MYOCARDIAL CELLS IN VITRO

BY

KENZO OHTSUKA

CONTENT

INTRODUCTION

Isolated cells from higher organisms have a low probability of growing singly in culture. Under usual culture conditions, less than 0.1 % of isolated embryo cells grow singly and divide repeatedly to form cell clones. The proportion of cells that grow and their rate of growth are increased, however, if the cells are cultured in large population; thus the inoculum density affects the subsequent growth of individual cells. This effect was early noted by Earle and his colleagues (9), and led to a view that a cell must condition the surrounding medium with metabolic products in order to grow. Under usual culture conditions where cell density is low, particularly when compared to the density in solid organs (about 10^8 cells/cm^3), the concentration of metabolites would be insufficient for cells to grow. Puck and his colleagues (35) developed a simple method for cloning single cells. This technique included the use of non-growing irradiated cells to increase the cell density and so act as "feeders" for small numbers of viable cells. Under such a condition with feeder cells, a high proportion of cells grow into colonies. Thus, the concept of medium conditioning has been very important in cell biology (reviewed in ref. 44). Medium that has been conditioned by exposure to cells of high density is called conditioned medium (CM). CM can influence such various phenomena as growth (8, 36, 40, 44, 45, 46), cell aggregation (30, 39), contact inhibition (56), adhesion to substrate (47) and differentiation (20, 22, 23,

24, 26, 41, 42, 48, 53). When mammalian cells were cultured, "microexudate carpet" was formed between cells and substrate (34, 38, 55). This microexudate carpet has been shown to exert significant effects on the adhesion properties of cells (4, 29, 33, 50, 51), the rate of DNA synthesis and division in cells attached to solid surfaces (1, 52, 55). This may be identical with the cell growth stimulating factors or so called conditioning factors secreted into culture medium by cells.

Trypsin-dissociated myocardial cells were first cultured in monolayer by Cavanaugh (2). Thereafter, the methods for the preparation of myocardial cells and the culture techniques were improved by DeHaan (5), Goshima and Tonomura (18) and others, and the conditions where high proportion of myocardial cells can beat have been established. Many pharmacological studies on the beating properties of cultured myocardial cells were reported, but there were few reports on the effects of medium conditioning on the beating properties of myocardial cells. Gordon and Wilde (14) reported that heart muscle-conditioned medium promoted the spreading and the contraction of myocardial cells. More recently, Gordon and Brice (12) found a spreading factor and a contractile factor in heart muscle-conditioned medium, and they suggested that the former was a substance containing protein and the latter a glycoprotein.

This paper consists of two parts; in the part I, the effects of the cell density and the conditioned medium on the beating properties of cultured myocardial cells are described. It is

also shown that various kinds of cells from various animals secrete into medium a factor(s) which accelerates the beating rate of myocardial cells. The part II described that a beating-stimulating factor(s) partially purified from conditioned medium and characterized as glycoprotein(s) with molecular weight of about $5\text{-}7 \times 10^4$ daltons.

PART I

EFFECTS OF CONDITIONING OF MEDIUM ON BEATING OF SINGLE MYOCARDIAL CELLS *IN VITRO*

ABSTRACT

Heart cells singly dissociated from mouse embryos were cultured at low (0.5×10^5 cells per plate) or high (4×10^5 cells per plate) density. After cultivation for one day, the percentages of beating cells were 51 % and 75 %, respectively, and the average beating rates were 39 beats/min (b/m) and 70 b/m, respectively. The average beating rate of single heart cells cultured at low density in the medium consisting of 3 parts of conditioned medium and 1 part of fresh medium was 72-85 b/m, while the value in the fresh medium was 40-57 b/m. Lung, kidney, skeletal muscle and L cells from mice, FL cells, chick embryonic cells and reptilian iguana heart cells were effective at high density in increasing both the percentage of beating cells and the average beating rate of mouse myocardial cells at low density.

Single heart cells from chick and mouse embryos dissociated with trypsin beat spontaneously in monolayer culture (2, 5, 18, 29). Norepinephrine (3, 10, 16 54) and dibutyryl cyclic AMP (17, 25) have been reported to accelerate the beating rate of cultured heart cells. Haraley and Slater (19) reported that various compounds (e.g., oligomycin, dinitrophenol and Ouabain) inhibit beating.

The growth rate (cell division, DNA synthesis and protein synthesis) of mammalian and avian cells cultured in vitro is known to be dependent on the cell population density and is promoted by 'conditioning factor' secreted into the culture medium by cells (8, 26, 37, 40, 44, 46, 52). There are, however, few reports on the effects of medium conditioning on the beating properties of single myocardial cells, i.e., the spontaneous beating activity and the beating rate. Using chick embryos, Gordon and Brice (11, 12, 14) reported that heart muscle-conditioned medium increased the survival and promoted the spreading and contraction of isolated chick heart cells. They (13) also reported that the contractile activity, the spreading on glass and the survival increased as the inoculum level rose, but they did not report the effects of medium conditioning on the beating rate.

In the present study, we examined the effects of cell density and conditioned medium on the percentage of beating cells (PBC) and the average beating rate (ABR) of single myocardial cells dissociated from mouse embryo. It was observed that both

the PBC and ABR were higher at high cell density than at low cell density and that ABR increased when cells were cultured in conditioned medium. We also cultured mouse heart cells at low density in an area surrounded by mouse heart cells or other cells at high density. Using this system, we showed that both the PBC and ABR of single myocardial cells from mice were increased remarkably in medium conditioned by various cells.

MATERIALS AND METHODS

Preparation of single heart cells and other cells. Heart cells were prepared by reported procedures (5, 18, 28) with slight modifications as follows. Heart ventricles were dissected from 14-17-day-old mouse embryos (ddo-strain), placed in Hanks' balanced salt solution and cut into pieces of about 0.1-0.2 mm length with scissors. The tissue was digested successibly with 0.1, 0.08 and 0.08 % trypsin for 15, 10 and 10 min, respectively, at 37°C, with gentle stirring using a magnetic stirrer. The supernatants from the second and third trypsin treatments were transfered to test tubes containing ice-cold Eagle's minimum essential medium (MEM) supplemented 10 % calf serum and centrifuged at 1,800 rpm for 8 min. The pellets were resuspended in MEM. The suspension was filtered through a Swinney filter with two sheets of tissue paper to remove cell clumps, and the product was used as the sample of heart cells.

Cells were also prepared from lungs, kidneys and skeletal muscle (thigh muscle) of 14-17-day-old mouse embryos by a proce-

dure similar to that used for heart cells. Chick embryonic cells were also prepared in the same way by digesting mixed tissue fragments of lungs, heart and skeletal muscles from 11-day-old embryos.

FL and L cells were kindly supplied by Prof. Y. Okada, Research Institute of Microbial Diseases, Osaka University. IgH-2 cells were purchased from Flow Laboratories Inc., Maryland, U.S.A. FL, L and IgH-2 cells were obtained by treatment for 5 min with 0.12 % trypsin and 0.05 % EDTA, 0.12 % trypsin and 0.12 % trypsin, respectively. After centrifugation, these cells were resuspended in MEM. Cells were counted on a hemocytometer slide.

Culture of heart and other cells. Gelatin-coated plates (35 mm in diameter) were inoculated with 4×10^5 heart cells (high density, HD) or 0.5×10^5 heart cells (low density, LD) that were suspended in 0.5 ml of MEM. Then, 1.3 ml of MEM and 0.2 ml of calf serum were added, and the plates were incubated under an atmosphere of 5 % CO_2- 95 % air at 37°C. The culture medium was changed daily.

Heart and other cells were inoculated at HD ($6\text{-}7 \times 10^5$ cells per plate) onto a plate (45 mm in diameter) with a cover glass (18 x 18 mm) placed on the plate. The plate was then incubated for 6-7h or 7-8h. The cover glass was removed, and single heart cells from another sample at LD ($0.7\text{-}0.8 \times 10^5$ cells per plate) were inoculated onto the plate without changing the medium. Thus, the LD-area previously occupied by the cover glass was surrounded by the HD-area. The cells were further cultured for

24h, and the PBC and ABR of single myocardial cells in the LD-area were measured. In the case of FL cells and lung cells, which differed morphologically from myocardial cells, it was verified that cells in the HD-area did not invade the LD-area under the present experimental conditions.

Preparation of conditioned medium. After cultivation of herat cells at HD for 0-1, 0-2 and 0-3 days, the medium were collected in test tubes and centrifuged at 1,800 rpm for 8 min to remove the floating cells and cell debris. The supernatant was stored at 4°C for use within two days.

