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CHICK EMBRYO CARDIOCYTES IN CULTURE SYNTHESIZE PROTEINS NOT PRESENT IN EMBRYONIC HEART

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SUMMARY - Proteins synthesized by cardiocytes isolated from 11d embryonic hearts and cultured in vitro have been compared with proteins present in the 11d embryonic heart. It is shown that cultured cardiocytes synthesize two new proteins, C_1 and C_2 with a molecular weight of 27 500 and pI of 6.35 and 6.05 respectively. The synthesis of these proteins is associated with the appearance of new RNA species. This induction is not related to the conditions of culture since it occurs with either aggregated cardioacytes grown in suspension or cardiocytes grown in monolayer. Finally no other embryonic cell types seem to synthesize these proteins.

Tissue culture of cells isolated from specific organs has been widely used over the past decades and is thought to be an important tool in the understanding of the mechanisms which control expression of specific programmes of differentiation. However, these tissue cultures can only serve as model systems as long as they are a true representation of the in vivo situation. In this communication we wish to report on chick embryo cardiocytes in culture. We will show that these cells, although they do express the major cardiac proteins also synthesize new proteins which are not found among the proteins accumulated in the heart tissue. However, these proteins are only synthesized by cultured cardiocytes and not by other chich embryo cell types.

MATERIALS AND METHODS

Cell culture - Cardiocytes were isolated from 11d chich embryonic heart according to De Haan (1). Hearts were dissected free of vessels, minced with scisors and subjected to three successive trypsinizations of 10 minutes each. Debris and undissociated tissue were eliminated by low speed centrifugation. Cells in suspension were seeded at 10^7 cells on 10 cm Falcon petri dishes. After two hours, the cells remaining in suspension were collected, and seeded at 2-3 x 10^6 cells per 5 cm Falcon petri dishes and incubated at 37°C in an air-CO_2 humidified incubator. Next morning the medium was changed, which eliminated debris and dead cells, and the culture was further incubated without changes of medium.

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Extraction of RNA and in vitro translation - RNA was extracted from cultured cardiocytes as already described (2). For 11d embryonic hearts, 1 g of frozen tissue was homogenized in 8 ml 6M urea/3 M LiCl in a Waring blender for three times 20 seconds. This homogenate was kept at 4°C for 48 hours. The RNA was extracted from the precipitate as described for RNA isolated from cultured cells (2). These RNAs were characterized by in vitro translation using the nuclease-treated reticulocyte lysate (3) as already described (4).

Two-dimensional gel electrophoresis - Cell extracts or products from in vitro translation were prepared for 2 D gel electrophoresis as already described (5) 2 D gel electrophoresis was performed according to O'Farrell (6) as previously described (5).

RESULTS AND DISCUSSION

Cardiocytes in culture synthesize new proteins - As shown in Figure 1, when a total cell extract of chick embryonic heart is analyzed by 2 D gel electrophoresis (Fig. 1A) one can easily detect most of the proteins which constitute the contractile apparatus of this muscle, such as cardiac myosin heavy chain, cardiac myosin light chains LC_1 and LC_2, α-actin and α-tropomyosin. Also recognizable are the α and β desmins and the β isozyme of creatine phosphokinase which migrates as two variants. It has to be noted that on the acidic side of LC_1 and with a slightly higher molecular weight is a protein, the nature of which is unknown, which is also present in other embryonic tissues. This protein will be further referred to as protein X.

When cardiocytes isolated from 11 d embryonic heart and maintained in culture are labelled with ^{35}S-methionine and the total cell extract analysed by 2 D gel electrophoresis (Fig. 1 B) most of the same proteins can also be detected. Among them are the already mentioned contractile proteins : cardiac myosin heavy chain, cardiac myosin light chains LC_1 and LC_2, α-actin and α-tropomyosin ; α and β desmin and the β isozyme of creatine phosphokinase. This result is in good agreement with previous reports which have shown that cultures of cardiocytes consist of differentiated cells which express heart specific proteins and which beat spontaneously (7-10).

