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RECEPTOR-MEDIATED FORMATION OF MULTILAYER AGGREGATES OF PRIMARY CULTURED ADULT RAT HEPATOCYTES ON LACTOSE-SUBSTITUTED POLYSTYRENE

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Summary: We used a lactose-substituted polystyrene, poly-N-p-vinylbenzyl-D-lactonamide (PVLA), as a substratum for adult rat hepatocytes in primary culture. Spherical-shaped hepatocytes attached on PVLA substratum formed stable multilayer aggregates anchored on substratum through the stimulation of epidermal growth factor (EGF). The cells required calcium ion essentially to form the aggregates. The formation of multilayer aggregates was inhibited by colchicine, but not by cytochalasin B. The inhibition was also observed by added PVLA molecules in the culture medium and by treating surfaces of PVLA-coated dishes with allo A lectin. It was suggested that adult rat hepatocytes attached on PVLA substratum required the specific interaction between asialoglycoprotein receptors on the cell surface and PVLA substratum to form anchored multilayer aggregates.

A primary culture of hepatocytes have been extensively used as a model system to investigate metabolisms in liver functions and to construct a hybrid artificial liver. A lot of attempts have been made to express highly differentiated functions and/or proliferation of hepatocytes in primary cultures on various solid surfaces and substrata. It has been reported that the hepatocyte attachment and the expression of their cellular functions were improved by using extracellular matrices such as collagen (1,2), fibronectin (3,4), laminin (5), proteoglycans (6) as substrata.

We reported in previous papers (7,8) that adult rat hepatocytes attached well to the dishes whose surfaces were coated with a lactose-substituted polystyrene, poly-N-p-vinylbenzyl-D-lactonamide (PVLA). We previously pointed out that the hepatocyte attachment to PVLA coated-dishes showed resemblance to the receptor-mediated clearance of asialoglycoproteins from the blood stream by hepatocytes in following respects. 1) β-D-galactose residues were specific signals in recognition and binding by hepatocytes (9). 2) Calcium ion was

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required essentially (10). 3) The uptake of asialoglycoproteins into hepatocytes was inhibited by colchicine, but not by cytochalasin B (11). In addition, spherical morphology of hepatocytes cultured on asialoceruloplasmin-coated dishes was retained in those cultured on PVLA-coated dishes as well (12). It was assumed that the hepatocyte attachment to PVLA-coated dishes was based on the specific interaction between asialoglycoprotein receptors on the cell surface and clustered β-D-galactose residues of PVLA substratum (13). Thus, PVLA may be regarded as a synthetic model of asialoglycoprotein.

Recently, the spherical-shaped hepatocytes attached on PVLA substratum were found to form stable multilayer aggregates anchored on substratum through the stimulation of epidermal growth factor (EGF) and insulin (14,15). The formation of multilayer aggregates depended on the concentrations of EGF and insulin. The cells in the aggregates expressed a long-term survivability, higher level of differentiated functions and lower ability of proliferation determined by [^{3}H]-thymidine uptake than those in monolayer cultures on collagen and fibronectin.

In this study, we attempted to clarify the role of the interaction between asialoglycoprotein receptors on the cell surface and PVLA substratum during the formation of multilayer aggregates.

MATERIALS AND METHODS

Preparation of culture dish. PVLA was synthesized as reported (16,17). An 1 ml aliquot of an aqueous PVLA solution (0.01%) was placed into 35-mm diameter polystyrene dish (Falcon 1008) at room temperature. After 10 min, the PVLA solution was decanted and the surface of dish was rinsed three times with a Dulbecco's phosphate buffer solution.