Measurement of the percentage of beating cells and the average beating rate of cultured myocardial cells. DeHaan et al.(6) reported that even when no contact was detected by light microscopy between two myocardial cells separated by 5-7 μ, they beat synchronously and that a cell process (about 0.1 μ in diameter) connecting the two cells was observed by electron microscopy. Only myocardial cells appearing singly by light microscopy were selected. Cells which beat synchronously with neighbouring cells were omitted from measurements to avoid the effect of direct cell-to-cell contact. The time required for 20 beats was measured, and the beating rate was expressed as the number of beats per min. When no beating was observed for 20-30 sec, the cell was regarded as not beating.

The percentage of beating cells (PBC) was calculated as

$$PBC = \frac{\text{No. of beating cells}}{\text{No. of total single myocardial cells}} \times 100\ (\%)$$

Myocardial cells were observed at 37°C, using an inverted phase contrast microscope (Nikon, Type MD) with a temperature regulated chamber. For microscopic observation, the medium was replaced by MEM buffered at pH 7.2 with 10 mM Tricine[N-Tris (hydroxymethyl) methyl glycine] and supplemented with 10 % calf serum to avoid a pH shift of the culture medium (7, 15).

The distribution of beating rates of single myocardial cells was usually asymmtric and did not fit the normal distribution curve (cf. Figs. 3A and B). Therefore, we replotted the percentage with indicated beat against the square root of the beating rate (cf. Figs. 3C and D). As shown in Fig. 1, the cumulative distribution plotted against the square root of the beating rate upon probability paper approximated a straight line, while the cumulative distribution plotted against the beating rate deviated from linearity. The level of statistical

---

Fig. 1.

---

significance (P) for the difference between the two ABR was calculated by the Student's t-test on the percentage against the square root of the beating rate.

RESULTS

Effect of cell density. As previously reported by Mark and Strasser (28), DeHaan (5) and Goshima and Tonomura (18), single heart cells obtained by trypsin treatment became attached to the

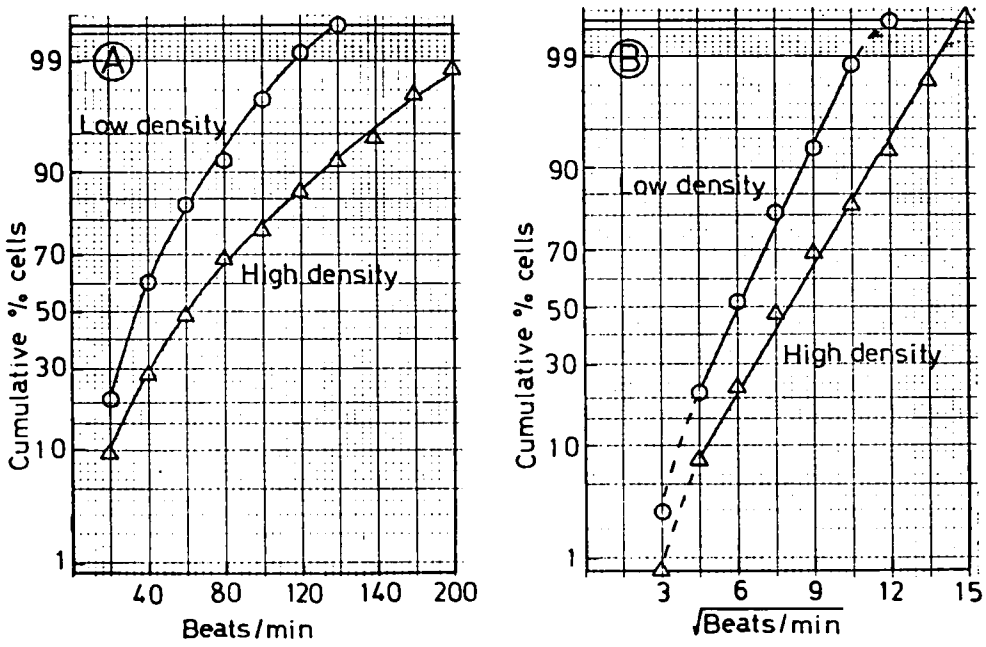

Fig. 1. Beating rates of single myocardial cells cultured for one day at LD and HD (shown in Fig. 3) are plotted against cumulative percentages. The square root of the beating rate is plotted in Fig. 1B.

surface of the gelatin-coated plate on cultivation for one day. Two morphological types of cells were observed: myocardial cells and fibroblastic cells. Myocardial cells were thick and highly refractile under phase optics. They had a dense cytoplasm, and their nuclei were often less distinct. Fibroblastic cells were very thin, well-spread and were transparent and poorly refractile. Only myocardial cells were observed beating. As shown in Fig. 2A, when 0.5×10^5 cells were inoculated per plate, most

---

Fig. 2.

---

cells were observed singly. On decreasing the inoculation to less than 0.5×10^5 cells per plate, the number of cells attached to the plate decreased, and the attached cells were mostly spherical. On the other hand, when 4×10^5 cells per plate were inoculated and cultured for one day, many cell groups were formed and a small number of single cells were observed among them (Fig. 2B). On increasing the cell inoculum to more than 4×10^5 cells per plate, the number of single cells decreased, and at very high cell density only cell groups and monolayer sheets were formed. Therefore, in subsequent experiments 4×10^5 cells and 0.5×10^5 cells per plate were selected as inoculums for high density (HD) and low density (LD), respectively.

Figs. 3A and B show the beating rate distributions of single myocardial cells at LD and HD, respectively, after cultivation for one day. Cells with beating rates of above 160 b/m were

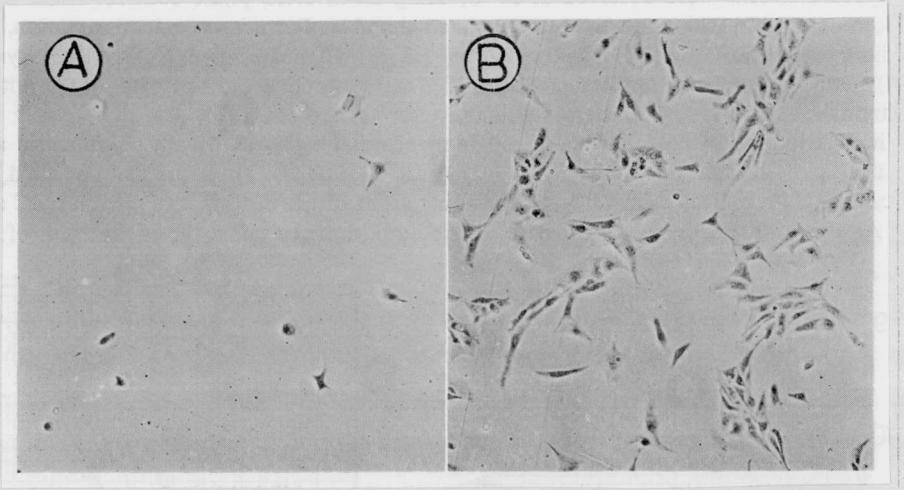

Fig. 2. Photographs of heart cells at low (A) and high (B) density after cultivation for one day. For low density, 0.5×10^5 cells were inoculated per plate. For high density, 4×10^5 cells were inoculated per plate. Phase contrast, x 230. See text for details.

Fig. 3.

rarely observed at LD. At HD about 2 % of cells (15 cells/888 cells) beat at rates of more than 200 b/m. At LD and HD the ABR were 39.6 and 70.5 b/m, respectively, while the PBC were 51 and 75 %, respectively. As mentioned in the methodology section, the distributions of beating rates were asymmetric (Fig, 3A and B). When the percentage beating rate was plotted against the square root of the beating rate, the distribution approached normality (Fig. 3C and D), and the significance (P) of the ABR difference between LD and HD was less than 0.001.

Figs. 4A and B show the time-cources of PBC and ABR, respectively, during cultivation. When heart cells were inoculated at

Fig. 4.

