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Original Article

Cytoskeletal Inhibitors, Anti-adhesion Molecule Antibodies, and Lectins Inhibit Hepatocyte Spheroid Formation

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We investigated the role of cytoskeletons, adhesion molecules, membrane-glycosylations, and proteoglycans in forming the shape of adult rat hepatocyte spheroids. Isolated hepatocytes were cultured on dishes coated with chondroitin sulfate phosphatidyl ethanolamine (CS-PE). Spheroid-forming ability was observed after adding cytoskeletal inhibitors (cytochalasin D, colchicine, okadaic acid, mycalolide B), anti-adhesion molecule antibodies (anti-E-cadherin, anti-connexin 32, anti-zo-1), a glycosphingolipid synthetic inhibitor (N-butyldeoxynojirimycin), a proteoglycan synthetic inhibitor (p-nitrophenyl-β-D-xylopyranoside), and several lectins. Localization of actin was studied using confocal microscopy after rhodamine-phalloidin staining. Adding cytoskeletal inhibitors on the initial day resulted in weakly clustered cell aggregates rather than smoothly formed spheroids. These effects disappeared at lower reagent concentrations. When reagents were added on day 3, after the formation of spheroids, only mycalolide B was associated with an irregular spheroid surface; the others had no effect. Adding the anti-E-cadherin, anti-connexin 32 on the initial day showed inhibition of spheroid formation, but anti-zo-1 and proteoglycan synthetic inhibitor had no effects. Among the several lectins, only Wheat Germ Agglutinin (WGA), Ricinus communis Agglutinin I (RCA-I), and Concanavalin A (ConA) showed inhibition. These results suggest that cytoskeletal conformation and some adhesion molecules are necessary to form spheroids. Based on the interactions between lectins and hepatocytes in the present study, hepatocytes appear to contain an N-linked complex or N-linked hybrid glycosylated chains.

Key words: hepatocyte spheroid, okadaic acid, mycalolide B, E-cadherin, lectins

We have previously studied adult rat hepatocyte spheroids and have determined their three-dimensional structure and highly maintained hepatocyte-specific functions [1, 2]. Based on these merits, several researchers have used hepatocyte spheroids for bioartificial experimental liver models [3-6].

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In our spheroid culture system, seeded hepatocytes begin to self-assemble and reorganize themselves into a three-dimensional cyto-architecture similar to that observed *in vivo* [1, 2, 7]. In such structures, cell-to-cell contacts are maximized, showing junctional complexes and bile canaliculi [8, 9], and the hepatocytes survive for a longer period of time while maintaining their liver-specific functions [10, 11]. Therefore, cytoskeleton molecules and adhesion molecules must play important roles in spheroid formation and function.

In the present study, we investigated the role of cytoskeletons, adhesion molecules, membrane-glycosylations, and proteoglycans in the formation of a spheroidal shape by using specific inhibitors or antibodies to these molecules. In addition, we studied whether or not the blockade of glycosylated chains by lectins [12] can inhibit spheroid formation.

Materials and Methods

Chemicals. Okadaic acid, cytochalasin D, and colchicine were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Mycalolide B from Wako Pure Chemical Co. (Osaka, Japan), and used as cytoskeletal inhibitors. Okadaic acid, a protein phosphatase inhibitor, has been shown to disrupt intermediate filaments [13-15]; cytochalasin D, which has been shown to inhibit actin polymerization [16, 17], was used as an inhibitor of microfilaments; colchicine was used as an inhibitor of microtubules [18].

Mycalolide B is an actin depolymerizing agent that has been shown to inhibit the Mg^{2+}-ATP aseactivity of native actomyosin [19, 20].

The imino sugar N-butyldeoxynojirimycin (Sigma) is an inhibitor of the ceramide-specific glycosyltransferase, and thus inhibits glycosphingolipid biosynthesis [21] as well as inhibit oligosaccharide trimming enzymes, α-glucosidase I and II. Finally, p-nitrophenyl-β-D-xylopyranoside (PNP-Xyl) (Sigma) has previously been used as a selective inhibitor of proteoglycan synthesis [22].

