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LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY

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IN VITRO STUDIES OF BEATING HEART CELLS IN CULTURE

XVI THE EFFECT OF A HEAT STABLE SERUM FRACTION

ON THE LEVEL OF MYOSIN ADENOSINETRIPHOSPHATASE¹

ISAAC HARARY, KASTURI DATTA, FRANK

HOOVER, BARABARA FARLEY AND ED SATO

LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY
AND THE MOLECULAR BIOLOGY INSTITUTE, UNIVERSITY OF
CALIFORNIA, DEPARTMENT OF BIOLOGICAL CHEMISTRY, UCLA
SCHOOL OF MEDICINE, LOS ANGELES, CALIFORNIA 90024

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Proofs to be sent to: Dr. Isaac Harary

Laboratory of Nuclear Medicine
and Radiation Biology

Developmental Biology Division

900 Veteran Avenue

Los Angeles, California 90024

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SUMMARY

A heat stable serum factor of low molecular weight maintains the myosin ATPase activity of cultured rat heart cells. Its action is directly on the heart cells and does not act by selection of heart muscle cells. It has no effect on heart muscle creatine phospho-kinase or lactic dehydrogenase nor on myosin in skeletal muscle cultures.

The myosin level in neo-natal rat heart cells in culture is affected by the serum level (10% fetal calf and 10% horse serum) of the medium (1). In the presence of serum, with normal cell growth, the myosin level is increased. In the presence of serum and colchicine, to inhibit cell division, the myosin level is maintained and in cultures with no serum, and consequently also no cell division, the myosin level drops drastically. Thus the myosin level is directly dependent on the presence of serum and in its presence the normal one-half life ranges from 3.5 to 6 days (2). The serum appears to contain multiple factors which increase the myosin level by increase of the cellular growth rate, or which maintain the level by stimulation of the synthesis rate or by inhibition of the degradation rate. Fractionation of the serum component has led to the separation of a heat stable fraction (HSF) which maintains the myosin level but does not support growth.

METHODS

Rat heart cells were isolated from neo-natal rats 2 to 4 days old (3) and cultured (5×10^6 cells per 20 x 100 mm plastic dish was used unless otherwise noted) in Ham's F10 (4) or CMRL - 1415 ATM (5) with 10% .

fetal calf and 10% horse serum and antibiotics (complete F10 medium or complete CMRL medium). The cells were also cultured in F10 or CMRL without serum and designated minimal F10 medium or minimal CMRL medium and in minimal medium with HSF (HSF - F10 medium or HSF - CMRL medium).

HSF is prepared by bringing the serum to 100°C, adding the heated serum to the minimal medium and filtering the complete medium. Alternatively the heated serum was centrifuged before addition of the serum supernatant fraction to the medium.

Ca-activated ATPase (Ca-ATPase) and protein were measured as previously described (2). Ca-ATPase activity as measured in this way, corresponds to the myosin level and is therefore designated as myosin-ATPase. Units are expressed as nanomoles of inorganic phosphate produced from ATP per minute. Lactic dehydrogenase was determined according to method of Schwartz and Bodansky (6) and creatine phosphokinase according to Rosalski (7). 5-Bromodeoxyuridine was used according to a modification (8) of the method of Puck and Kao (9).

Rat skeletal muscle cells were obtained from Dr. D. Schubert, San Diego and were a subculture of a line originally isolated by Yaffe (10). They were cultured on Dulbecco modified Eagles medium (DME) (11) with 20% fetal calf serum (complete DME medium). Papain was obtained from Sigma Co. Redistilled reagent grade chloroform and methanol were used in the extraction of HSF.

RESULTS

Action of heat stable serum factor (HSF)

Serum affects the myosin level in three ways; by stimulation of growth (1) by stimulation of synthesis; and by inhibition of degradation (2). To deal with each of these factors separately the fractionation of the serum was attempted.

A heat stable fraction of the serum was prepared which supported the myosin level in the absence of whole serum but which did not support growth. The total activity remained constant in the presence of the heat stable factor, decreased in the absence of serum and increased in the presence of serum (fig. 1a). Growth proceeded normally in complete medium but there was no growth either with or without HSF added to minimal medium (fig. 1b). As a result the relative specific activity (total activity/total protein) was maintained best in the presence of HSF and fell drastically in the absence of serum (fig. 1c). These results have been repeated in 14 experiments.