HD, the mean numbers of cells per unit field (1.1 mm^2) were 334, 378 and 407 at the first, second and third day of cultivation, respectively. Even at the third day, several hundred myocardial cells per plate remained single. At HD single, attached myocardial cells did not start to beat until about 7h after inoculation. As shown in Fig. 4A, at HD the PBC increased from 44.8 to 75.5 % with an increase in cultivation time from 10 to 25h, and then remained nearly constant for two days. On the other hand, at LD the PBC increased slowly from 46.5 to 69.0 % with an increase in

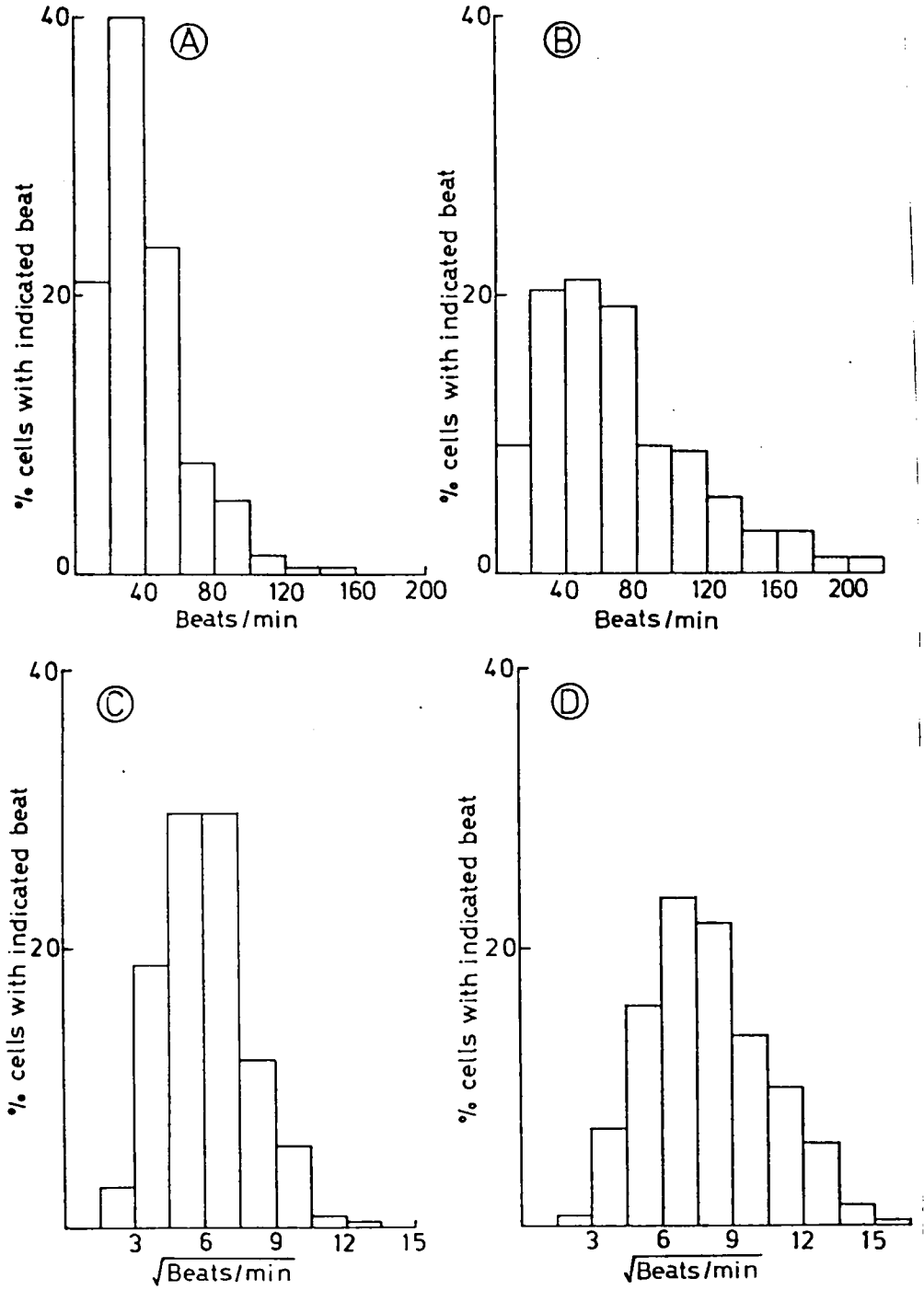

Fig. 3. Distributions of beating rates of single myocardial cells cultured for one day at LD (Figs. 3A and C) and HD (Figs. 3B and D). The number of beating myocardial cells examined was 248 for Fig. 3A and 273 for Fig. 3B.

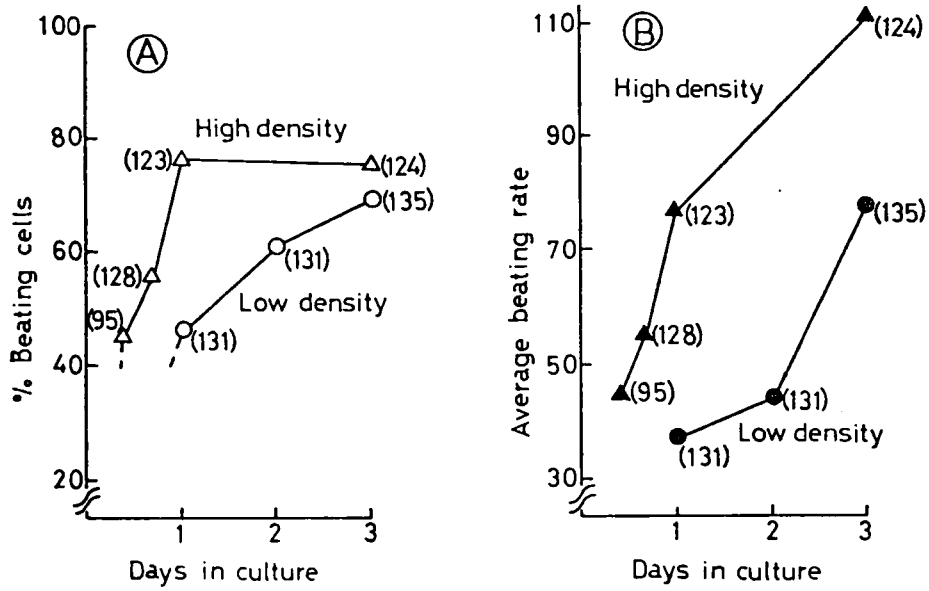

Fig. 4. The time-course of PBC (Fig. 4A) and ABR (Fig. 4B) of single myocardial cells during cultivation at HD (△,▲) and LD (○,●). Values in parentheses indicate the number of single beating myocardial cells examined.

cultivation time from one to three days. At HD the ABR increased almost linearly from 45.0 to 74.0 b/m with an increase in cultivation time from 10 to 25h, and increased still further to 111.0 b/m by the third day. At LD the ABR increased only slightly from 37.0 to 44.2 b/m with an increase in cultivation time from one to two days, and then increased considerably to 78.6 b/m on the third day (Fig. 4B).

Effect of conditioned medium on the beating of single myocardial cells. As shown in Fig. 3 and 4, the PBC and ABR of single myocardial cells were both higher at HD than at LD and increased during cultivation. Thus, these phenomena seemed to be due to the conditioning of the medium. Therefore, the effects of so-called conditioned medium on the beating of single myocardial cells were examined. Single heart cells were cultured at LD in a medium consisting of definite proportions of conditioned medium (CM) and fresh medium (FM). After cultivation for one day, the medium was replaced by Tricine buffered MEM supplemented with 10 % calf serum, and PBC and ABR were measured. No difference in the cell number was found between the CM and FM plate. As listed in Table 1, the ABR did not increase significantly in media containing ratios of CM to FM of 1:3 and 3:1 ($P>0.5$ and ~ 0.11) at 1 day (Experiment 1). The ABR increased from the control value of 40-48 b/m to 72-84 b/m in the medium containing a ratio of CM to

TABLE 1

TABLE 1. EFFECTS OF CONDITIONED MEDIUM ON THE BEATING OF SINGLE MYOCALDIAL CELLS

Experiment	CM[a]: FM	Beating cells (%)	Average beating rate (beats/min)	No. of beating cells observed	P
1	0 : 1	50.8	46.9	56	
	1 : 3	70.5	48.0	64	>0.5
	3 : 1	65.6	56.4	57	0.11
2	0 : 1	62.3	39.7	102	
	1 : 3	63.7	58.2	106	<0.001
	3 : 1	62.3	71.6	101	<0.001
3	0 : 1	57.3	48.3	100	
	1 : 3	74.6	72.9	102	<0.001
	3 : 1	59.9	83.9	101	<0.001
4	0 : 1	61.4	56.7	103	
	1 : 3	65.4	65.4	100	0.15
	3 : 1	66.9	84.8	101	<0.001

[a] CM, conditioned medium; FM, fresh medium. Medium in which heart cells cultured at high density for one day (Experiment 1), two days (Experiments 2 and 3) or three day (Experiment 4) was used as the conditioned medium.

FM of 3:1 ($P<0.001$) at 2 days (Experiment 2 and 3). The ABR also increased in the medium containing CM of 3 days (Experiment 4).

Beating of single myocardial cells at LD surrounded by heart cells at HD. In experiments at HD (Fig. 3B), many single myocardial cells were located near cell groups, and the effects of cell-to-cell contact on beating could not be excluded, although as mentioned in the methodology section, cells beating synchronously with neighbouring cells were excluded.

Therefore, the beating properties of single myocardial cells at LD surrounded by heart cells at HD were examined (Fig. 5). The

---

Fig. 5.

---

cell number in the control plate was almost equal to that in the LD-area of the test plate. As summarized in Table 2, the ABR in

---

TABLE 2.