However when figure 1B is compared with figure 1A a few differences clearly show up : firstly protein X is not synthesized, and secondly two new proteins with approximately the same molecular weight (about 27 500) and appa-

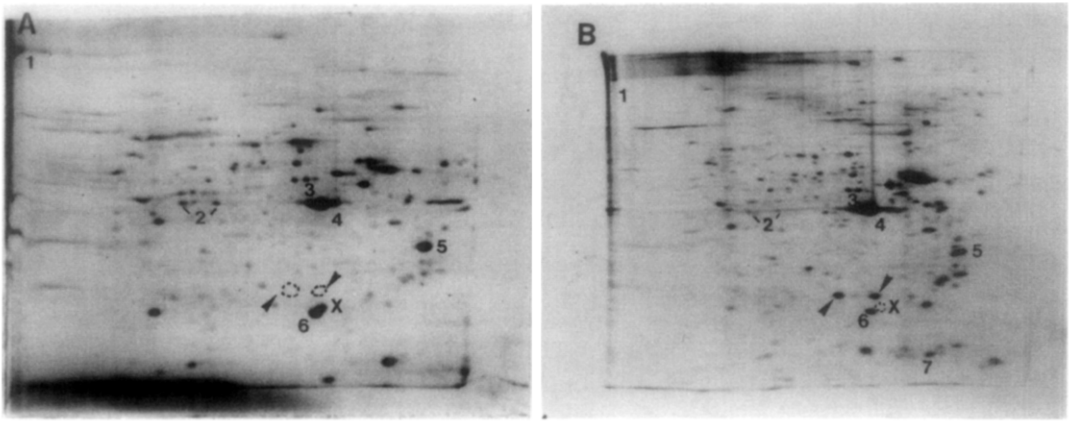

Figure 1 : 2 D gel electrophoresis of proteins present in heart extract or synthesized by cultured cardiocytes

The extract in (A) was prepared by homogenization of 11d. embryonic heart as described elsewhere (D. Montarras et al., to be published) and was subjected to electrophoresis on isoelectric focussing gel over pH range 5-8, followed by SDS poylacrylamide gel electrophoresis on 12.5 % acrylamide gel. The gel was stained with Coomassie brilliant blue. In (B) cardiocytes were isolated from 11d embryonic heart and maintained in culture. After 2 days cells were labelled for 2 hours with ^{35}S-methionine (50 µCi/ml) in a methionine deficient medium. Total cell extracts were prepared as previously described (5) and analysed as in (A). The gel was dried and subjected to autoradiography.

Numbers refer to : (1) - myosin heavy chain ; (2) - B isozyme of creatine kinase ; (3) - β and α-desmin ; (4) - α-actin ; (5) - α-tropomyosin ; (6) myosin light chain LC_1 (7) - myosin might chain LC_2.

rent pI of 6.35 and 6.05 are synthesized by cardiocytes in cultures but are not present in embryonic heart. For convenience we have called these proteins C_1 and C_2.

In vitro translation of messenger RNA present in heart and cultured cardiocytes

Since in one case, the heart extract, we are dealing with stable proteins while in the other we are looking at proteins labelled during a pulse of two hours, it is possible that the difference we are detectingis due to the fact that C_1 and C_2 are unstable proteins which never accumulate. To test this hypothesis we have analysed by in vitro translation both eübryonic heart mRNA and cultured cardiocyte mRNA. Total RNA was isolated from 11d embryonic heart and cytoplasmic RNA was isolated from cardiocytes cultured for 24 hours. These two sets of RNA were translated in vitro using nuclease-treated reticulocyte lysate (3-4)