Preparation and primary culture of hepatocytes. Adult rat hepatocytes were prepared from 5-week old female Sprague-Dawley rats (150-200 g) by the method of Seglen (18) with a slight modification. The basal medium was William's medium E containing 100 U/ml penicillin, 100 U/ml streptomycin and 1 μg/ml fungizone. The hormone-supplemented medium was made by supplementing EGF (Takara Shuzo, Japan), insulin (Sigma Chemical Co., US) and dexamethasome (Sigma Chemical Co.) to the basal medium at final concentration of 50 ng/ml, 10^{-9} M, and 10^{-9} M, respectively. The isolated hepatocytes were finally suspended at a concentration of 5×10^5 cells/ml in the basal medium. Aliquots (2.0 ml) of the cell suspension were placed into PVLA-coated dishes and incubated for 4 h in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After incubation, the medium and dead hepatocytes were removed and placed with the hormone-supplemented medium containing cytochalasin B (Sigma Chemical Co.), colchicine (Sigma Chemical Co.), PVLA molecules, aprotinin (Sigma Chemical Co.), and fetal calf serum (GIBCO, US) at various concentrations, and then cultured for 48 h.

Treatment with allo A lectin. Hepatocytes seeded to PVLA-coated dishes were incubated for 4 h in the basal medium. After incubation, the medium and dead hepatocytes were removed and placed with the hormone-supplemented medium containing various concentrations of allo A lectin (Allomyrina dichotoma

lectin, COSMO BIO Co., Japan) which can specifically bind β-D-galactose, and then incubated for 20 min at 37°C in order to block exposed β-D-galactose residues of PVLA substratum, and then cultured for 48 h.

Morphological observation and measurement of aggregate diameter. Morphological observation was carried out using a phase-contrast microscope. Anchored multilayer aggregates of hepatocytes cultured on PVLA substratum were gently removed from the dish with a silicon rubber scraper. The average diameter of one thousand of the aggregates was measured with electronic particle-size analyzer (MULTISIZER II, Coulter Electronic Ltd., UK).

RESULTS AND DISCUSSION

When freshly isolated adult rat hepatocytes, spherical single cells, were cultured on PVLA substratum in the basal medium, most of the cells did not spread and retained their spherical shape (Fig. 1A). In the hormone-supplemented medium, the cells migrated gradually to assemble with each other, and then formed anchored multilayer aggregates with average diameter of 80-100 μm within 48 h of culture (Fig. 1B). In contrast, the formation of anchored multilayer aggregates was not observed on adhesive proteins such as collagen, fibronectin and laminin. The morphological features of the cells cultured on PVLA substratum was a striking contrast to that of the cells flattened and formed continuous monolayer on adhesive proteins. On the other hand, hepatocytes cultured in the hormone-supplemented medium excluded calcium ion,