Since the heart cell culture is a primary preparation which contains other than muscle cells it was not clear whether HSF functioned as a necessary factor for the maintenance of myosin during the incubation period or whether HSF functioned by enhancing the selective attachment of heart cells. To distinguish between these two possibilities the cells were attached in HSF - F10 medium and incubated for 3 days. On the third day the cultures were divided, one part remaining in HSF-F10 medium and the other shifted to minimal F10 medium. The cells remaining in HSF - F10 medium maintained their myosin level while the others showed a drastic loss (fig. 2a).

Other cells plated in complete F10 medium were shifted to HSF - F10 medium and minimal F10 medium. Here too the cells incubated on HSF - F10 medium maintained their activity as compared to the loss in the cells shifted to minimal F10 medium (fig. 2B). Independent of the medium used for cellular attachment and initiation of the culture, the presence of HSF was necessary for the maintenance of myosin.

The question also arises whether HSF could act by selecting and maintaining active muscle cells by inhibiting the growth of non muscle cells. The inability of minimal F10 medium to support myosin in cells plated in HSF - F10 medium would seem to rule this out since there is no cell division in this medium. To test this further, cultures were treated with BUDR for 3 days and irradiated to kill all dividing cells. Cultures treated this way leave functioning heart cells alive which beat and maintain their myosin (8). In addition, for four days post irradiation, there is no cellular increase even in complete growth medium. On the 3rd day immediately upon irradiation, the cells were shifted to HSF - F10 medium, minimal F10 medium and complete F10 medium. Even after being treated with BUDR to remove all dividing cells, the cultures required either HSF or whole serum to maintain the myosin level. (Table 1). Since BUDR treated cultures contain only non-dividing beating cells these results also indicate that HSF directly support the myosin maintenance in beating heart cells. Complete F10 medium supported myosin but not growth in BUDR treated cultures. The serum effect on BUDR cultures was similar to the effect of HSF on intact primary cultures.

To assure further that HSF does not enhance activity by inhibition of division of non muscle cells the action of BUDR on primary cultures was tested directly. BUDR did not prevent the loss which occurred in minimal medium (Table 2), and as such it was not acting like HSF.

Other Proteins

It was of interest to test the effect of HSF on other enzyme activities in the heart cells. Lactic dehydrogenase was chosen as an example of a protein that is generally present in all tissues. There was an increase in total activity in complete F10 medium which depended upon growth. In the minimal medium there was a loss of activity and this loss was not affected to any extent by HSF. Neither the total or relative activity was too different (Table II).

As an example of an enzyme relatively specific for muscle, creatine phosphokinase activity was measured under the same experimental conditions (Table IV). Total enzyme activity increased with growth. In the minimal F10 medium the activity decreased. HSF had little effect on this decrease. Whatever changes occurred in the total activity seemed to depend upon growth since the relative specific activity was very similar under all conditions.

Skeletal muscle cells

Skeletal muscle cells in culture derived from a clone (10) were also tested for the effect of HSF on the myosin level (fig. 3). In contrast to the heart cell, serum had no effect on the maintenance of the myosin level.

Serum was necessary for fusion and growth of the cells. Therefore the increase in total myosin depended upon the presence of serum. In the cells shifted to the minimal DME medium the increase in myosin ceased abruptly but the level was maintained. In cells on HSF - DME medium the myosin was somewhat greater than in cells on serumless medium. However since the relative specific activity was the same in all these cases, it can be concluded that the myosin level paralleled the growth and serum had no role in maintaining the level other than, indirectly, by controlling the growth rate.

Properties of HSF

Some preliminary attempts have been made to ascertain the nature of the heat stable factor. Although the factor is heat stable it did not withstand ashing. It appeared to be non protein in nature since papain (12) did not inactivate it (Table V). It appeared to be water soluble and not soluble in lipid solvents (Table IV) (13,14) and could be dialyzed through dialyzing membrane (Table VII) with a pore size of 24A⁰.

DISCUSSION

It was clear from previous findings that the serum was necessary to maintain the myosin-ATPase level in non dividing cultures (1). When the turnover of myosin was measured directly it was also seen that serum functioned by stimulating its synthesis and protecting against

its degradation (2). Most of the ability to support the myosin level seems to reside in a heat stable fraction of the serum which contains a heat stable non lipid, non protein, dialyzable factor.

It also seems probable that the increase in myosin which depends upon cell division is an indirect result of the presence in the serum of factors which promote the growth and development of the heart muscle cells.