---

the LD-area surrounded by the HD-area was 73-99 b/m, while it was 43-52 b/m in the LD-area not surrounded by a HD-area ($P<0.001$).

Effects of other cells at HD on the beating of single mouse myocardial cells at LD. The technique shown in Fig. 5 was used to determine whether the effects of high cell density on the beating properties of single myocardial cells were organ-specific or species-specific. To test whether the effects were organ-specific,

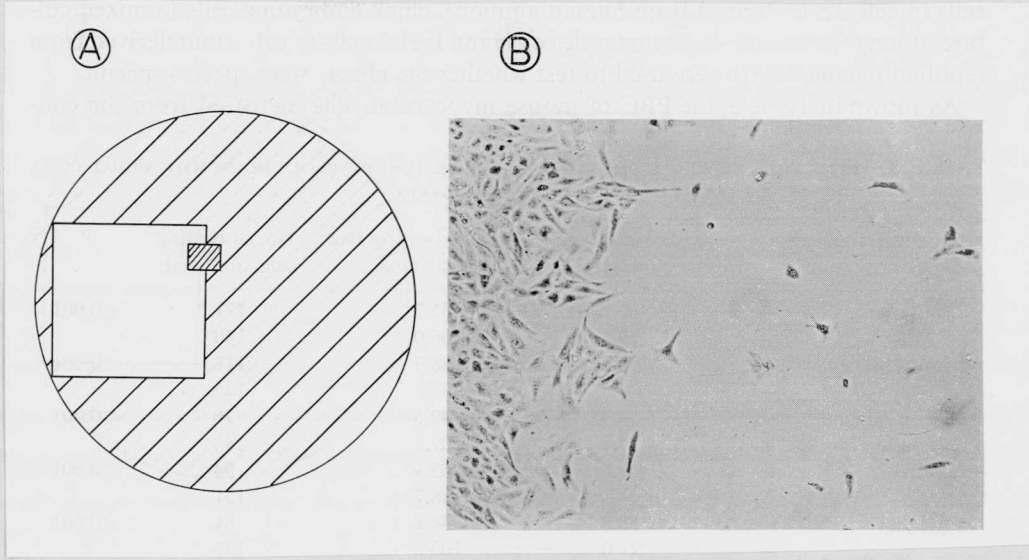

Fig. 5. Myocardial cells at LD surrounded by an area of cells at HD. Fig. 5A, A cover glass (18 x 18 mm) was placed on a plate (45 mm in diameter), and cells were inoculated onto the plate at HD (6-7 x 10^5 cells) and cultured for 7-8h. Then, the cover glass was removed gently, and single mouse heart cells were inoculated at LD (0.7-0.8 x 10^5 cells) without changing the medium. The plate was incubated further for 24h.
Fig. 5B, Photograph of the boundary area between the LD and HD area. Phase contrast. x 215.

TABLE 2. EFFECTS OF HEART CELLS AT HIGH DENSITY (HD) ON THE BEATING OF SINGLE MYOCARDIAL CELLS AT LOW DENSITY (LD)

Experiment	HD-area	Beating cells (%)	Average beating rate (beats/min)	No. of beating cells observed	P
1	–	64.3	52.4	102	<0.001
	+[a]	60.4	86.0	103	
2	–	56.8	50.7	101	<0.001
	+	67.9	99.0	102	
3	–	50.0	43.2	52	<0.001
	+	80.0	73.2	104	

[a] Heart cells were inoculated at HD onto a plate with a cover glass placed on the plate. The plate was then incubated for 7-8 h. The cover glass was removed, and single heart cells from another sample were inoculated at LD onto the plate. The beating properties of single cells in the area previously occupied by the cover glass were measured.

we used cells dissociated from the lungs, kidneys and skeletal muscle (thigh muscle) of 14-17-day-old mouse embryos and L cells (a cell strain derived from mouse connective tissue), FL cells (a cell strain derived from human amnion), chick embryonic cells (a mixed culture of lung, herat and skeletal muscle cells) and IgH-2 cells (a cell strain derived from reptilian iguana heart) were used to test whether the effects were species-specific.

As shown in Table 3, the PBC of mouse myocardial cells increased from the control value of 42-57 % to 70-80 % in all tested

TABLE 3.

cells. The ABR also increased 1.8-2.5 times the control value in all cells examined($P<0.001$). The effects of cells at HD on spontaneous beating activity and the beating rate of mouse myocardial cells were neither organ-specific nor species-specific.

DISCUSSION

Single heart cells prepared from 14-17-day-old mouse embryos were cultured in monolayers, and the percentage of beating cells (PBC) and the average beating rates (ABR) of single myocardial cells were measured at high density (HD) and low density (LD). Both beating indices were higher at HD than at LD (Fig. 3) and increased with the duration of cultivation (Fig. 4). The ABR increased when cells were cultured in the conditioned medium (Table 1) and increased when cells were surrounded by an area of

TABLE 3. EFFECTS OF VARIOUS CELLS AT HD ON THE BEATING OF SINGLE MOUSE MYOCARDIAL CELLS IN LD-AREA

Cells in HD-area[a]		Beating cells (%)	Average beating rate (beats/min)	No. of beating cells observed	P
Mouse lung	–	57.1	55.7	131	<0.001
	+	77.3	106.0	140	
Mouse kidney	–	56.0	48.7	118	<0.001
	+	80.0	98.9	145	
Mouse skeletal muscle	–	52.3	42.5	76	<0.001
	+	77.1	107.9	144	
L cells	–	48.6	53.9	94	<0.001
	+	—	118.7	151	
FL cells	–	56.8	44.8	81	<0.001
	+	71.0	103.8	143	
Chick embryo cells	–	42.2	44.2	80	<0.001
	+	70.4	87.5	145	
IgH-2 cells	–	41.6	46.2	50	<0.001
	+	72.1	83.1	138	

[a] Cells were cultured at HD for 6-7 h, before inoculation of heart cells at LD.

heart cells at HD (Table 2). But PBC did not increase under these conditions. The reason why PBC did not increase in these case remains to be clarified. From these results, it was concluded that ABR of single myocardial cells increased during conditioning of the medium by heart cells without direct cell-to-cell contact. We could not exclude the possibility that slowly beating cells are more adhesive together when heart cells are cultured at HD or in CM. However, this possibility seems rather improbable, since the number of single cells per unit field did not change during the conditioning of medium.

Previously, Mark and Strasser (28) and Goshima and Tonomura (18) concluded that the beating rates of cell groups are determined by the rate of the fastest beating cell, since they observed that the beating rates of cell groups were higher than those of single cells. On the other hand, DeHaan and Hirakow (7) reported that when two myocardial cells with different beating rates came into contact, the synchronized beating rate of the two cells was not necessarily determined by the cell with the higher beating rate. Moreover, The ABR of single myocardial cells was increased by conditioning of the medium, as mentioned above. Therefore, it seems possible that the beating rate of each cell in a cell group is influenced by adjacent cells.

There are few reports on tissue and organ specificity of conditioned medium. Paran et al. (32) reported that human spleen-conditioned medium induced colony formation with human and rodent bone-marrow cells, whereas rodent spleen-conditioned medium

induced colony formation with rodent bone-marrow but not with human cells, and they stated that the inducer had some species specificity, apparently in one direction. On the other hand, Takahashi and Okada (46) reported that muscle, skin, kidney, liver and lung-conditioned medium enhanced the plating efficiency and the growth rate of lung secondary cells which indicates the absence of tissue specificity in the conditioned medium. In the present study, we found that PBC and ABR of single mouse myocardial cells in the LD-area increased remarkably when various cells were cultured at HD around the area of LD (Table 3). Therefore, these experiments strongly suggest that the effects of conditioning the medium by various cells on the beating properties of single mouse myocardial cells are neither organ-specific nor species- specific.

It seems probable that some substance(s) formed by cells and released in the medium regulates the beating of single myocardial cells. In preliminary experiments, we found that the conditioned medium increased the PBC of single myocardial cells even after dialysis in a cellophane bag against MEM supplemented with calf serum (unpublished data). The stimulatory substance(s) may specifically stimulate PBC and/or ABR of single mouse myocardial cells or may promote metabolic processes,such as protein synthesis and increase the PBC and ABR as secondary effects. Recently, Gordon and Brice (11, 12) reported that two factors appeared to be present in serum-free heart muscle-conditioned medium that independently effected contraction and spreading.

They suggested that the contractile factor is a proteoglycan and that the spreading factor is a protein containing compound. It will be interesting to see whether their factors and our substance(s) affecting the beating properties of single myocardial cells are identical.

