Department of Anatomy (Prof. Y. SANO), Kyoto Prefectural University of Medicine,
Kyoto, Japan

Self-Differentiation of the Heart Rudiment of the Newt (*Cynops pyrrhogaster*)

Fumio IDE

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Summary. In a previous study, the cultured heart rudiment of the Japanese newt embryo of stage 15 (early neurula) could be differentiated into beating myocardial cells in a ROSE's chamber filled with BARTH and BARTH's inorganic solution with the addition of 10% calf-serum. With the use of the inorganic solution free of serum, however, even the material after stage 22 (early tail-bud) could not be differentiated.

In the present work, culture of the heart rudiment of the embryo from stage 16 to 22 was performed in fine glass capillary tubes filled with a BARTH and BARTH's salt solution free of calf-serum. At stages 21 and 22 the heart rudiment differentiated successfully into beating myocardial cells. It was thus known that the heart rudiment at stage 21 already attains its self-differentiating potentiality, because there was obviously no effect of other cellular substances in this case.

In the culture performed in the glass capillary, diffusion of the metabolites of the cultured cells was limited since the culture medium was very small in quantity. Therefore, differentiation is believed to have been brought about by the medium thus conditioned in the capillary.

In a strict sense self-differentiation of an organ rudiment means that the rudiment can differentiate into a mature organ without any kind of influence from other tissues surrounding it. It means, namely, the differentiation of the organ rudiment by itself.

Self-differentiation of mesodermal organs has generally been investigated by three experimental methods: 1) transplantation method, 2) explantation method with an ectoderm covering, called "a ball culture," and 3) common tissue culture method.

In the transplantation method, influence from surrounding tissue is not negligible for organ differentiation. On the other hand, in the explantation method with an ectoderm covering, the covering epidermis may have influence upon the mesodermal organ rudiment. Because of this the most suitable method seems to be the ordinary tissue culture method in which the organ rudiment itself can be observed to differentiate. However, culturing amphibian mesodermal tissue is not easily accomplished successfully.

AMANO and IDE (1969) obtained successful results in which the heart rudiment of the Japanese newt differentiated into myocardial cells with rhythmic contractions. In this case, however, the medium consisted of BARTH and BARTH's solution containing 10% calf-serum. A possible effect of the calf-serum was not excluded in the differentiation of the heart rudiment.

The aim of the present experiments, therefore, was to determine whether or not the heart rudiment differentiates into myocardial cells in a medium of BARTH and BARTH's solution free of calf-serum.

BARTH and BARTH's (1959) salt solution with no organic substances such as calf-serum was used as the culture medium. The heart rudiment was inserted in a fine glass capillary in order to limit the culture medium, because according to TWITTY's experiment (1944), limitation of the culture medium is the most effective for the differentiation of amphibian embryonic cells. It was reported that the embryonic heart cells cultured in the fine glass capillary differentiated into myocardial cells with the ability to contract rhythmically.

Materials and Methods

Embryos of Japanese newts (*Cynops pyrrhogaster*) from early neurula (stage 16) to early tail-bud (stage 22) were used as materials. These developmental stages were referred to in OKADA and ICHIKAWA's standard table (1949). Extirpation of the heart rudiment was carried out according to the methods outlined in the previous report by AMANO and IDE (1969). The heart rudiment consisted of 80–100 cells.

The procedures of cultivation in the fine glass capillary were as follows: The inner surface of the fine glass capillary was coated with silicon oil in order to prevent the sticking of cultured cells to the glass surface. The heart rudiment was sucked into glass capillary (0.3 mm in diameter, 20 mm in length) with BARTH and BARTH's salt solution.

Results

The heart rudiment of the newt embryo from stages 16 to 22 was cultured in BARTH and BARTH's solution in a ROSE's chamber as the control experiment in the present study. In all cases the cells became a cluster within 20–30 min and clustered cells, especially in early stages, dispersed 24 hrs after operation. In later stages, these cells remained in loose contact with one another without dispersion. After 3-day's cultivation, however, every cell in the culture was dead.