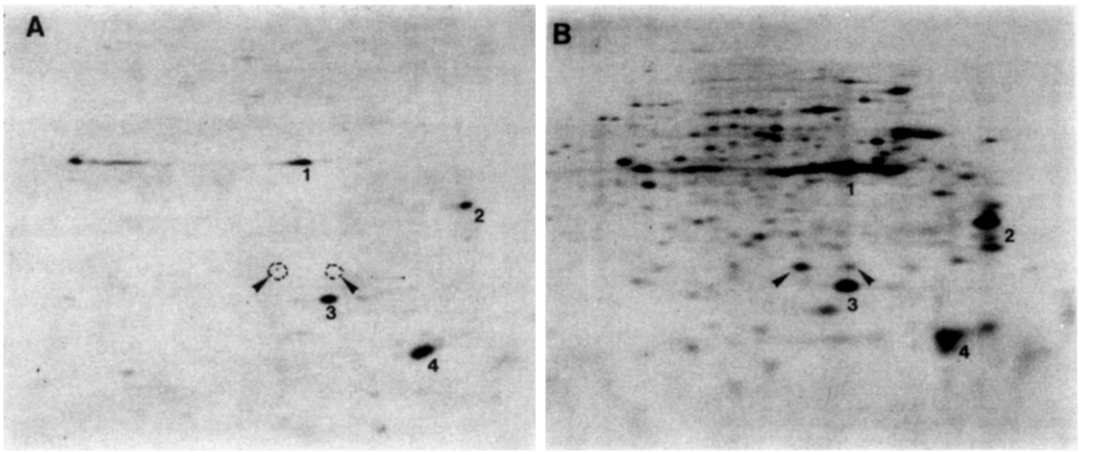

Figure 2 : In vitro translation analysis of 11d embryonic heart RNA and cultured cardiocytes RNA.

RNA isolated from either 11d embryonic heart (A) or cultured cardiocytes (B) was translated in vitro with the use of reticulocyte lysate as previously described (4). The products were analysed by 2 D gel electrophoresis. The gels were dried and subjected to autoradiography.

Numbers refer to : (1) - α-actin ; (2) α-tropomyosin ; (3) myosin light chain LC_1 (4) myosin light chain LC_2.

and the products synthesized in vitro were analysed by 2 D gel electrophoresis. Fig. 2 A presents the analysis of the products translated from heart RNA while fig. 2B presents the products translated from cultured cardiocyte RNA. When the two autoradiograms are compared it is evident that the general pattern of the in vitro translation products is simpler in the case of embryonic heart RNA than in the case of cardiocyte RNA. In the former, there are essentially four major products : α-actin, cardiac myosin light chains LC_1 and LC_2 and α-tropomyosin, while in the latter, whilst these four proteins are indeed among the major products, they are surrounded by numerous others. The reason for this difference is not clearly understood although it could simply reflects the fact that in embryonic heart there is a much larger accumulation of messenger RNAs coding for the major contractile proteins. Among the products synthesized by messenger RNAs are the two proteins C_1 and C_2 present in cultured cardiocytes. These proteins are not detected among the in vitro products translated from heart RNA even after a much longer period of exposure than the one which is presented. When one compares the autoradiograms presented in figure 2B and 1B

it is striking to note that, in culture, the rate of synthesis of the two proteins is identical (fig. 1B) while the amount of mRNA does not seem to be the same (fig. 2B). This suggests either that there is some kind of translational control or that C_1 and C_2 are two forms of the same protein, with C_2 being a modified form of C_1. In that case the modification would have to be partially made in the reticulocyte lysate. Which ever interpretation is correct, this experiment further strengthens the conclusion that cardiocytes in culture synthesize proteins which are not normally present in the embryonic heart.

Presence of C_1 and C_2 in relation with different culture conditions - One possible explanation for such an induction of new proteins could reside in the new organization that the cells have to acquire when they are cultured *in vitro* as compared to the situation which exists in the tissue. For example, on a Petri dish, the cells are growing as a monolayer and therefore the spatial organization and the cell to cell contacts are completely different from those in a three dimensional structure such as in a tissue. To test this hypothesis we have compared cardiocytes grown under normal conditions and cardiocytes which have aggregated and have been either maintained in suspension or allowed to settle on a Petri dish. These different cultures were labelled 24 or 48 hours later and total cell extracts were analysed by 2D gel electrophoresis. The amount of radioactivity corresponding to C_1 and C_2 was determined after elution of the appropriate regions of the gels. The result of this experiment is presented in Table 1.

24 hours after seeding, cardiocytes cultured under normal conditions synthesize C_1 and C_2 with the same rate of synthesis. The amount of radioactivity incorporated in C_1 or C_2 represents approximately 0.5 % of the radioactivity incorporated into total proteins. The same is true when the analysis is performed after 48 hours. When cardiocytes are allowed to aggregate and maintained in suspension not only do they synthesize C_1 and C_2 but the rate of synthesis is identical to the one obtained with cardiocytes cultured on Petri dishes. Lastly, when the aggregate are allowed to settle, they also

TABLE I

	time (hrs)	C_1 cpm	C_1 %	C_2 cpm	C_2 %
Cardiocytes monolayer	24	5010	0.50	4805	0.48
	48	4575	0.46	4600	0.46
Aggregates maintained in suspension	24	5105	0.51	5110	0.51
	48	4800	0.48	4750	0.47
Aggregates allowed to attach to tissue culture Petri dish	24	4870	0.49	4780	0.48
	48	4650	0.46	4680	0.47

Synthesis of C_1 and C_2 by cardiocytes under various conditions of culture

11d embryonic hearts were used to isolate cardiocytes according to De Haan (1). One third of these cardiocytes were plated on Petri dishes and incubated at 37°C in an air-CO_2 humidified incubator. Cells were pulse labelled for 2 hours with ^{35}S-methionine (50 µCi/ml) either 24 or 48 hours after seeding.