PART II

CARACTERIZATION AND PARTIAL PURIFICATION OF FACTOR(S) STIMULATING THE BEATING OF CULTURED MYOCADIAL CELLS FROM CONDITIONED MEDIUM

ABSTRACT

We previously reported that the beating rate of single myocardial cells cultured in conditioned medium (CM) was higher than that of those cultured in fresh medium. We characterized and partially purified the beating-stimulating factor(s) of cultured myocardial cells from a conditioned medium of mouse embryonic cells.

The beating-stimulating factor(s) had stable activity for more than one month when it was freeze-dried in 0.1 M sucrose and stored at -20°C. The factor(s) was non-dialyzable and heat-labile. It was inactivated by treatment with trypsin, pronase or hyaluronidase but not neuraminidase. These results strongly suggested that the beating-stimulating factor(s) is glycoprotein(s) When concentrated CM was filtered on Sephadex G-200, a main peak of activity appeared in almost the same fractions as those of hemoglobin. The SDS-polyacrylamide gel electrophoretogram of crude CM showed more than 20 protein bands and about 10 bands stainable by periodic acid-Schiff (PAS). On the other hand, the electrophoretogram of the active peak on Sephadex G-200 showed about 10 protein bands and one PAS-stainable band.

Trypsin-dissociated myocardial cells of mammalian and avian embryo and neonate beat spontaneously in culture (5, 18, 28, 31). Gordon and Wilde (14) reported that when dissociated myocardial cells of chicken embryo were cultured in serum- and protein-free chemically defined medium, less than 1 % of the cells were contractile and less than 2 % spread on glass, while about 80 % of them were contractile and the percentage of spreading cells increased remarkably when the cells were cultured in conditioned medium. More recently, Gordon and Brice (11, 12) found a spreading factor and a contractile factor in heart muscle-conditioned medium, and suggested that the former was a substance containing protein and the latter a glycoprotein.

We previously reported (31) that the medium conditioning increased beating rate of single myocardial cells, and its effect showed no tissue or species specificity. In this study, we characterized and partially purified the beating-stimulating factor(s) of myocardial cells from CM of mouse embryonic cells. Our results suggest strongly that the beating-stimulating factor(s) is glycoprotein(s) with a molecular weight of about 50,000-70,000 daltons.

MATERIALS AND METHODS

Preparation of conditioned medium (CM). Mouse embryos 14 to 16 days old were cut into small pieces with scissors after their heads had been cut off, and subjected to stepwise

trypsinization. Dissociated cells were collected by gentle centrifugation and resuspended in Eagle's MEM. Plates, 90 mm in diameter, were inoculated with 2.5 ml cell suspension containing about 2-3 x 10^7 cells. Next, 6 ml of Eagle's MEM and 1.5 ml of calf serum were added and the plates were incubated under an atmosphere of 5 % CO_2 - 95 % air at 37°C for 2 days. The cells were subcultured in 10 ml of Eagle's MEM supplemented with 10 % calf serum for 1 day. These cells were washed three or four times with Hanks' balanced salt solution to remove the serum, then cultured in 10 ml of serum-free Eagle's MEM for 4 days. This culture medium was called the crude conditioned medium (crude CM). The amount of protein in crude CM increased from 5-7 μg to 40-70 μg during the 4 days of culture. The crude CM was concentrated by ultrafiltration (UK-10, Toyo) or precipitation with dialysis against saturated ammonium sulfate. The concentrated crude CM was dialyzed against Hanks' solution for about 20 h at 5°C, then subjected to activity measurement.

Pretreatment of CM with enzymes. Concentrated crude CM was pretreated with trypsin (Worthington Biochem.), pronase (Kaken, Japan), hyaluronidase (Sigma, Type I) or neuraminidase (Sigma, Type V) as follows: 0.05 ml trypsin solution (1.5 mg/ml), 0.05 ml pronase solution (0.9 mg/ml), 0.05 ml hyaluronidase solution (3 mg/ml) or 0.15 ml neuraminidase solution (1 mg/ml) was added to 1.5 ml CM solution (300-500 μg/ml protein), which was then incubated at 37°C for 0-90 min. Trypsin digestion was stopped by adding 0.1 ml trypsin inhibitor solution (2 mg/ml).

The reactions of pronase, hyaluronidase and neuraminidase on CM were stopped by adding 0.4 ml calf serum, which we found was effective.

Procedures for measuring beating-stimulating activity. As previously reported (31), heart cells were prepared by stepwise trypsinization of heart ventricles of 14-16-day-old mouse embryos. Gelatin-coated plates (35 mm in diameter) were inoculated with 0.3 ml portions containing 1 x 10^5 cells, then 1.0 ml of Eagle's MEM and 0.2 ml of calf serum were added. Hanks' solution (0.5 ml) was added to the control plate, and CM solution (0.5 ml), which had been sterilized by being passed through a Millipore filter (Type HA, 0.45 μ), was added to the test plate. These plates were incubated under an atmosphere of 5 % CO_2 - 95 % air at 37°C for 20 h. They were washed with Hanks' solution, then 1.5 ml of Tricine-buffered Eagle's MEM (10 mM Tricine, pH 7.4) supplemented with 10 % calf serum was added to the plates for microscopic observation. In this medium, the beating rate of myocardial cells was stable at least for 3 h. The rate was observed at 36-37°C using an inverted phase contrast microscope (Nikon, Type MD) with a temperature-regulated chamber. Beating rates of at least 30 single myocardial cells were observed in the test and control plates. The ratio of the average beating rate of cells in the test plate (ABR_{test}) to the average beating rate of cells in the control plate (ABR_{cont}) indicates the beating-stimulating activity of the CM solution:

$$ABR(relative) = \frac{ABR_{test}}{ABR_{cont}}$$

We found that stimulation of the beating rate was proportional to the amount of CM protein added (see Fig. 1). Therefore, from the observed increase in the beating rate, i.e., ABR(relative) - 1, we could easily calculate the increase when 0.1 mg of CM protein was added to the plate, and this value was also used as the activity per unit protein of a CM preparation.

Disc-gel electrophoresis. The procedures of Weber and Osborne (49) were used for disc gel electrophoresis [7.5 % acrylamide-bisacrylamide gel with 0.2 % sodium dodecyl sulfate (SDS) in 0.1 M sodium phosphate buffer, pH 7.0]. Protein samples for electrophoresis were prepared in a mixture of 2 % SDS and 10 % β-mercaptoethanol then boiled for 3 min. The gels were stained to detect protein with Coomassie brilliant blue. To detect carbohydrates, the gels were stained by a method using periodic acid-Schiff (PAS) reaction (21). Immediately following electrophoresis, the gels were fixed for 1 h in 12.5 % TCA in test tubes. Next, they were subjected to sample oxidation with 1 % periodic acid for 2 h, then washed for 2 h against 15 % acetic acid. These steps were all carried out at room temperature. The gels were placed in clean test tubes containing Schiff's reagent and stored in a refrigerator (in the dark) for 2 h. They were destained using 7 % acetic acid with several changes

over a 24-h period.

Protein measurement. Protein was measured by the method of Lowry et al. (27).

RESULT

Properties of the beating-stimulating activity in conditioned medium. The beating-stimulating activity in crude CM was almost unchanged by dialysis against Hanks' solution for more than 1 day. When crude CM was passed through an ultrafilter membrane (UK-10, Toyo), the activity remained in the retentate not the filtrate (Table 1). When crude CM concentrated by ultrafiltration

---

TABLE 1

---

was fractionated with 0-33, 33-65 and 65-100 % saturated ammonium sulfate solution, the activity was observed in all fractions, and the ratio of the activity per unit protein was 2:1:2. As shown in Table 1, when concentrated crude CM was precipitated with 100 % saturated ammonium sulfate, all the activity was observed in the precipitate.

The beating-stimulating activity of crude CM concentrated by ultrafiltration was measured within two days after the CM had been collected. As shown in Fig. 1, the relationship between the activity and the amount of protein added was linear. Thus, the ABR increased two and three times over that of the

TABLE 1. EVIDENCE FOR THE MACROMOLECULAR NATURE OF THE BEATING-STIMULATING FACTOR(S) IN CONDITIONED MEDIUM

Treatment of CM	Activity after treatment [a]
Dialysis	+ +
Ultrafiltration (UK - 10) retentate	+ +
filtrate	-
Saturated ammonium sulfate precipitate	+ +
supernatant	-

See the text for details about CM preparation and assay procedures of the beating-stimulating activity.

[a] + + : full activity; - : no activity

control, when 200 and 400 μg of CM protein were added to a plate, respectively.

Fig. 1

When the concentrated crude CM was stored at 4°C, its activity decreased gradually, as shown in Fig. 2. The activity on the 36th day was about 10 % of that on the first day. But when the concentrated crude CM was freeze-dried in 0.1 M sucrose on the seventh day, the activity showed no further decrease and remained constant for more than one month.