These conclusions are supported by a comparison of the effects of HSF on intact primary cultures with the effect of serum on BUDR treated cultures. HSF - medium does not support growth or cell division in primary cultures although it maintains myosin. Preparation of HSF results in the inactivation of the growth factors, thus HSF acts only on the myosin producing cells and the cells capable of division do not interfere. On the other hand complete medium supports myosin but no growth in BUDR treated cultures because, while both HSF and the growth factors are present in the serum, the growth factors are rendered inoperable by elimination of their target, the dividing cell.

The indication is that HSF acts directly on the heart cells during the incubation period and does not maintain myosin as a result of a selection of cells. Support for these arguments is seen in the fact that BUDR did not replace HSF and in the observation that HSF was active on cells independent of the possible differences brought about by selective attachment of cells in different media.

Serum, containing a multitude of factors, is required for many cell culture systems. In the heart cell culture we have shown that serum lipids are required for beating (13) although they can be substituted

for by fatty acids. It has long been known that serum factors play a role in the contractility of the heart (15, 16). In addition the protein fractions of the serum can support the growth of the heart cells (13) and growth factors for cultured cells (17) have been isolated. Growth hormone and thyroxin have been shown to be important for the growth of the heart (18).

It has also been reported that a serum sulfation factor stimulates the incorporation of amino acids into muscle protein (19) and that insulin and somatotropin stimulate the development of skeletal muscle cells in culture (20). Little work has been done on the control of myosin synthesis and the effect of serum. Thyroxin increases the Ca^{++} activated ATPase activity in the heart (21) but since it has no effect on the K^{+} activated activity it is not clear whether the affect is on enzyme activity or on protein synthesis.

Many factors affect heart cell function. It is clear that identifying them and investigating their mechanism will lead us to better understanding both of how the multicellular organism maintains the integrity of specialized organs and perhaps the factors in the development of the organ. Of all these factors for the heart the most important for function are those that affect the structural protein and those that affect the maintenance of the membrane and its spontaneous activity.

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Table 1. Comparison of HSF and whole serum on BUDR treated heart cells

Total myosin-ATPase per plate				
	Exp. I		Exp. II	
	Day 4	8	4	7
<u>Medium</u>				
HSF	46.3	27.1	44.8	18
Minimal	46.3	5.9	44.8	9.1
Complete	46.3	34.8	44.8	30.7

Rat heart cells were attached and incubated for one day in complete F10 medium after which all cells were shifted to complete F10 medium plus 10^{-3} M BUDR. On the 4th day this medium was replaced with complete F10 medium and irradiated under cold white fluorescent lamp at a 6" distance for 30 min. The cultures were analyzed for enzyme activity and put on the test media indicated until time for enzyme analysis.

Table II. Effect of BUDR on heart cells in minimal medium

<u>Medium</u>	Total Myosin-ATPase per plate		
	Day 3	5	7
Complete + BUDR	45.1	27.7	23.4
Minimal	45.1	6.8	0
Minimal + BUDR	45.1	9.6	3.1

Heart cells were plated directly on complete F10 medium plus 10^{-3} BUDR for 3 days replaced with complete F10 medium and irradiated as in Table I, after which the cells were incubated in the indicated test media.

Table III. Lactic Dehydrogenase in rat heart cells

	Total activity				Relative activity			
	Day 2	5	6	7	2	5	6	8
<u>Medium</u>								
Complete	47.5	333.3	709.5	429.7	121.1	565	774	436
Minimal	47.5	59.2	44.2	14.6		227	211	96.6
HSF	47.5	52.2	75.6	18.0		266	399	120

Cells were plated in complete F10 medium and shifted on the 2nd day to experimental media. For enzyme determination the cells were scraped with 0.067M phosphate buffer in the cold and washed 2 times with the same buffer. The cells were homogenized with a Virtis in the same buffer and centrifuged at 15,000 rpm for 15 minutes and the supernatant was used as the enzyme source. The enzyme activity was determined in an incubation mixture of 0.02 M phosphate buffer pH 7.4, 0.3m M sodium pyruvate, 0.1 μ M NADH and the change in optical density was measured at 340 m μ . Units are expressed as μ Moles per minute.