Antibodies to adhesion molecules. Rat anti-mouse-E-cadherin antibody and rat anti-mouse-zo-1 antibody, which reacts with rat zo-1, were purchased from Chemicon International Inc. (Temecula, CA, USA). The cadherins are a family of glycoproteins involved in the formation of Ca^{2+} dependent cell-cell adhesion [23-25], and zo-1 is a high molecular weight tight junction-associated protein [26, 27]. Connexin 32 is a gap junction-associated protein, and mouse anti-rat-connexin 32 antibody [28, 29] was used for the present experiment.

Lectins. A lectin-screening kit containing Dolichos biflorus Agglutinin (DBA), Peanut Agglutinin (PNA), Soybean Agglutinin (SBA), Ulex europaeus Agglutinin- I (UEA-I), Concanavalin A (ConA), Wheat Germ Agglutinin (WGA) and Ricinus communis Agglutinin I (RCA-I) was purchased from Vector Laboratories (Burlingame, CA, USA)

Isolation and Culture of hepatocytes. Hepatocytes were isolated from male Wistar rats (body weight, 180 to 200 g) by collagenase perfusion, and purified by differential centrifugation. Isolated cells were cultured by serum-free hormonally defined medium (HDM) consisting of William's E medium containing epidermal growth factor (EGF), insulin, penicillin and streptomycin [30]. Hepatocytes were seeded at a density of 3×10^5 cells/ml of culture medium on dishes coated with chondroitin sulfate-phosphatidyl ethanolamine (CS-PE) [31, 32]. Chemicals or antibodies were added to the culture medium on the initial day in order to determine their effects on spheroid formation. The concentrations studied were as follows: for okadaic acid, 30 nM, 3 nM, 0.3 nM); for cytochalasin D, 10 μM, 1 μM; for colchicine, 5 μM, and 0.5 μM; for p-nitrophenyl-β-D-xylopyranoside, 5 mM, 0.5 mM; for N-butyldeoxynojirimycin, 2 mM, 0.2 mM, and for lectins, 1 μM, 0.1 μM. The dilution rates of the antibodies were as follows: for anti-zo-1, $\times$ 10, $\times$ 100; for anti-connexin 32, $\times$ 10, $\times$ 100; and for anti-E-cadherin, $\times$ 10, $\times$ 100. To investigate the effect of rat anti-mouse-E-cadherin-antibody, hepatocytes from mouse liver were isolated from six- to seven-week-old male BALB/c mice.

The study was performed in accordance with the Guidelines for Animal Experiments of Okayama University Medical School.

Estimation of spheroid formation. On culture day 4, cells were estimated as the number of spheroids or aggregates, and spheroid formations were examined by phase-contrast microscopy using a low-power field and classified as good, irregular, or poor. The viability of the hepatocytes failing to form spheroids was determined by trypan blue staining.

Immunofluorescence microscopy and confocal microscopy. Spheroids were incubated for 45 min at 37 °C on type-III collagen-coated slides and then fixed for 20 min with 10% buffered formalin. After fixation, slides were washed with phosphate-buffered saline (PBS) for 5 to 10 min and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA), which binds with high affinity to F-actin [9], for 1 h at 37 °C. Slides were then washed 3 times with PBS, and observed by immunofluorescence microscopy and confocal microscopy in order to determine the actin structure of spheroids [33, 34]. To check its effect, the actin depolymerizing agent mycalolide B was added into the

culture medium on day 3, after normal spheroids had already formed, and spheroid shapes were observed 24 h later by confocal microscopy and phase-contrast microscopy.

