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A method for rapid preparation of single-cell suspensions from rat hepatocyte primary cultures on collagen substratum and the mechanism of cell dissociation.

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A method for rapid preparation of single-cell suspensions from rat hepatocyte primary cultures on collagen substratum and the mechanism of cell dissociation.*

Masahiro Miyazaki, Yasunori Suzuki, and Jiro Sato

Abstract

A method has been developed for the rapid preparation of single-cell suspensions from rat hepatocyte primary cultures on collagen substratum. Hepatocytes were adequately dissociated into single cells when the cultures were first treated with a combination of trypsin and ethylenediaminetetraacetic acid (EDTA) and then with collagenase. However, when the order was reversed, hepatocytes were inadequately dispersed. The possible mechanism of cell dissociation is discussed on the basis of the experimental data obtained.

KEYWORDS: primary culture, rat hepatocytes, collagen substratum, cell dissociation

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—Brief Note—

A Method for Rapid Preparation of Single-Cell Suspensions from Rat Hepatocyte Primary Cultures on Collagen Substratum and the Mechanism of Cell Dissociation

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A method has been developed for the rapid preparation of single-cell suspensions from rat hepatocyte primary cultures on collagen substratum. Hepatocytes were adequately dissociated into single cells when the cultures were first treated with a combination of trypsin and ethylenediaminetetraacetic acid (EDTA) and then with collagenase. However, when the order was reversed, hepatocytes were inadequately dispersed. The possible mechanism of cell dissociation is discussed on the basis of the experimental data obtained.

Key words : **primary culture, rat hepatocytes, collagen substratum, cell dissociation**

Collagen, one component of the extracellular matrix, is known to promote attachment and spreading of hepatocytes in serum-free primary culture (1). Various studies using hepatocyte primary cultures often require the dissociation of cultured hepatocytes into a single-cell suspension, *e. g.*, for determination of the cell number. Hepatocytes cultured on standard plastic substratum can be easily dissociated into single-cell suspension by incubation at 37°C for a few minutes with a combination of trypsin and ethylenediaminetetraacetic acid (EDTA) (2). On the other hand, hepatocytes cultured on collagen substratum are not dispersed in such a short time. In this communication, we describe a method for the rapid preparation of single-cell suspensions from rat hepatocyte primary cultures on collagen substratum, and discuss the possible mechanism of cell dissociation.

As reported previously (2), hepatocytes, having an initial viability of 85% to 90% as measured by trypan blue exclusion, were isolated from Donryu male rats (3-month-old) by liver perfusion with type I collagenase (Sigma Chemical Co., St. Louis, MO., USA). The culture medium used was DM-160 (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) supplemented with penicillin (Meiji Seika Co. Ltd., Tokyo, Japan) at 100 U/ml, streptomycin (Meiji Seika) at 100 μg/ml, fungizone (Sankyo Co. Ltd., Tokyo, Japan) at 1 μg/ml, dexamethasone sodium phosphate (Banyu Pharmaceutical Co. Ltd., Tokyo, Japan) at 10 μM and insulin (Sigma)

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at 10 μg/ml. Rat tail collagen (RTC, type I) gels were prepared by a slight modification of the method of Michalopoulos and Pitot (3), and used for coating plastic dishes (60 mm in diameter, Falcon, Oxnard, CA, USA). After sterilizing 1 gram of RTC fibers by incubation in 80% ethanol for 3 h, the fibers were then dried under UV irradiation for 1 to 2 h. The fibers were then dissolved in 300 ml of 0.017 M acetic acid by stirring at 4℃ for 48 h, and the solution was centrifuged at $1,670 \times g$ for 10 min. The supernatant solution was used as the stock RTC solution. Plastic dishes were flooded with 1 ml of the solution and left to stand for 1 h at room temperature. The excess solution was then aspirated off, and the dishes were dried overnight at 37℃. Before use for cell culture, the RTC-coated dishes were rinsed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS).

The isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells in 4 ml of medium onto RTC-coated dishes, and maintained in a humidified atmosphere of 5% CO_2 at 37℃. By 24 h after inoculation, hepatocytes attached themselves firmly to RTC-coated dishes and formed monolayers. Such 24-h cultures were used for the dissociation experiments. The solutions used for the dissociation experiments were: PBS, 0.02% EDTA (Sigma) in PBS, 0.1% trypsin (1 : 250, Difco, Detroit, Mich., USA) in PBS, 0.1% trypsin in PBS containing 0.02% EDTA, 0.05% collagenase in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution supplemented with 5 mM $CaCl_2$ and 10 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma) (0.05% collagenase solution, pH 7.5), and 0.025% collagenase solution containing 0.05% trypsin and 0.01% EDTA.

The tested procedures for cell dissociation are summarized in Fig. 1. All the cultures were first washed once with 4 ml of PBS. Some cultures were further washed once with 4 ml of 0.02% EDTA. Then the cultures were treated at 37℃ with various

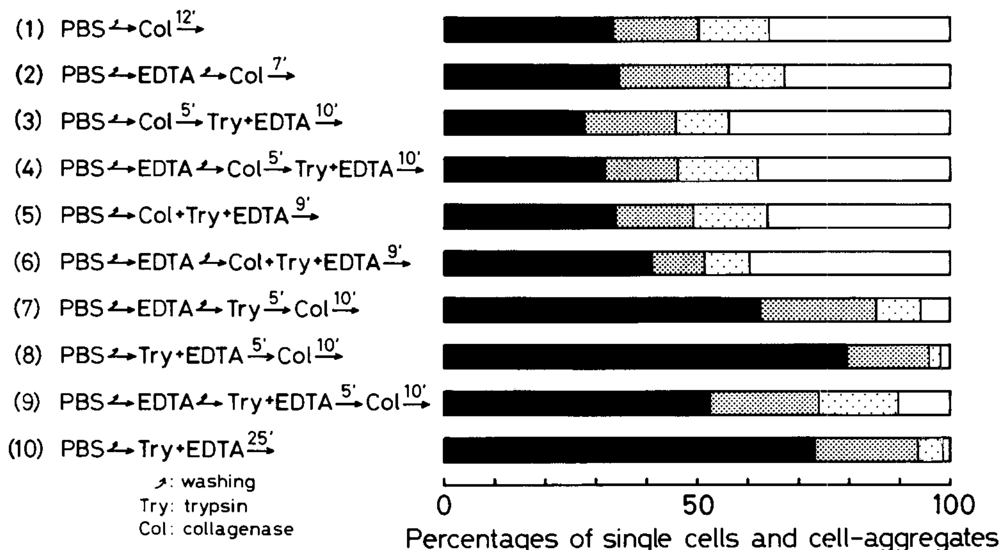

Fig. 1 Percentages of single cells and cell-aggregates in suspensions prepared by various procedures from rat hepatocyte primary cultures on collagen substratum. The numbers above the arrows show the incubation time in min. Results are expressed as the mean of three experiments. ■, Single cells; ▒, 2-cell aggregates; ░, 3-cell aggregates; □, aggregates formed of more than 3 cells.

enzyme solutions. Two ml of each enzyme solution was added per dish. The final enzyme treatment in each procedure was continued until the hepatocyte monolayer was detached by gently swaying the dish. Immediately after the final enzyme treatment, 2 ml (procedures 1, 2, 5, 6 and 10) or 4 ml (procedures 3, 4, 7, 8 and 9) of DM-160 medium supplemented with 20% bovine serum (prepared in this laboratory) was added to each dish to inhibit further proteolytic activity of the enzymes. Then the suspensions were gently pipetted to disperse the hepatocytes. The numbers of single cells and cell aggregates in suspensions were counted and summed to determine the percentages.