Fig. 2

As shown in Table 2, when the concentrated crude CM was heated at 60°C for 30 min or at 100°C for 5 min, its activity decreased to about 30 % of that before the heating (Exp. 1). The activity was completely destroyed by heating at 100°C for 15 min (Exp. 2). When concentrated CM was treated with 0.025 mg trypsin and 0.015 mg pronase for 30 min, its activity decreased to about 30 and 50 %, respectively (Exp. 2, 3, 4). However, the activity disappeared almost completely upon treatment with trypsin or pronase for 60 min (Exp. 2, 4).

TABLE 2

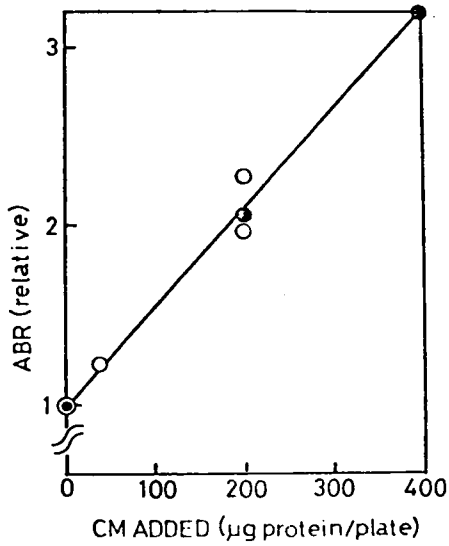

Fig. 1. Dependence of ABR on the concentration of the conditioned medium added. The abcissa gives the amount of CM protein. See the text for explanation of ABR measurements. ○, crude CM concentrated by ultrafiltration (UK-10, Toyo); ●, crude CM precipitated by saturated ammonium sulfate.

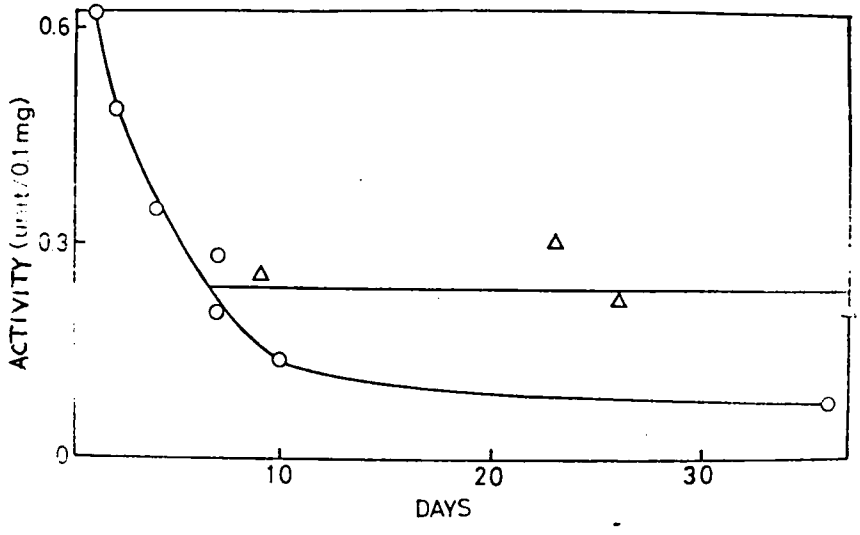

Fig. 2. Stability of beating- stimulating activity in the conditioned medium. The ordinate gives the activities per 0.1 mg protein per plate. See the text for details about activity measurements. ○, CM stored in Hanks' solution at 4°C; △, CM freeze- dried on the 7th day and stored at -20°C.

TABLE 2. ACTIVITIES OF THE CONDITIONED MEDIUM AFTER HEATING AND DIGESTION WITH PROTEASES

CM added (0.5 ml/plate)	ABR (relative)			
	Experiment 1	2	3	4
None	1.00(0)[a]	1.00(0)	1.00(0)	1.00(0)
Untreated	1.97(100)	1.57(100)	1.91(100)	1.56(100)
Heated at 60°C for 30 min	1.30(31)			
Heated at 100°C for 5 min	1.26(26)			
for 15 min		1.006(1)		
Digested with 0.025 mg trypsin				
for 0 min		1.48(84)	1.66(73)	
30 min		1.16(28)	1.32(35)	
60 min		1.04(7)		
Digested with 0.015 mg pronase				
for 0 min				1.53(95)
30 min				1.26(47)
60 min				1.07(12)

Relative values of average heating rate [ABR(relative)] representing the ratio of ABR in the presence and absence of CM. See the text for details about the conditions of trypsin and pronase treatments.

[a] The values in the parentheses indicate the percent activity, i.e.,
(ABRtested - 1)/(ABR of untreated CM - 1) x 100.

As shown in Table 3, when concentrated CM was treated with 0.05 mg hyaluronidase for 60 min, the activity disappeared completely (Exp. 1, 2). On the other hand, when concentrated CM was treated with 0.05 mg neuraminidase for 90 min, its beating-stimulating activity was almost unaffected (Exp. 3, 4).

TABLE 3

These results strongly suggested that the beating-stimulating factor(s) was macromolecule(s) of a complex of protein with carbohydrate.

Gel chromatography. When concentrated crude CM was gel filtered on Sephadex G-50, all the beating-stimulating activity was eluted at void volume. When concentrated CM was filtered on Sephadex G-200 (Fig. 3), the activity was eluted at void

Fig. 3

volume and fraction numbers 6-12, where hemoglobin (M.W. 65,000) was also eluted. Protein was eluted with a large peak at fractions slightly after void volume. Also, a small peak appeared at fraction number 15 and a shoulder at fraction number 10. The highest activity per 0.1 mg protein (fraction number 8) obtained by gel chromatography was 1.1, i.e., the ABR increased to 2.1-fold of the control on addition of 0.1 mg protein, and the activity at void volume was about half that

TABLE 3. ACTIVITIES OF THE CONDITIONED MEDIUM AFTER TREATMENTS WITH HYALURONIDASE AND NEURAMINIDASE

CM added (0.5 ml/plate)	ABR (relative)			
	Experiment 1	2	3	4
None	1.00(0)[a]	1.00(0)	1.00(0)	1.00(0)
Untreated	1.69(100)	1.66(100)	1.46(100)	1.50(100)
Treated with 0.025 mg hyaluronidase				
for 0 min	1.64(93)	1.61(92)		
30 min	1.60(87)	1.34(52)		
60 min	1.01(1)	0.98(-3)		
Treated with 0.04 mg neuraminidase				
for 0 min			1.54(74)	—
60 min			1.53(72)	1.45(90)
90 min			—	1.50(100)

Relative values of average beating rate [ABR(relative)] show the ratio of ABR in the presence and absence of CM. See the text for details about the conditions of hyaluronidase and neuraminidase treatments.

[a] The values in parentheses show the percent activity, i.e.,

(ABR tested - 1)/(ABR of untreated CM - 1) x 100.

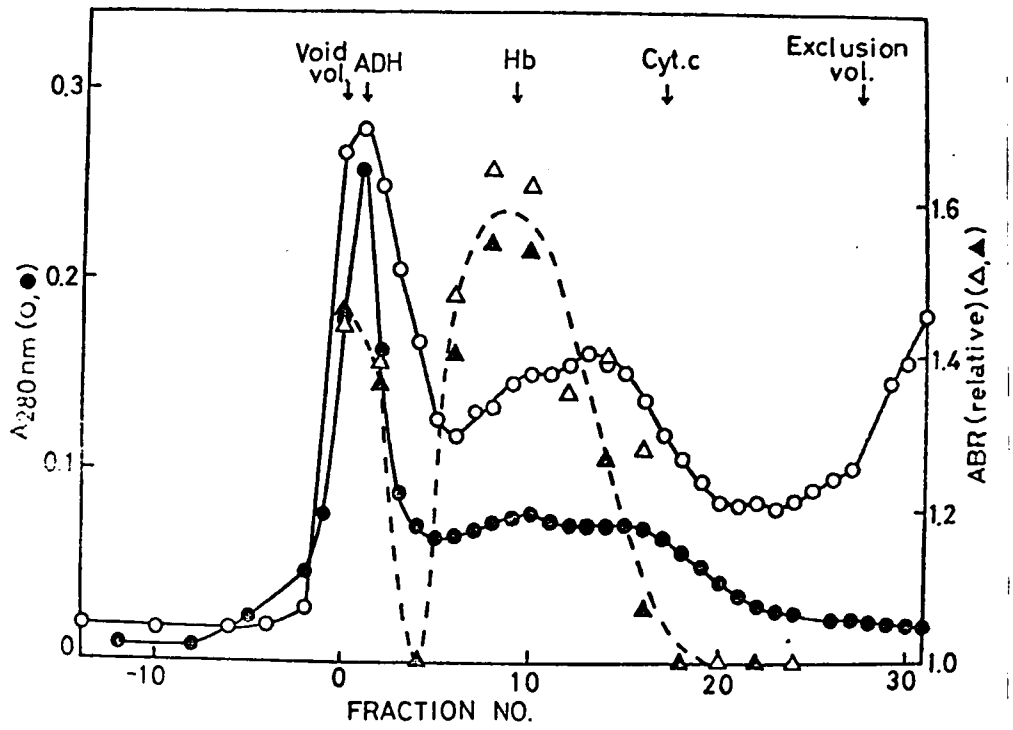

Fig. 3. Gel chromatography of concentrated CM on Sephadex G-200. Two independent experiments are represented in this figure. Void volume was chosen as fraction number 0. In one series of experiments (○,△), a colunm of 2.5 x 46 cm was used and the volume of each fraction was 5.0 ml. In the other series of experiments (●,▲), a column of 2.8 x 35 cm was used, and the volume of each fraction was 4.5 ml. The positions of marker proteins are given for the latter column. ADH, alcohol dehydrogenase (M.W. 151,000); Hb, hemoglobin (M.W. 65,000); Cytc, cytochrome c (M.W. 12,000).

of fraction number 8. The void volume fraction might have contained aggregates of the beating-stimulating factor(s), which had a molecular weight of about 50,000-70,000 daltons.