Results

Effects of cytoskeletal inhibitors. In control cells, spheroids had successfully completed formation by 3 or 4 days (Fig. 1A). Okadaic acid, cytochalasin D, and colchicine were used as cytoskeletal inhibitors. At a concentration of 30 nM, okadaic acid caused inhibition of spheroid formation, and isolated cells formed irregular aggregates (Fig. 1B, Table 1). At a lower concentration (0.3 nM) of okadaic acid, spheroid formation was not disturbed.

With cytochalasin D (10 μM) isolated cells formed similar aggregate shapes (Fig. 1C, Table 1). But when less than 1 μM of cytochalasin D was added to medium, spheroids were formed successfully. Colchicine (5 μM) induced hepatocytes to form irregular aggregates, but at

Table 1 Effects of cytoskeletal inhibitors on spheroid formation

	No. of spheroids	Spheroid formation
Normal control	7～15/low power field	++
Okadaic acid		
30 nM	0	−
3 nM	0	±
0.3 nM	5～14	++
Cytochalasine D		
10 μM	0	−
1 μM	7～13	++
Colchicine		
5 μM	0	−
0.5 μM	7～15	++

Estimated numbers of spheroids by phase-contrast microscopy per low power field (× 100) on day 4. Spheroid formation was classified into 3 grades: good, ++; irregular aggregates, ±; poor (small numbers of irregular aggregates are included), −.

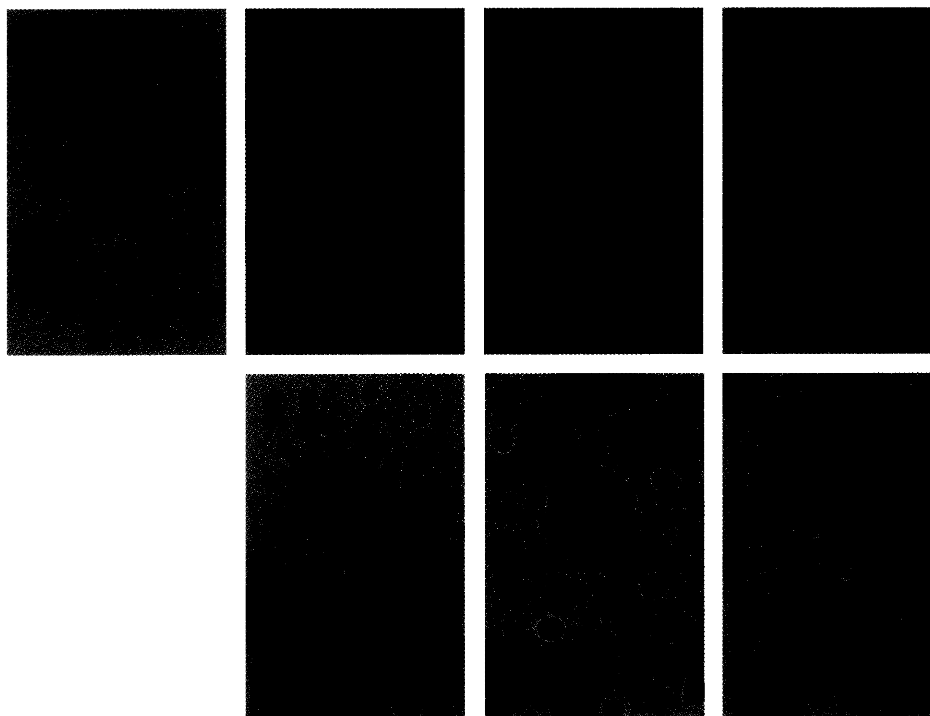

Fig. 1 Failure of spheroid formation due to cytoskeletal inhibitors. Phase-contrast microscopy of hepatocyte culture on day 3. (**A**) Normal control (without inhibitors); (**B**) okadaic acid treated (30 nM); (**C**) cytochalasin D treated (10 μM); (**D**) colchicine treated (5 μM). × 400. Spheroid formation was successful at lower concentrations of these chemicals. (**E**) Okadaic acid treated (0.3 nM); (**F**) cytochalasin D treated (1 μM); (**G**) colchicine treated (0.5 μM). × 400.

a lower concentration (0.5 μM) induced clear spheroid formation (Table 1). More than 80% of hepatocytes were viable even at the highest concentration of chemicals used for this experiment.