On the other hand, the cultured heart rudiment in the fine glass capillary became a compact cell mass 20–30 min after operation (see Fig. 1). After 24 hrs the outer part of the compact cell masses began to disperse gradually due to their own moving activity with the aid of pseudopodia. The number of moving cells in the early

stages was larger than those in the late stages.

Fig. 1. Compact mass (A) of heart rudiment in a fine glass capillary, 1 hr after isolation. $\times 100$

Fig. 2. A cluster (A) and dispersed cells (B) in a fine glass capillary, 3 day culture. $\times 100$

Fig. 3. Beating pace maker tissue (A) and myocardial tissue (B) with pericardium tissue (C). $\times 150$

In the glass capillary culture of the heart rudiment from stages 16–20, many dispersed cells lost their moving activity and died in 48–72 hrs. Even larger aggregated cells of the later stages became inert after 72 hrs. Thus, the heart rudiments from stage 16–20 could not show any structural sign of muscular differentiation.

On the contrary, the heart rudiments from stages 21 and 22 showed successful differentiation in terms of the contraction of myocardial cells (see Table 1). The myocardial cells developed from the initial cell aggregation (Fig. 2). In the case of stage 21, the myocardial cells began to beat on the 9th day of culture, and in stage 22, they began to beat on the 4th and 5th days, respectively (Fig. 3).

As shown in Table 1, only the heart rudiments of stage 21 and 22 embryos could be self-differentiated into contractive myocardial cells in 70 experiments performed in this study for stages from 16 to 22. Although the ratio of the successful results was 21% (3/14) for stage 21 and 25% (2/8) for stage 22, these percentages were considered to be highly significant.

Furthermore, two groups of different myocardial cells were found in the culture in 4 out of 5 successful cases; one with its independent beating ability, the other with

Table 1.

Stages	Number of experiments	Beating myocardial cells	Non-beating myocardial cells
16	2	0	2
17	12	0	12
18	11	0	11
19	11	0	11
20	7	0	7
21	17	3	14
22	10	2	8
	70	5	65

a dependent one. However the rhythmic contraction of the latter depended upon the regulating potentiality of the former. In the remaining one case, a cell group without beating (C in Fig. 3) was observed besides the two groups (A, B in Fig. 3).

Discussion

How did the heart rudiment differentiate in an inorganic salt solution?

First, TWITTY (1944) succeeded in differentiating the pigment cells from the amphibian neural plate by culture in fine glass tubes filled with a physiological salt solution. SANFORD et al. (1948) cultured mouse connective tissue in narrow glass tubes and succeeded in differentiating it into clonal strains. These results suggested that a small amount of culture solution is suited for the life of cultured cells. The result in the present investigation confirmed this fact.

EAGLE and PIEZ (1962) demonstrated that low molecular metabolites synthesized in cultured cells diffuse into the medium surrounding the cells. The success of TWITTY (1944) in the cell differentiation using fine glass capillary tubes containing inorganic salt solution indicates that the media in a capillary will be conditioned with the metabolites issued by the cultured cells. In the present experiment the heart rudiments could differentiate into myocardial cells in the inorganic salt solution conditioned with the metabolites of cultured cells.

Whenever stage 25 heart rudiments, in which the fate of cells should be more clearly destined than in the present materials, were cultured in the open state of ROSE's chamber containing BARTH and BARTH's solution, they could not survive more than 3 days. This fact suggests that their own metabolites are necessary for cell differentiation of newt heart rudiments beyond than stage 21.

Our previous experiments (AMANO and KOBAYASHI 1966a, b, IDE and AMANO 1969) indicated that the organic differentiation usually is conditioned by an interaction between organ rudiment and surrounding tissue. The influence from surrounding tissue is generally termed "the induction" and the alteration inside the organ rudiment is understood as "the determination". In mesodermal organs this kind of determination is known as "gradual determination".