Furthermore, concentrated crude CM was applied to DEAE-cellulose column (1.5 x 5 cm) and eluted stepwise with Hanks' solution of various concentrations. The activities per unit protein at fractions eluted by 0.085 M and 0.107 M Hands's solution were more than twice that of crude CM before column chromatography. A combination of gel filtration and ion exchange column chromatography could have been used to obtain purer factor(s) but large amounts of starting materials were not available.

SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, the SDS-polyacrylamide gel electrophoretogram of concentrated crude CM showed more than 20 protein bands (A) and about 10 PAS-

---

Fig. 4

---

stainable bands (B). On the other hand, the gel electrophoretogram of a high activity fraction of Sephadex G-200 (fraction numbers 6-12 in Fig. 3) showed about 10 protein bands (C) and one PAS-stainable band (D). The one PAS-stainable band seen in Fig. 4D may be the beating-stimulating factor, although other possibilities can not be excluded, as there were about 10 protein bands in C which might include glycoprotein(s) with a carbohydrate moiety that was undetectable by the PAS-staining method used. Gel electrophoretograms of calf serum shown in E and F of the protein

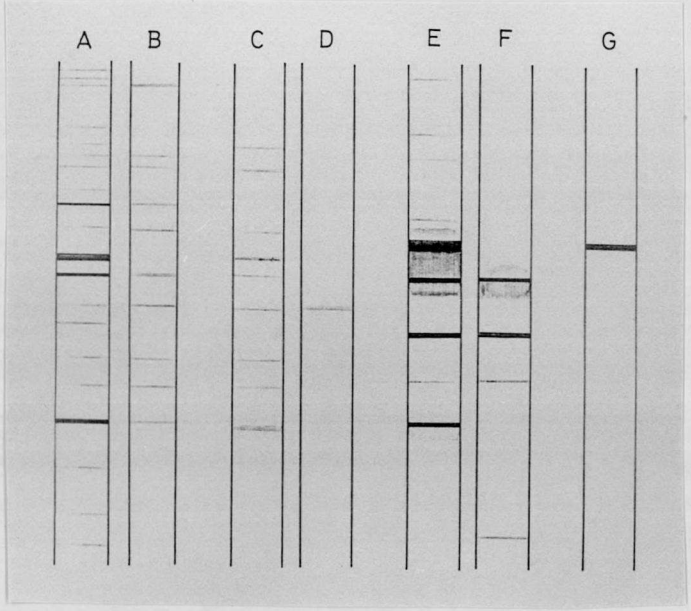

Fig. 4. SDS-polyacrylamide gel electrophoretograms of the conditioned medium. A, C, E, G, stained with Coomassie brilliant blue; B, D, F, stained by PAS reaction.
A, B, concentrated crude CM; C, D, active fractions obtained by Sephadex G-200 gel chromatography (fraction numbers 6-12 in Fig. 3); E, F, calf serum; G, bovine serum albumin.

band and the PAS-stainable band, respectively, had remarkably different patterns from those of crude CM.

DISCUSSION

The results summarized in Tables 1, 2 and 3 strongly suggest that the beating-stimulating factor(s) in conditioned medium (CM) of mouse embryonic cells is a complex of protein with carbohydrate [glycoprotein(s)]. As reported by Gordon and Brice (12), the contractile factor which promotes the percentage of beating cells is also a protein-carbohydrate complex. While our beating-stimulating factor(s) was completely inactivated by heating at 100°C for 15 min, their contractile factor was fairly heat-stable. Our factor(s) resembled theirs with respect to other properties, such as inactivation by trypsin and hyaluronidase and resistance to neuraminidase.

The possibility that the beating-stimulating factor(s) was derived from calf serum was very unlikely. If it existed in calf serum, myocardial cells cultured in higher concentration of calf serum should have shown higher ABR. However, the ABR was nearly constant in 5-30 % calf serum. Furthermore, the gel electrophoretogram of crude CM was remarkably different from that of calf serum (Fig. 4) and almost all proteins in CM were produced by cells, as described in "MATERIALS AND METHODS".

When norepinephrine (3, 10, 16, 54) or dibutyryl cyclic

AMP (17, 25) is added to cultured myocardial cells, the beating rate increases immediately. However, in our study, the effect of concentrated CM was not observed for 2-3 h after it had been added to cultured myocardial cells. The factor(s) in CM may affect the excitation processes of the cell membrane or promote metabolic processes in cells and increase the beating rate as secondary effects, after being incorporated into the cell membrane. As already described in "RESULTS" (cf. Fig. 3), the most active fraction obtained by gel filtration increased the ABR to twice its level when 85 μg of its protein was added per plate. This active fraction contained only one PAS-stainable band (cf. Fig. 4), and the protein content of this band was estimated to be about 6 % of the total protein of the active fraction. Therefore, if we assume that this band is actually the active component, we can conclude that 5 μg protein of the active factor per plate increases the ABR to twice its level. This suggests that the function mechanism of the beating-stimulating factor is very specific. Its effect on myocardial cells remains to be clarified. We also do not known whether it affects other cell functions. The beating-stimulating factor(s) must be purified further before attempting to clarify these problems.

ACKNOWLEDGEMENT

The author is indebeted to Prof. Y. Tonomura of Faculty of Science, Osaka University and Prof. K. Goshima of Faculty of Science, Nagoya University for their stimulating discussions during the course of this work. This work was supported by grants from Muscular Dystrophy Association, Inc. and the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. BOLUND, L., Z. DARZYKIEWICZ and N.R. RINGERTZ, Cell concentration and the staining properties of nuclear deoxyribonucleoprotein. Exp. Cell Res. 62, 76-89, 1970
2. CAVANAUGH, M.W. Pulsation, migration and division in dissociated chick embryo heart cells in vitro. J. Exp. Zool. 128, 573-589, 1955
3. CHANG, T.D. and G.R. CUMMING. Chronotropic responces of human heart tissue cultures. Circ. Res. 30, 628-633, 1972
4. CULP, L.A. Substrate-attached glycoproteins mediating adhesion of normal and virus-transformed mouse fibroblast. J. Cell Biol. 63, 71-83, 1974
5. DEHAAN, R.L. Regulation of spontaneous activity and growth of embryonic chick heart cells in tissue culture. Dev. Biol. 16, 216-249, 1967
6. DEHAAN, R.L., J.O. DUNNING, R. HIRAKOW, T.F. McDONALD, J. RASH, H. SACHS, J. WILDES and H.K. WOLF. Pacemaker rate control and mitotic activity in the embryonic heart. Carnegie Institution Annual Report of the Director Department of Embryology. pp. 66-84, 1970-1971
7. DEHAAN, R.L. and R. HIRAKOW. Synchronization of pulsation rates in isolated cardiac myocytes. Exp. Cell Res. 70, 214-220, 1972
8. DULAK, N.C. and H.M. TEMIN. A partially purified polypeptide fraction from rat liver cell conditioned medium with

multiplication-stimulating activity for embryo fibroblasts. J. Cell Physiol. 81, 153-160, 1973

9. EARLE, W.R., K.K. SANFORD, V.J. EVANS, H.K. WALTZ and J.E. SHANNON. The influence of inoculum size on proliferation in tissue cultures. J. Natl. Can. Inst.12, 133-153, 1951

10. ERTEL, R.J., D.E. CLARKE, J.C. CAHO and F.R. FRANKE. Autonomic receptor mechanisms in embryonic chick myocardial cell culture. J. Pharmacol. Exp. Ther. 178, 73-80, 1971,

11. GORDON, H.P. and M.C. BRICE. Intrinsic factors influencing the maintenance of contractile embryonic heart cells in vitro.
I. The heart muscle conditioned medium effect. Exp. Cell Res. 85, 303-310, 1974

12. GORDON, H.P. and M.C. BRICE. Intrinsic factors influencing the maintenance of contractile embryonic heart cells in vitro.
II. Biochemical analysis of heart muscle conditioned medium. Exp. Cell Res. 85, 311-318, 1974

13. GORDON, H.P. and M.C. BRICE. Intrinsic factors influencing the maintenance of contractile embryonic heart cells in vitro.
III. The effect of inoculum level. Exp. Cell Res. 87, 409-412, 1974

14. GORDON, H.P. and CH. E. WILDE. "Conditioned" medium and heart muscle differentiation: Contrast between explants and disaggregated cells in chemically defined medium.