Effects of anti-adhesion molecule antibodies. Tight junctions or gap junctions were previously demonstrated in the hepatocytes of normal liver tissue or spheroids [8]. Antibodies to cell adhesion molecules (E-cadherin, zo-1 and connexin 32) were used irrespective of whether blocking these molecules could disrupt spheroid formation. As shown in Table 2, the addition of anti-E-cadherin or anti-connexin 32 was associated with poor spheroid formation. But when anti-zo-1 was added, spheroids were successfully formed.

Effects of glycosphingolipid or proteoglycan synthetic inhibitors. When isolated cells were incubated with N-butyldeoxynojirimycin or with p-nitrophenyl-*β*-*D*-xylopyranoside spheroid formation was as complete as in control cells (Table 3).

Effects of lectins. Incubation with different lectins led to different outcomes (Table 4, Fig. 2). Four lectins, DBA, PA, SBA, and UEA-I, had no influence on spheroid formation. ConA and WGA inhibited sphe-

Table 2 Effects of anti-adhesion molecule antibodies on spheroid formation

	No. of spheroids	Spheroid formation
Mouse		
Normal control	5~10/low power field	++
Anti-E-cadherin × 10	0	−
Anti-E-cadherin × 100	0	±
Rat		
Normal control	5~12	++
Anti-zo-1 × 10	6~13	++
Anti-zo-1 × 100	5~12	++
Anti-connexin32 × 10	0	−
Anti-connexin32 × 100	0	−

Estimated numbers of spheroids by phase-contrast microscopy per low power field (× 100) on day 4. Spheroid formation was classified into 3 grades: good, ++; irregular aggregates, ±; poor (small numbers of irregular aggregates are included), −.

Table 3 Effects of glycosphingolipid or proteoglycan synthetic inhibitor on spheroid formation

	No. of spheroids	Spheroid formation
Normal control	5~14/low power field	++
N-butyldeoxynojirimycin		
0.2 mM	5~12	++
2 mM	5~12	++
P-nitrophenyl-β-d-xylopyranoside		
0.5 mM	5~18	++
5 mM	5~18	++

Estimated numbers of spheroids by phase-contrast microscopy per low power field (× 100) on day 4 are shown. Spheroid formation classified into 3 grades: good, ++; irregular aggregates, ±; poor (small numbers of irregular aggregates are included), −.

Table 4 Effectes of various lectins on spheroid formation

Lectins	Sugar chains to bind	Concentration	No. of spheroids	Spheroid formation
Normal control		—	5~14/low power field	++
DBA	GalNAc	1 μM	7~12	++
		0.1 μM	7~13	++
PNA	Galβ1-3GalNAc	1 μM	8~11	++
		0.1 μM	7~11	++
SBA	GalNAc	1 μM	7~11	++
		0.1 μM	9~15	++
UEA-I	L-Fuc	1 μM	7~11	++
		0.1 μM	7~13	++
ConA	Mannose, Methyl-α-D-Mannose	1 μM	0	−
		0.1 μM	0	±
WGA	GlcNAc	1 μM	0	−
		0.1 μM	0	±
RCA-I	Galactose, Lactose	1 μM	0	−
		0.1 μM	0	−

Estimated numbers of spheroids by phase-contrast microscopy per low power field (× 100) on day 4. Spheroid formation classified into 3 grades: good, ++; irregular aggregates, ±; poor (small numbers of irregular aggregates are included), −.

roid formation at a concentration of 1 μM, but not at a concentration of 0.1 μM. RCA-I inhibited spheroid formation both at 1 mM and at 0.1 mM.