The remainder was allowed to aggregate in a gyrotory bath according to the technique described by McDonald et al. (11). 18 hours later the aggregates were collected by low speed centrifugations ; half of the populations was further incubated in suspension while the other half was allowed to attach to the bottom of tissue culture Petri dishes. These aggregates were pulse labelled for 2 hours with ^{35}S-methionine (50 µCi/ml) at the same time as the attached cardiocytes.

Total cell extracts containing 10^6 cpm were analysed by 2 D gel electrophoresis Proteins C_1 and C_2 were located by autoradiography, cut out of the dried gels, soaked in 50 µl of water for 60 minutes and incubated overnight in a scintillation cocktail containing 10 % NCS. The radioactivity was determined using an Intertechnique Scintillation Counter. Each point was corrected (1) for background level and (2) for the efficiency of elution as already described (5).

synthesize C_1 and C_2 with the same rate of synthesis. Therefore it does not seem that the induction of these new proteins is related to the conditions under which cardiocytes are cultured. However, it could reflect some kind of reaction of the cells to the disruption of their original environment.

Induction of C_1 and C_2 is restricted to cardiocytes - An alternative hypothesis would be that these proteins belong to another cell specific program or represent merely an induction which takes place every time an embryonic cell is taken from the embryo and set into culture. To test the first part of this hypothesis we have checked whether heart fibroblasts, which represent the major contamination of cardiocyte cultures, also synthesize C_1 and C_2. To do this experiment, we compared the synthesis of C_1 and C_2 with either cardiocytes or the cells which have attached during the preplating step. As shown in

TABLE II

	% cells stained with the antiserum	C_1		C_2	
		cpm	%	cpm	%
Cardiocytes	75 ± 10	4800	0.48	4950	0.5
Heart fibroblasts	15 ± 5	510	0.05	400	0.04

Synthesis of C_1 and C_2 by cardiocytes and heart fibroblasts

Cardiocytes were prepared as described in Materials and Methods and the fibroblastic culture consisted in the cells which attached during the preplating step.

Both cultures were used on the second day after seeding. Cells to be stained with the antiserum were fixed in a mixture of ethanol-acetone (1/1) at 0°C and processed as described elsewhere (12).

For the measure of C_1 and C_2, the cultures were labelled for two hours with ^{35}S-methionine (50 µCi/ml) and processed as described in the legend of table 1. In each case 10^6 cpm were loaded on the gel for isoelectric focussing.

Table II, the cardiocyte culture contains 80 % cells which can be labelled with an antiserum prepared against cardiac myosin. In this culture C_1 or C_2 represent approximately 0.5 % of the total proteins synthesized during a two hour pulse. On the other hand, the cells which attach very rapidly contain approximately 10-15 % cells which react with the antiserum and C_1 or C_2 represent less than 0.05 % of the total proteins synthesized during a two hour pulse. This experiment therefore strongly suggests that cardiocytes, and not fibroblasts are the only cells which significantly contribute to the synthesis of C_1 and C_2. As for the second part of the hypothesis, we have up to now been unable to detect the synthesis of C_1 or C_2 with other embryonic cell types such as hepatocytes, smooth muscle cells isolated from gizzard or aorta, skeletal myoblasts or various types of fibroblasts (data not presented).

In conclusion we have provided evidence that cardiocytes in culture synthesize two proteins which have been called C_1 and C_2 which are not accumulated in 11d embryonic heart. The synthesis of these proteins is corrolated in cultured cardiocytes but not in embryonic heart with the presence of specific messenger RNA as evidenced by in vitro translation assay. Finally the synthesis of these proteins is not related to the culture condition of the cardiocytes,

and it seems safe to assume that only cardiocytes synthesize these proteins; Experiments are now in progress to understand more completely the origin and function of C_1 and C_2.

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