Table IV. Creatine phosphokinase activity in rat heart cells

	Total activity				Relative activity		
	Day 2	5	6	7	2	6	7
<u>Medium</u>							
Complete	3.65	12.05	12.9	13.6	13.0	10.8	13.0
Minimal	3.65	2.41	1.84	2.3	13.0	5.4	12.0
HSF	3.65	3.13	2.41	3.75	13.0	4.0	8.6

Conditions same as in Table III. Cells from several culture plates were scraped and pooled for each determination. Cells were scraped and washed 2 times in 0.154M KCl. The cells were homogenized in 0.131M KCl, 0.025M Tris buffer pH 7.4 in a Virtis for 30 seconds and centrifuged for 15 minutes at 16,000 g. The supernatant was assayed for enzyme activity (7). The incubation mixture for enzyme activity contained 0.037M $MgCl_2$, 0.001M ADP, 0.02M glucose, 0.008M NADP, 0.01M AMP, 0.005 M cysteine HCl, 0.05M Tris pH 6.8, 0.6 I.U./ml hexokinase, 0.3 I.U./ml glucose-6-phosphate dehydrogenase and 0.01M creatine phosphate. Change in optical density at 340 m μ was measured and the units were defined as μ Moles per minute.

Table V. Effect of papain on HSF

	Total Myosin-ATPase per plate		
	Day 5	6	7
<u>Medium</u>			
Complete	37.2	58.8	67.9
Minimal	5.9	-	3.65
HSF	37.2	44.2	41.7
HSF + papain	17.8	44.5	46.7

5×10^6 Heart cells were attached and incubated in complete F10 medium for 2 days and then shifted on the 2nd day to indicated media. For papain treatment (13) 200 ml of serum was boiled and cooled to room temperature. 2.0 ml cysteine was added to bring the molarity to 0.02M and the pH adjusted to 6.0. 1 ml of papain (3.0 mg/ml) was added and after 1 hour at 37°C the reaction was stopped by boiling. The mixture was used as such to make up the papain treated HSF - F10 medium.

Table VI. Effect of lipid solvent on HSF.

<u>Medium</u>	Total Myosin ATPase Activity	
	Day 7	8
Complete	67.2	58.5
Minimal	11.2	-
HSF	21.3	28.5
Minimal + HSF-lipid	14.2	12.2
Minimal + lipid free HSF	27.5	25.2
Minimal + Combined	23.4	-

Rat heart cells were incubated in complete F10 medium and changed to experimental media on the 2nd day. For lipid extraction, the supernatant obtained from centrifuging the heated serum was lyophilized and the powder was extracted with chloroform-methanol (2:1 v/v) at 25°C three times, washed with the same solution and then extracted with ether two times (14). The residue was dried in a vacuum dessicator for 24 hours and this fraction was designated the lipid free heat stable fraction (lipid free HSF). The chloroform-methanol and ether fractions were combined, evaporated to dryness, washed with water, evaporated once more, taken up in a small quantity of ether and added directly to the culture medium. The ether was removed by passing an air current through the medium. This fraction was designated the lipid extract of the heat stable fraction (HSF-lipid).

Table VII. Effect of Dialysis on HSF

	Total myosin-ATPase per plate		
	Day 5	6	7
<u>Medium</u>			
Complete	59	61.9	69.7
Minimal	36.1	29.5	29.5
HSF	59.9	50.9	38.5
Minimal + inside HSF	35.7	25.1	31.3
Minimal + outside HSF	53.6	50.9	58.1

Heart cells were plated in complete CMRL medium and transferred to the designated medium on the 2nd day. The dialysis was performed on a lyophilized sample of HSF dissolved in 40.0 ml of minimal CMRL medium pH 7.0, and dialyzed against 1 liter of minimal CMRL medium for 24 hours at 4°C. The solution remaining within the dialysis bag was designated as inside HSF and that in the dialysate as outside HSF.

Fig. 1. Rat heart cells were incubated in complete CMRL medium and shifted on the second day to fresh complete CMRL medium (closed circles), minimal CMRL medium (open circles) and to HSF-CMRL medium (open triangles). The experimental media were renewed every two days. Myosin-ATPase activity was expressed as total units per plate, protein as mgm per dish, and relative specific activity as total enzyme activity per total protein.

Fig. 2 (a) Rat heart cells were attached and incubated for three days in HSF-F10 medium and shifted to HSF-F10 medium (closed circles) and to minimal F10 medium (open circles). (b) Cells were attached in complete F10 medium and after one day shifted to HSF - F10 medium (closed circles) and to minimal F10 medium (open circles).

Fig. 3. Skeletal muscle cells were plated in Dulbecco's modified Eagles medium with 20% fetal calf serum and incubated in 5% CO_2 at 37°C . On the seventh day the medium was changed to minimal DME medium (open circles) HSF-DME medium (open triangles) and complete DME medium (closed circles). The media was changed every 2 days. The first signs of fusion occurred on the 4th day. a) Total myosin activity per dish b) Relative specific activity (total activity/total protein).

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