Immunofluorescence microscopy and confocal microscopy. Rhodamine-phalloidin was used to identify the microfilaments of F-actin, which are generally recognized as stress fibers in monolayer culture [35]. In the spheroids, actin staining was observed on the surface of each cell, and intensely observed at the adhesion belts in cellular contact regions (Figs. 3A, B). The intensely stained structures inside the cells probably corresponded to the pericanalicular microfilament sheaths. After spheroid formation on day 3 and treatment with mycalolide B for 24 h, actin staining was seen in the center of the spheroids, but was absent from the outer surface of the spheroids' peripheral hepatocytes (Fig. 3C). The surfaces of the mycalolide B-treated spheroids became irregular as shown by phase-contrast microscopy

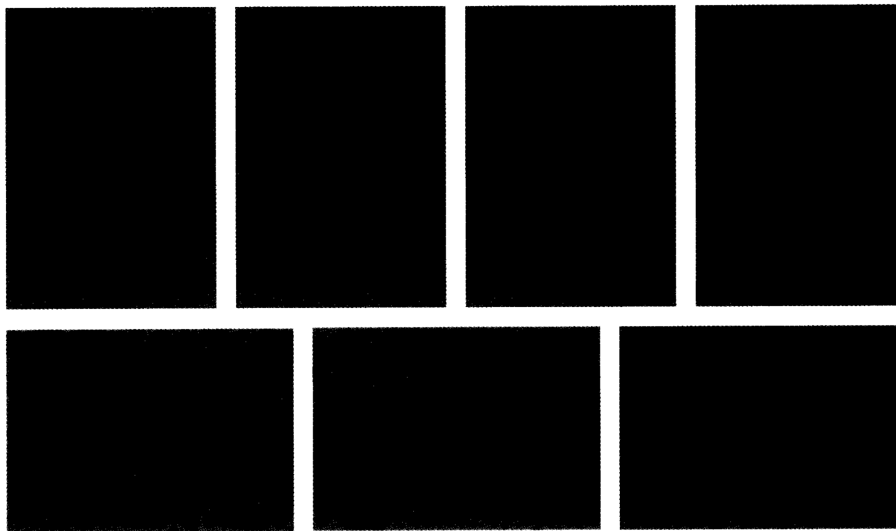

Fig. 2 Effect of lectins on spheroid formation. Phase-contrast microscopy of hepatocyte culture on day 3. (A) WGA; (B) RCA-I; (C) ConA; (D) DBA; (E) PNA; (F) SBA; (G) UEA-I treated (1 μM each). $\times$ 400.