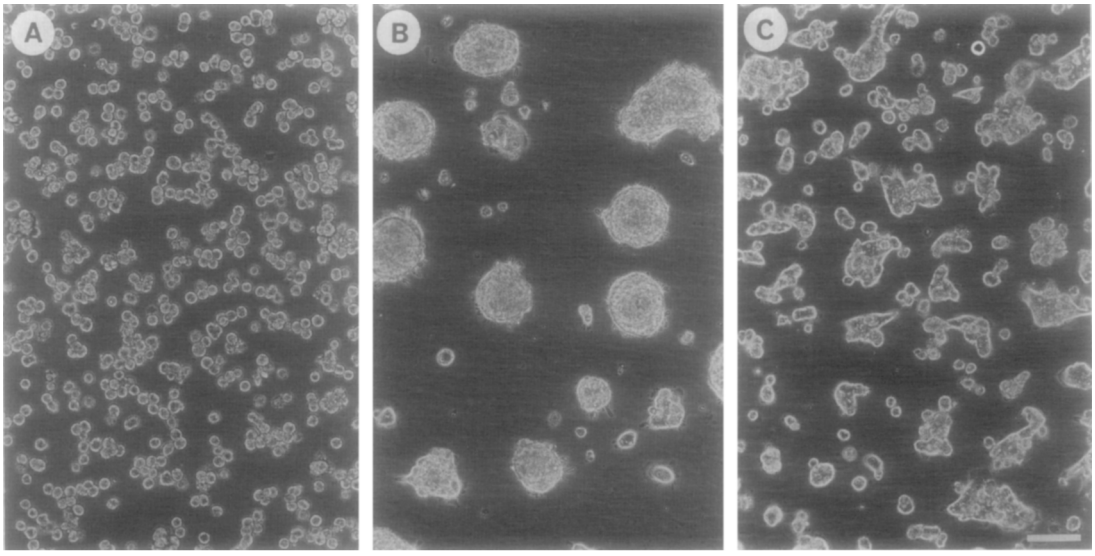

Fig. 1. Phase-contrast microscopic features of adult rat hepatocytes cultured on PVLA substratum for 48 h. (A) In the basal medium. (B) In the hormone-supplemented medium (50 ng/ml of EGF). (C) In the hormone-supplemented medium excluded calcium ion. The scale bar represents 100 μm.

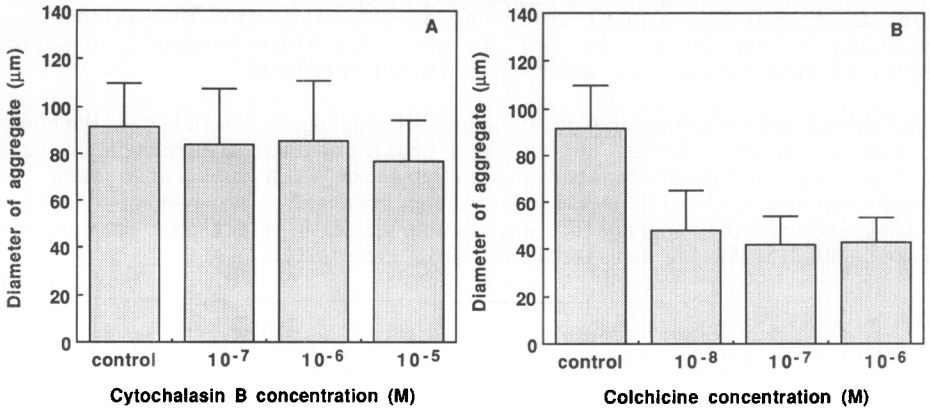

Fig. 2. Effects of cytochalasin B and colchicine on the formation of multilayer aggregates of adult rat hepatocytes on PVLA substratum. The cells were cultured in the hormone-supplemented medium for 48 h. (A) The culture medium contained various concentrations of cytochalasin B. (B) The culture medium contained various concentrations of colchicine. The values of mean ± SD were calculated from one thousand multilayer aggregates.

spread slightly and could not form stable multilayer aggregates (Fig. 1C). Hepatocytes required calcium ion essentially to form multilayer aggregates on PVLA substratum. The requirement of calcium ion was the same as the uptake of asialoglycoproteins into hepatocytes (10).

The influence of cytoskeletons on the formation of multilayer aggregates of hepatocytes cultured on PVLA substratum was examined using inhibitors such as cytochalasin B and colchicine. As shown in Fig. 2A, the average diameter of multilayer aggregates was scarcely influenced by cytochalasin B which is an inhibitor of actin filament functions. On the other hand, the average diameter of the aggregates was remarkably decreased by colchicine which is an inhibitor of microtubule functions (Fig. 2B). It was reported that the uptake of asialoglycoproteins into hepatocytes which was mediated by asialoglycoprotein receptors was inhibited by colchicine, but not by cytochalasin B (11). This suggested that the formation of multilayer aggregates of hepatocytes cultured on PVLA substratum may be related to receptor-mediated interaction as well as the uptake of asialoglycoproteins into hepatocytes.