JACOBSON (1961) mentioned that the determination of newt heart is carried out by some outer influences over a long period. We consider, however, that the determination must be caused by an internal alterant of the organ rudiment, i.e. certain

physiological changes of the rudiment itself.

We further advocate three critical steps in the development of newt heart rudiments. In the first step the heart rudiment of the lateral mesoderm at the gastrula stage, just after invagination, could not differentiate into beating myocardial cells without the effect of the side wall of the archenteron, even though it is situated in the ectodermal encystation (AMANO and KOBAYASHI 1966, b, JACOBSON and DUNCAN 1968). When the embryo becomes neurula, the heart rudiment is able to differentiate in the ectodermal encystation without the effect of endodermal archenteron side wall (AMANO and IDE 1969). This state is regarded as the second step. In the early stage of the tail-bud, the heart rudiment cultured in an inorganic salt solution alone can differentiate into myocardial cells that are able to beat by themselves without any influence from the outside. This solution is believed to have been conditioned with the metabolites of the cultured cells.

Self-differentiation means that rudiment cells differentiate into certain organ cells only by the effect of their interaction. The present work proved that at the early stage of tail-bud the rudiment of the newt can self-differentiate into beating myocardial cells. The differentiation of the heart rudiment of stage 21 is free of inductive influence from the endodermal archenteron or ectodermal epidermis surrounding it, and fully deserves the designation of self-differentiation.

Although the actual number of cells of the heart rudiments of all the stages adopted in the present work was not estimated, it seems likely that the 80–100 cells cultured in our experiment may be enough for self-differentiation into the whole heart. The same result is believed to be obtainable from materials of much earlier developmental stages under the same conditions even if much larger rudimental fragments were used than those studied in the present work. Besides the above mentioned point, if a greater number of cells were used for culturing, other organ rudiments may possibly begin to differentiate at the same time.

Non-beating cells were observed throughout this experiment and are labeled C in Figure 3. The same cells were observed also in the study by IDE and AMANO (1969) and assumed to become pericardial cells. The same cells were also detected by JACOBSON (1961) who showed some non-beating cells differentiated in connection with beating myocardial cells. He ascribed the occurrence of these cells to the fact that the movement of mesodermal prospective heart cells could not be controlled well by the ectodermal epidermis covering.

In Figure 3, the beating rate of A and B was different from each other. Considered from the beating number, the cells of part "A" are believed to act as the pacemaker. This fact is considered to suggest that the determination of partial differentiation of the heart already occurs earlier than stage 21.

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イモリの心臓原基の自律分化についての研究

井手章夫

イモリの心臓原基を ROSE の培養器を用いて BARTH の無機塩液に仔牛の血清を加えた培養液の中で培養すると、ステージ 15 (神経胚初期) 以降であれば、原基のみで搏動する心筋細胞に分化し得る。しかし血清を除いた無機塩培養液の中では、少なくともステージ 22 (尾芽胚中期) に至っても搏動する心筋細胞に分化することはできない。

本実験は血清を含まない BARTH の無機塩のみの培養液で、ガラス細管の中でステージ 16 から 22 の心臓原基の培養を行なった。その結果、ステージ 21 と 22 で搏動する心筋細胞に分化することがわかった。この場合原基以外の細胞からの物質的影響がないことは明らかであり、ステージ 21 の心臓原基がすでに自律分化能を確立しているといえる。

またガラス細管を用いることによって、培養液を極端に少なくした環境では、原基細胞の出す代謝産物の拡散が僅少にとどまり、培養液がコンディションされるため、組織の分化が進むものと考えられる。

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井手章夫
〒602 京都市上京区河原町通店小路
京都府立医科大学
第一解剖学教室

Fumio IDE
Department of Anatomy
Kyoto Prefectural University of Medicine
Kawaramachi-Hirokoji
Kyoto, 602 Japan