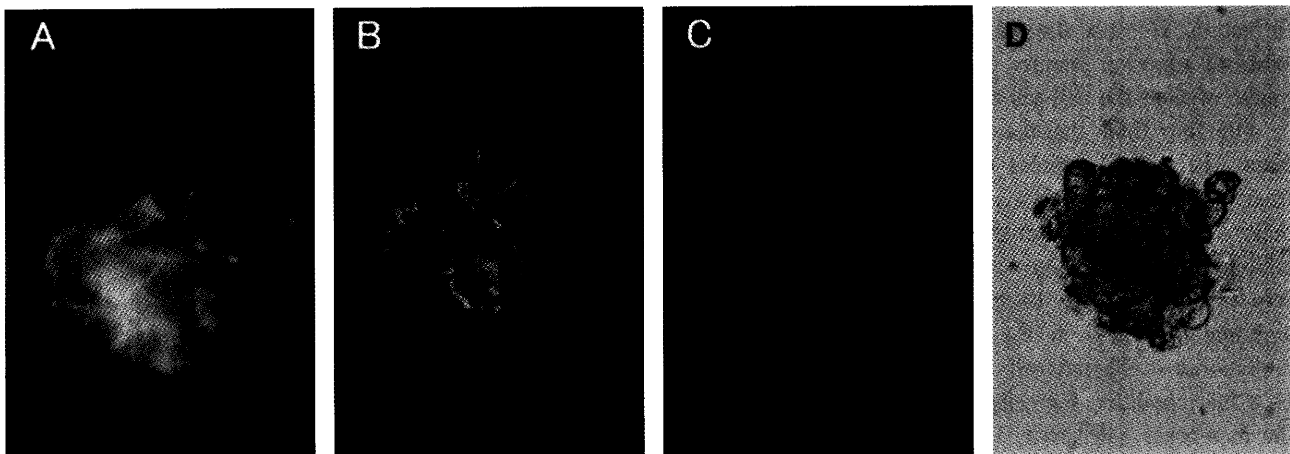

Fig. 3 Immunofluorescence microscopy and confocal microscopy of spheroids. (A) Visualization of a rhodamine-phalloidin-stained normal spheroid by immunofluorescence microscopy. (B) Visualization of a rhodamine-phalloidin-stained normal spheroid by confocal microscopy. (C) Visualization of rhodamine-phalloidin-stained mycalolide B treated spheroids by confocal microscopy. (D) Phase-contrast microscopy of mycalolide B treated spheroid. $\times$ 400.

(Fig. 3D).

Discussion

By our previously reported methods, isolated hepatocytes can form spheroids within 3 or 4 days [1, 2, 7]. In this study, we used CS-PE-coated dishes [31, 32] to induce earlier and more uniform spheroid formation. Hepatocytes formed spheroids in 3 days and the diameter of the spheroids ranged from 90-120 μm. These hepatocyte spheroids show as high albumin production as those formed by previous methods [1, 2, 7] (data not shown).

Okadaic acid is a protein phosphatase inhibitor. At a concentration of 30 nM, it was shown to disrupt intermediate filaments without influencing the cell viability of cultured rat hepatocytes [14]. We started the experiment at a concentration of 30 nM to avoid cell death during the culture period. We also chose the concentration of cytochalasin D and colhicine for the same reason [14, 18]. Each of the 3 cytoskeletal inhibitors used (okadaic acid; intermediate filaments, cytochalasin D; microfilaments, colchicine; microtubules) disrupted spheroid formation (Table 1, Fig. 1). Cell-cell attachments were weak and hepatocytes showed irregular aggregates. No spheroids were formed. Our results indicate that all 3 cytoskeleton components are necessary for spheroid formation. These effects were concentration-dependent. Lower concentrations of these cytoskeletal inhibitors did not inhibit spheroid formation. These results indicate that successful spheroid formation requires cytoskeletal conformation. Because the cytoskeleton is one of the components of adhesion plaque, disruption of the cytoskeleton might weaken the cell-cell attachments.

One may think that the irregular aggregates shown by Figs. 1B, C, D express a low level of membrane refractivity. But the purpose of these experiments is to determine whether or not these chemicals (as cytoskeletal inhibitors, not as cytotoxic agents) can inhibit the spheroid forming process during the initial days. Irregular aggregates formed in the consequence of cytoskeletal inhibition lost effective cell-cell interaction. As this should be a bad condition for cells, it might be reasonable that the membrane refractivity of cells on day 4 seemed low. Similar images were shown by anti-adhesion molecule antibodies or lectins (Fig. 2). More than 80% of the cells forming irregular aggregates were seen to viable, further investigations were performed. EGF or insulin in the hormonally defined medium [30] might have the effects to keep these cells' viability.

These inhibitors had an effect on spheroid formation when applied on the first day, but had no effect on day 3, after spheroid formation was complete. Furthermore, adding these inhibitors to monolayer cultured hepatocytes on day 3 resulted in detached cells (data not shown). Thus, hepatocyte spheroids showed resistance to these chemicals. Walker *et al.* [36] also showed that spheroids appeared less sensitive to exposure to methotrexate.