When the cultures were treated with collagenase alone (procedure 1), the hepatocyte monolayer was detached from the RTC-coated dishes, but the cells were not dispersed adequately. Neither washing with EDTA before treatment with collagenase (procedure 2) nor treating with a combination of trypsin and EDTA after collagenase treatment (procedure 3) affected cell dissociation. Even treatment with a combination of collagenase, trypsin and EDTA resulted in inadequate dissociation of hepatocytes (procedure 5). In other words, when the collagenase treatment preceded or progressed simultaneously with the trypsin treatment, most of the hepatocytes in monolayer detached from dishes as a sheet, and the detached cells formed large aggregates. These cell aggregates could not easily be dissociated into single cells by subsequent trypsin treatment and pipetting. On the other hand, when treatment of the cultures with trypsin alone or in combination with EDTA preceded the collagenase treatment, adequate dissociation of hepatocytes occurred (procedures 7-9). Although treatment of the cultures with the combination of trypsin and EDTA alone (procedure 10) also caused cell dissociation as efficiently as procedure 8, it took a longer time (25 min). It is known that trypsin damages the plasma membrane of hepatocytes more severely than collagenase when they are used to isolate cells from liver (4, 5). Also, in the present study, hepatocytes dissociated by procedure 10 exhibited much more frequent bleb formation on their plasma membranes and much higher uptake of trypan blue than those dissociated by procedure 8 (data not shown). Thus, procedure 8, which includes a 5-min treatment with the trypsin and EDTA mixture followed by a further 10-min treatment with collagenase, was best for dissociation of primary hepatocytes cultured on collagen substratum.

From the above observations, it is obvious that hepatocytes cultured on collagen substratum can be dissociated satisfactorily into single cells when the trypsin treatment precedes the collagenase treatment. Therefore, it is conceivable that trypsin first severs the attachment between adjacent hepatocytes in the monolayer and then the attachment between the cells and collagen substratum. On the other hand, collagenase seems to sever only the latter attachment. Incidentaly, it is known that collagenase preparations (type 1) are contaminated by trypsin and other proteolytic enzymes (6). This seems to be the reason why hepatocytes were partly dissociated into single cells by collagenase treatment alone.

It is known that Ca^{2+} plays an important role in cell adhesion and that the removal of Ca^{2+} facilitates the subsequent separation of hepatocytes from liver by collagenase perfusion (6). The effect of removing Ca^{2+} is irreversible, and seems to be due to the detachment and washout of the Ca^{2+}-dependent adhesion factor, namely the central plaque material of desmosomes (6, 7). Removal of Ca^{2+} is accomplished either with chelators, such as EDTA and ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA), or by efficient washing with Ca^{2+}-free buffer (6). Thus, in the

present study, EDTA promoted cell dissociation when it was added together with trypsin.

As described above, procedure 8, *i. e.*, treatment with a combination of trypsin and EDTA followed by collagenase treatment, is a excellent method for the rapid preparation of single-cell suspensions from hepatocyte primary cultures on collagen substratum.

References

1. Seglen PO and Fossa J: Attachment of rat hepatocytes *in vitro* to substrata of serum protein, collagen or concanavalin A. Exp Cell Res (1978) **116**, 199-206.
2. Miyazaki M, Handa Y, Oda M, Yabe T, Miyano K and Sato J: Long-term survival of functional hepatocytes from adult rat in the presence of phenobarbital in primary culture. Exp Cell Res (1985) **159**, 176-190.
3. Michalopoulos G and Pitot HC: Primary culture of parenchymal liver cells on collagen membranes. Morphological and biochemical observations. Exp Cell Res (1975) **94**, 70-78.
4. Grisham JW, Thal SB and Nagel A: Cellular derivation of continuously cultured epithelial cells from normal rat liver; in Gene Expression and Carcinogenesis in Cultured Liver, Gerschenson and Thompson eds, Academic Press, New York-San Francisco-London (1975) pp 1-23.
5. Miyazaki M: Primary culture of adult rat liver cells. I. Preparation of isolated cells from trypsin-perfused liver of adult rat. Acta Med Okayama (1977) **31**, 351-360.
6. Seglen PO: Preparation of isolated rat liver cells. Methods Cell Biol (1976) **13**, 29-83.
7. Amsterdam A and Jamieson JD: Studies on dispersed pancreatic exocrine cells. I. Dissociation technique and morphologic characteristics of separated cells. J Cell Biol (1974) **63**, 1037-1056.

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