Exp. Cell Res. 40, 438-442, 1965

15. GOSHIMA, K. A study on the preservation of the beating rhythm of single myocardial cells in vitro. Exp. Cell Res. 80, 432-438, 1973

16. GOSHIMA, K. Initiation of beating in quiescent myocardial cells by norepinephrine, by contact with beating cells and by electrical stimulation of adjacent FL cells. Exp. Cell Res. 84, 223-234, 1974

17. GOSHIMA, K. Antagonistic influence of dibutyryl cyclic AMP and dibutyryl cyclic GMP on the beating rate of cultured mouse myocardial cells. J. Mol. Cell Cardiol. 8, 713-725, 1976

18. GOSHIMA, K. and Y. TONOMURA. Synchronized beating of embryonic mouse myocardial cells mediated by FL cells in monolayer culture. Exp. Cell Res. 56, 387-392, 1969

19. HARALEY, I. and E.C. SLATER. Studies in vitro on single beating heart cells. VIII. The effect of oligomycin, dinitrophenol and Ouabain on the beating rate. Biochim. Biophys. Acta. 99, 227-233, 1965

20. ICHIKAWA, Y., D.H. PLUZNIK and L. SACHS. In vitro control of the development of macrophage and granulocyte colonies. Proc. Natl. Acad. Sci. USA. 56, 488-495, 1966

21. KAPITANY, R.A. and E.J. ZEBROWSKI. A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal. Biochem. 56, 361-369, 1973

22. KONIGSBERG, I.R. Clonal analysis of myogenesis. Science 140, 1273-1284, 1963

23. KONIGSBERG, I.R. Diffusion mediated control of myoblast fusion. Dev. Biol. 26, 133-152, 1971

24. KONIGSBERG, I.R. and S.D. HAUSHKA. Cell and tissue interaction in the reproduction of cell type. In Reproduction: Molecular Subcellular and Cellular., ed. LOCKE, M., Academic Press, New York, 243-291, 1965

25. KRAUSE, E.G., W. HALLE and A. WOLLENBERGER. Effect of dibutyryl cyclic GMP on cultured beating rat heart cells. In Adv. Cyclic Nucleotide Res., ed. GREENGARD, P. and G.A. ROBINSON, Raven Press, New York, Vol, 1. 301-305, 1972

26. LANDAU, T. and L. SACHS. Charaterization of the inducer required for the development of macrophage and granulocyte colonies. Proc. Natl. Acad. Sci. USA. 68, 2540-2544, 1971

27, LOWRY, O.H., N.J. ROSENBROUGH, A.L. FARR and R.J. RANDALL. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275, 1951

28. MARK, G.E. and F.F. STRASSER. Pacemaker activity and mitosis in cultures of newborn rat heart ventricle cells. Exp. Cell Res. 44, 217-233, 1966

29. MASLOW, D.E. and L. WEISS. Cell exudation and cell adhesion. Exp. Cell Res. 71, 204-208, 1972

30. MOSCONA, A.A. Surface specification of embryonic cells: Lectin receptors, cell recognition and specific cell ligands. In The cell surface in development.,ed. MOSCONA,A.A.,

John Wiley & Sons, New York, 67-99, 1974

31. OHTSUKA, K. and Y. TONOMURA. Effects of conditioning of medium on beating of single myocardial cells in vitro. Cell Struc. Funct. 1, 11-21, 1975

32. PARAN, M.L L. SACHS, Y. BARAK and P. RESNITZKY. In vitro induction of granulocyte differentiation in hematopoietic cells from leukemic and non-leukemic patients. Proc. Natl. Acad. Sci. USA. 67, 1542-1549, 1970

33. POSTE, G. Tissue dissociation with proteolytic enzymes. Adsorption and activity of enzymes at the cell surface. Exp. Cell Res. 65, 359-367, 1971

34. POSTE, G., L.W. GREENHAM, L. MALLUCCI, P. REEVE and D.J. ALEXANDER. The study of cellular "microexudates" by ellipsometry and their relationship to the cell coat. Exp. Cell Res. 78, 303-313, 1973

35. PUCK, T.T. and P.I. MARCUS. A rapid method for viable cell titration and clone production with HeLa cells in tissue culture: The use of X-irradiated cells to supply conditioning factors. Proc. Natl. Acad. Sci. USA. 41, 432-437, 1955

36. REIN, A. and H. RUBIN. Effects of local cell concentration upon the growth of chick embryo cells in tissue culture. Exp. Cell Res. 49, 666-678, 1968

37. REIN, A. and H. RUBIN. On the survival of chick embryo cells at low concentrations in culture. Exp. Cell Res. 65, 209-214, 1971

38. ROSENBERG, M.D. Microexudates from cells grown in tissue culture. Biophys. J. 1, 137-159, 1960

39. ROTH, S. Studies on intercellular adhesion selectivity. Dev. Biol. 18, 602-631, 1968

40. RUBIN, H. A substance in conditioned medium which enhances the growth of small numbers of chick embryo cells. Exp. Cell Res. 41, 138-148, 1966

41. SCHACTER, L.P. Effect of conditioned media on differentiation in mass cultures of chick limb bud cells. I. Morphological effects. Exp. Cell Res. 63, 19-32, 1970

42. SHACTER, L.P. Effect of conditioned media on differentiation in mass cultures of chick limb bud cells. II. Metabolic and biochemical effects. Exp. Cell Res. 63, 33-42, 1970

43. SOLURSH, M. and S. MEIER. A conditioned medium (CM) factor produced by chondrocytes that promotes their own differentiation. Dev. Biol. 30, 279-289, 1973

44. STOKER, M. Contact and short-range interactions affecting growth of animal cells in culture. In Curr. Top. Dev. Biol. ed. MOSCONA, A.A. and A. MONROY, Academic Press, New York, Vol. 2, 107-128, 1967

45. STOKER, M. G.D. CLARKE and M. THRONTON. Density dependent stimulation of thymidine incorporation in BHK21 cells by active material released from the same cells. J. Cell Physiol. 78, 345-354, 1971

46. TAKAHASHI, K. and T.S. OKADA. An analysis of the effect of "conditioned medium" upon the cell culture at low density.

Dev. Growth Differ. 12, 65-77, 1970

47. TAKEICHI, M. Changes in the properties of cell-substrate adhesion during cultivation of chicken fibroblasts in vitro in a serum-free medium. Exp. Cell Res. 68, 88-96, 1971

48. WATANABE, K. Changes in capacity for clonal growth and differentiation in vitro of the vertebral cartilage cells with embryonic development. II. Vitalizing effect of conditioned medium of the cells of younger embryos. Dev. Growth Differ. 13, 107-118, 1971

49. WEBER, K. and M. OSBORN. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406-4412, 1969

50. WEISS, L. Studies on cellular adhesion in tissue culture. IV. The alteration of substrata by cell surfaces. Exp. Cell Res. 25, 504-517, 1969

51. WEISS, L. Studies on cellular adhesion in tissue culture. XI. Some effects of Ouabain. Exp. Cell Res. 71, 281-288, 1972

52. WEISS, L., G. POSTE, A. MACKEARNIN and K. WILLETT. Growth of mammalian cells on substrates coated with cellular microexudates. I. Effect on cell growth at low population densities. J. Cell Biol. 64, 135-145, 1975

53. WHITE, N.K. and S.D. HAUSHKA. Muscle development in vitro: a new conditioned medium effect on colony differentiation. Exp. Cell Res. 67, 479-482, 1971

54. WOLLENBERGER, A. Rhythmic and arrhythmic contractile activity of single myocardial cells cultured in vitro. Circ. Res. 14 and 15, Suppl. 2, 184-201, 1964

55. YAOI, Y. and T. KANASEKI. Role of microexudate carpet in cell division. Nature, New Biology. 237, 283-285, 1972

56. YEH, J and H.W. FISHER. A diffusible factor which sustains contact inhibition of replication. J. Cell Biol. 40, 382-394, 1969