The interaction between hepatocytes and PVLA substratum during the formation of multilayer aggregates was examined by adding PVLA molecules into the culture medium in order to block asialoglycoprotein receptors on the cell surface, and by treating PVLA-coated dishes with allo-A lectin in order to block exposed β-D-galactose residues of PVLA substratum. The formation of multilayer aggregates was effectively inhibited by added PVLA molecules in the culture medium. As shown in Fig. 3A, the average diameter of the aggregates

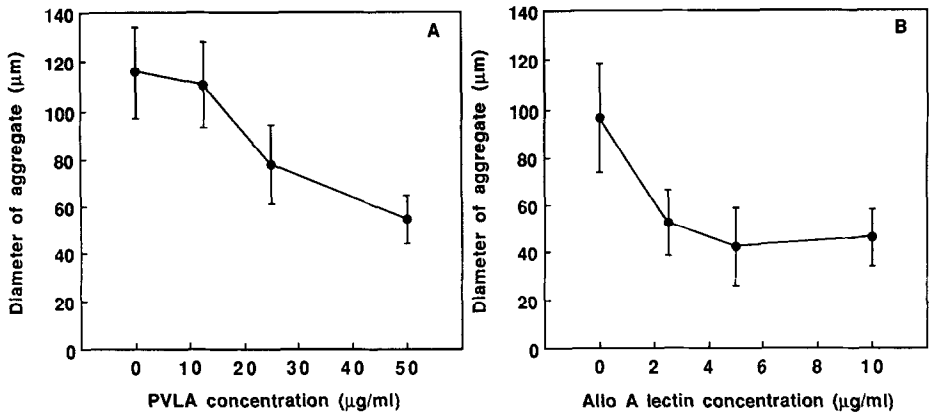

Fig. 3. Effects of PVLA molecules and allo A lectin on the formation of multilayer aggregates of adult rat hepatocytes on PVLA substratum. The cells were cultured in the hormone-supplemented medium for 48 h. (A) The culture medium contained various concentrations of PVLA molecules. (B) PVLA-coated dishes were used after the treatment with various concentrations of allo A lectin solutions as described in Materials and Methods. The values of mean ± SD were calculated from one thousand multilayer aggregates.

decreased with increasing concentration of added PVLA molecules. Higher concentration of PVLA molecules brought about spreading of hepatocytes in 48 h of culture. In cultures treated with allo A lectin, the formation was also inhibited by the treatment with more than 2.5 μg/ml of allo A lectin (Fig. 3B). PVLA molecules in the culture medium can compete with PVLA substratum for receptor binding, and allo A lectin can bind specifically to exposed β-D-galactose residues of PVLA substratum. These results suggested that the formation of multilayer aggregates on PVLA substratum was inhibited by blocking of asialoglycoprotein receptors on the cell surface, and by blocking of exposed β-D-galactose residues of PVLA substratum. It is likely that the interaction between hepatocytes and PVLA substratum plays a major role in the formation of multilayer aggregates.

It was reported (19-21) that adult rat hepatocytes cultured on some surfaces formed floating spheroidal aggregates (spheroids). The formation of floating spheroids can be completely inhibited by adding aprotinin which is a protease inhibitor, and calf serum containing several protease inhibitors. It is of interest to compare multilayer aggregates formed on PVLA substratum with these spheroids. We examined whether the formation of multilayer aggregates was influenced by aprotinin and calf serum. The average diameter of multilayer aggregates formed in the hormone-supplemented medium alone, with 0.12 μg/ml of aprotinin, with 5% of calf serum, with 0.12 μg/ml of aprotinin and 5% of calf serum were 88 ± 24 μm, 86 ± 28 μm, 94 ± 23 μm and 91 ± 21 μm, respectively. The formation of multilayer aggregates on PVLA substratum was scarcely influenced by aprotinin and calf serum. These results suggested that

anchored multilayer aggregates of hepatocytes cultured on PVLA substratum were formed by a different mechanism from the floating spheroids.

In summary, observations described in this study suggested that adult rat hepatocytes attached on PVLA substratum required the specific interaction between asialoglycoprotein receptors on the cell surface and PVLA substratum to form stable multilayer aggregates anchored on substratum. EGF was also found to be able to trigger the receptor-mediated formation of multilayer aggregates. Further studies using hepatocyte culture system described here are in progress to reveal the mechanism of receptor-mediated regulation in the cell behaviors.

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