Mycalolide B is a strong actin-depolymerizing agent. So, we used mycalolide B after spheroid formation to check the actin depolymerizing effect. Even when mycalolide B was added to the culture after the completion of spheroid formation on day 3, the surface of the spheroids became irregular (Fig. 3D). As mentioned by Sato *et al.* [20], mycalolide B can sever F-actin and have a strong actin depolymerizing effect, whereas cytochalasin D cannot completely depolymerize F-actin. These 2 chemicals' different functions may lead to the resistance of spheroids to cytochalasin D. Using mycalolide B, actin filaments of the peripheral hepatocytes of spheroids seemed to be destroyed, but central areas were still resistant to the chemical. This status was well demonstrated by rhodamine-phalloidin staining and confocal microscopy (Fig. 3C). In normal spheroids, cell surfaces, including peripheral hepatocytes, are stained strongly (Figs. 3A, B). In the spheroids treated with mycalolide B, the outer sides of peripheral hepatocytes were not stained with rhodamine-phalloidin (Fig. 3C). Stress fibers were not seen in any hepatocytes, as distinct from the findings of Tranter *et al.* [35] in monolayer cultured hepatocytes.

The cadherins are a family of glycoproteins involved in the formation of Ca^{2+} dependent cell-cell adhesion [23-25]. Zo-1 is a high molecular weight tight junction-associated protein [26, 27] and connexin 32 is a gap junction-associated protein [28, 29]. Antibodies to cell adhesion molecules (anti-E-cadherin, anti-zo-1 and anti-connexin 32) were used irrespective of whether blocking these molecules could disrupt spheroid formation. Among these antibodies, anti-E-cadherin and anti-connexin 32 showed inhibitory effects, whereas anti-zo-1 induced no inhibition of spheroid formation (Table 2). These results showed that cell-cell interactions by cadherins or gap junctions are necessary to form spheroids. Tight junctions may not play important roles for spheroid formation, or the anti-zo-1 antibody used cannot block tight junction

formation.

Neither p-nitrophenyl-β-D-xylopyranoside (proteoglycan synthesis inhibitor) nor N-butyldeoxynojirimycin (glycosphingolipid synthetic inhibitor) had an effect on spheroid formation (Table 3). Proteoglycans or glycosphingolipids may not be necessary for spheroid formation, or cells may utilize already-existing proteoglycans or glycosphingolipids. N-butyldeoxynojirimycin is also an α-glucosidase I, II inhibitor. It is the inhibitor of oligosaccharide trimming, while biosynthesis of oligosaccharides are not inhibited. This might be one reason why N-butyldeoxynojirimycin did not block hepatocyte spheroid formation.

Among the lectins studied, DBA, PA, SA, and UEA-I did not influence spheroid formation, whereas ConA, WGA, and RCA-I inhibited spheroid formation (Table 4, Fig. 2). ConA binds to Mannose or Methyl-α-D-mannose, WGA binds to GlcNac (N-acetylglucosamine), and RCA-I binds to Galactose or Lactose. If these 3 lectins blocked the same sugar chain, the sugar chain which showed important features for cell-cell interaction might be N-linked complexes or N-linked hybrid glycosylated chains.

Weigel *et al.* [37, 38] or Weisz and Schnnar [39, 40] have shown that rat hepatocytes bind to galactosyl groups in a sugar-specific manner. Weisz and Schnnar showed that rat hepatocytes adhere galactose-derived gels via the rat hepatic lectins (RHLs). Weigel *et al.* concluded that galactose-hepatocyte binding is mediated by asialoglycoprotein receptor [38]. These reports are based on cell-matrix interaction. But same event may happen In cell-cell-interaction. Inhibition of spheroid formation by lectins might be caused in the same manner, in which lectins directly block the ligands to bind receptors. To prove this, further studies may be necessary.

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